National Library of Canada

Acquisitions and Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada

Direction des acquisitions et des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

Canada

UNIVERSITY OF ALBERTA

DOPAMINE D1 REGULATION OF GROWTH HORMONE RELEASE IN THE GOLDFISH

by

ANDERSON ON-LAM WONG

A thesis submitted to
the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**.

DEPARTMENT OF ZOOLOGY
EDMONTON, ALBERTA
SPRING, 1993

ISBN 0-315-82074-8

UNIVERSITY OF ALBERTA

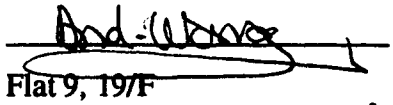
RELEASE FORM

NAME OF AUTHOR: **ANDERSON ON-LAM WONG**

TITLE OF THESIS: **DOPAMINE D1 REGULATION OF GROWTH HORMONE RELEASE IN THE GOLDFISH**

DEGREE: **DOCTOR OF PHILOSOPHY**

YEAR THIS DEGREE GRANTED: **1993**



Flat 9, 19/F
San Woon House
San Wai Court, Tuen Mun
N.T., Hong Kong

Date : 22/4/93

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled : **DOPAMINE D1 REGULATION OF GROWTH HORMONE RELEASE IN THE GOLDFISH** submitted by **ANDERSON ON-LAM WONG** in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY**.

(Dr. R.E. Peter, Supervisor)

(Dr. J.P. Chang, Co-Supervisor)

(Dr. L.C.H. Wang, Committee Member)

(Dr. S. Harvey, Committee Member)

(Dr. G. Tannenbaum, External Examiner)

(Dr. N.E. Stacey, Chairman and Examiner)

Date : 23 April 93

This thesis is
dedicated
to
my
father's
80th birthday.

Abstract

The role of dopamine (DA) on growth hormone (GH) secretion in the goldfish was investigated in the present study. DA stimulated GH release from goldfish pituitary fragments and pituitary cells. Results from studies using pharmacological probes for DA D1 and D2 receptors indicate that DA-stimulated GH release in the goldfish is mediated via the D1 receptors at the pituitary cell level. The GH response to DA could be blocked by somatostatin, but was additive to that of salmon gonadotropin-releasing hormone. The responsiveness of the goldfish pituitary to DA stimulation was found to be seasonal, being the highest in sexually regressed fish, intermediate in recrudescent fish, and the lowest in sexually mature (=prespawning) fish. Castration in prespawning goldfish significantly enhanced the GH responses to DA, indicating that the observed seasonality of DA actions on GH release was induced by the gradual development of the gonads. In goldfish undergoing gonadal recrudescence, serum GH levels were suppressed by intraperitoneal injection of the D1 antagonist SCH23390. Dietary supplementation with the DA agonist apomorphine, on the contrary, increased serum GH levels and body growth of the goldfish. The binding properties of DA D1 receptors in the goldfish pituitary cells were also characterized. The binding affinity and pharmacological profiles of these goldfish D1 receptors were comparable to those of the mammalian D1 systems. Moreover, these DA D1 receptors were found to be localized in the proximal pars distalis, overlapping with the distribution of goldfish somatotrophs. The post-receptor mechanisms of this newly identified D1 system in the goldfish were also examined. Results from *in vitro* studies suggest that activation of the adenylate cyclase-cAMP-protein kinase A pathway, followed by the entry of extracellular Ca^{2+} through voltage sensitive Ca^{2+} channels, mediates the GH-releasing actions of DA. Protein kinase C, however, was not involved in this DA D1 action. Taken together, the present study has demonstrated that DA, through its actions on DA D1 receptors in the pituitary, functions as a physiological GH-releasing factor in the goldfish.

Acknowledgement

I would like to express my gratitude for the support and excellent supervision of my two supervisors, Drs. R.E. Peter and J.P. Chang. I also wish to acknowledge the members of my supervisory and examination committees, Drs. G.S. Tannenbaum, S. Harvey, L.C.H. Wang, J.I. Goldberg and N.E. Stacey for their comments regarding this work.

I would like to thank the graduate students, postdoctoral fellows and visitors in our laboratory, especially C.K. Murthy, W. Ge, K.C. Yu, K. Suzuki, M. [illegible]chi, G. Van der Kraak, B.D. Sloley, C. Peng, R.M. Jobin, B. Himick, F. Van Goor and V.L. Trudeau for their inputs and discussions throughout the research project. I am also grateful to C.S. Nahorniak for her daily assistance and friendly support in the laboratory.

This research was sponsored by grants from the Natural Sciences and Engineering Research Council of Canada to Drs. R.E. Peter and J.P. Chang. The financial support from the Department of Zoology, University of Alberta, in the form of a Graduate Student Teaching Assistantship is also acknowledged.

Table of Contents

Appendix III

Seasonality of Salmon Gonadotropin-Releasing Hormone-Stimulated Growth Hormone Release from Perifused Goldfish Pituitary Fragments

List of Tables

List of Figures

Chapter 1

General Introduction

1.1 Introduction

Growth hormone (GH) is an important pituitary hormone regulating body growth (Holder *et al.*, 1981; Skottner *et al.*, 1989), body metabolism (Schwartz *et al.*, 1992; Towns *et al.*, 1992), tissue differentiation and proliferation (Barnard *et al.*, 1991; Okada *et al.*, 1992), reproduction (Jia *et al.*, 1986; Chatelain *et al.*, 1991) and immune responses in vertebrates (Kelley, 1990). In lower vertebrates, especially in teleosts, GH is also involved in osmoregulation (Yamauchi et al., 1991; Suzuki *et al.*, 1991). Our current understanding of the regulation of GH release is mainly based on studies using the mammalian animal models (for review, see Muller, 1987), and also to a lesser extent using the avian models (for review, see Harvey, 1983). In contrast to the case of mammals and birds, very little is known concerning the regulation of GH release in lower vertebrates.

In mammals, GH release is under the control of the hypothalamus through GH-releasing hormone (GHRH) and somatostatin (SRIF). GHRH stimulates, whereas SRIF inhibits GH release from the anterior pituitary (Frohman and Jansson, 1986). The asynchronous release of these two hypothalamic peptides determines the pulsatile pattern of basal GH release (Tannenbaum and Ling, 1984). In the past decades, increasing evidence suggests that other neuroendocrine factors are also involved in the control of GH release from the pituitary. These factors include the excitatory amino acids, neuropeptides, and the classical neurotransmitters, such as dopamine and norepinephrine (for reviews, see Muller, 1987; Root, 1989). In general, it is accepted that the regulatory actions of these neuroendocrine factors on GH release are often exerted indirectly by modulating GHRH and SRIF release from the hypothalamus.

Dopamine (DA) is one of the predominant catecholamines in the central nervous system of vertebrates (for review, see Fuxe *et al.*, 1985). In mammals, DA is involved in the regulation of body movement (Riederer and Wuketich, 1976; Jellinger, 1986),

emotional stability (Seeman, 1987a; Carlsson, 1988), pituitary hormone release (Fuxe *et al.*, 1985; Thorner and Vance, 1989) and various cardiovascular activities (Hahn and MacDonald, 1984; Hoshino *et al.*, 1986). In teleosts, DA also functions as a hormone-release inhibitor for gonadotropin (GTH) (for review, see Peter *et al.*, 1986) and α melanocyte stimulating hormone (α-MSH) (Omeljaniuk *et al.*, 1989). In the goldfish, the anterior pituitary is innervated by a preoptico-hypophyseal dopaminergic pathway (Kah *et al.*, 1987), whereas the neurointermediate lobe (NIL) is innervated by DA fibers from the periventricular area of the hypothalamus (Fryer *et al.*, 1985; Kah *et al.*, 1987). More recently, it has been shown that DA inhibits GTH-releasing hormone (GnRH) release *in vitro* from goldfish hypothalamic fragments (Yu *et al.*, 1991; Yu and Peter, 1992), indicating that a part of the DA inhibitory actions on GTH release is indirectly through a suppression on GnRH secretion. Taken together, the anatomical and endocrinological evidence strongly suggests that DA, besides its role as a neurotransmitter, also functions as a neurohormone regulating pituitary hormone release in teleosts.

In this thesis, the role of DA as a GH-releasing factor in the goldfish and the mechanisms by which it stimulates GH release from goldfish pituitary cells have been studied. In this chapter, the current literature in the physiology of DA actions, GH regulation in mammals, and the signal transduction pathways involved in GH release is reviewed. A brief overview of GH regulation in teleosts, especially in the goldfish, is also included to provide background information for the present study.

1.2 Physiology of Dopamine Actions in Mammals

1.2.1 Dopamine Receptor and its Signal Transduction Pathways

In mammals, the biological actions of DA are mediated primarily by two DA receptor subtypes, the D1 and D2 receptors (Kebabian *et al.*, 1984). These two DA receptors are distinct membrane-bound proteins (for review, see Strange, 1987) with the molecular structure containing seven transmembrane domains, typical of the G protein-coupled receptor family (Bunzow *et al.*, 1988; Sunahara *et al.*, 1990). However, the pharmacological properties of these two DA receptors are quite different (for a recent review, see Neumeyer *et al.*, 1992). The D1 receptors have a high binding affinity for

1-phenyl benzazepines, e.g., SCH23390 and SKF38393; whereas the D2 receptors are more selective for substituted benzamides and butyrophenones, e.g., (-)sulpiride and spiperone (Niznik, 1987; Stoof and Tilders, 1989). These two DA receptor subtypes are also known to be stereo-specific, with the D1 receptors being more selective for the (+)enantiomers of 1-phenylbenzazepines, e.g., (+)SCH23390, and the D2 receptors for the (-)enantiomers of substituted benzamides, e.g (-)sulpiride (for reviews on the stereo-specificity of DA receptors, see Kaiser, 1983; Iorio *et al.*, 1986). Furthermore, high and low affinity binding states for D1 and D2 receptors have been reported (MacKenzie and Zigmond, 1984; Seeman *et al.*, 1985; Seeman *et al.*, 1986). The high affinity binding state can be converted into the low affinity state in the presence of non-hydrolyzable GTP analogs (Sibley *et al.*, 1982; Seeman *et al.*, 1985). It is generally accepted that these two receptor binding states represent the differential coupling of DA receptors with the membrane-bound regulatory G proteins (Sibley and Creese, 1983; Senogles *et al.*, 1987).

The signal transduction pathways associated with the two DA receptor subtypes are also different. The D1 receptors are positively coupled to the cAMP-producing enzyme adenylate cyclase via a stimulatory G protein (Gs) (Battaglia *et al.*, 1986b; Monsma *et al.*, 1990). Activation of D1 receptors by DA and D1 agonists, e.g., SKF38393, often leads to a rapid accumulation of cAMP. This has been demonstrated in rat striatal slices (Battaglia *et al.*, 1986a,b), human neuroblastoma (Sidhu and Fishman, 1990), and some cultured cell lines, including the LLC-PK1 cells (Grenader and Healy, 1991) and COS-1 cells (Steffey *et al.*, 1991). Recently, down-regulation of the D1 receptor-coupled adenylate cyclase activity by cAMP analogs has been reported in the primary cultures of opossum kidney cells (Bates *et al.*, 1991), suggesting the possible existence of an intracellular feedback mechanism in the D1 receptor-associated cAMP-dependent pathway. The D1 receptors are also known to be coupled with the stimulation of phospholipase C activity (Felder *et al.*, 1989), formation of inositol phosphates (Undie and Friedman, 1990), and activation of protein kinase C (McMillian *et al.*, 1992).

In contrast to the signal transduction mechanism of D1 receptors, the D2 receptors are negatively coupled with adenylate cyclase through an inhibitory G protein (Gi) (Enjalbert and Bockaert, 1983; Elazar *et al.*, 1989). Inhibition of cAMP synthesis by

DA and D2 agonists, e.g., LY171555 and bromocryptine, has been demonstrated in striatal fragments (Battaglia *et al.*, 1985) and pituitary cells of the rat (Meunier and Labrie, 1982; Borgundvaag and George, 1985). In other studies, inhibition of inositol phosphate turnover (Pizzi *et al.*, 1987; Morra *et al.*, 1991) and arachidonic acid production (Canonico, 1989) via the D2 receptors has been reported, suggesting that DA D2 receptors may be associated with phospholipid metabolism. The D2 receptors are also coupled to K^+ channels, activation of which initiates outward K^+ current and subsequent membrane hyperpolarization (Lledo *et al.*, 1990a). More recently, activation of D2 receptors has been shown to reduce intracellular Ca^{2+} levels (Login *et al.*, 1990) and Ca^{2+} entry through voltage-sensitive Ca^{2+} channels (Bigornia *et al.*, 1990; Lledo *et al.*, 1990b), and a part of these inhibitory actions is independent of the D2 activation of K^+ channels (Lledo *et al.*, 1991).

Based on the techniques of molecular biology, multiple DA receptor subtypes beyond the D1/D2 classification have been identified (for review, see Andersen *et al.*, 1990). The first member of the DA receptor family cloned was the D2 receptor, the gene sequence of which reveals the presence of at least 8 exons separated by intervening introns, coding for a G protein-coupled receptor with a long third cytoplasmic loop and a short C-terminus (Bunzow *et al.*, 1988). By alternative splicing of a single 87 bp exon from the mRNA, the D2 gene can give rise to two D2 receptor isoforms, namely the $D2_A$(long) and $D2_B$(short) receptors. The $D2_B$ receptors are the truncated form of the $D2_A$ receptors with the absence of a 29 amino acid insert in the third cytoplasmic loop (Dal Toso *et al.*, 1989; Rao *et al.*, 1990). Activation of the $D2_B$ receptors is more effective than the $D2_A$ receptors in inhibiting cAMP accumulation (Hayes *et al.*, 1992), and the expression of these two DA D2 receptor isoforms can be differentially regulated by sex steroids (Kukstas *et al.*, 1991).

After the cloning of D2 receptors, the gene sequence of D1 receptors has been elucidated in the rat (Zhou *et al.*, 1990) and human (Gingrich *et al.*, 1991). In contrast to the gene of D2 receptors, the D1 gene contains no introns and codes for a G protein-coupled receptor with a small third cytoplasmic loop and a long C-terminus. Recently, the gene sequences of other novel forms of DA receptors, designated as D3 (Sokoloff *et al.*, 1990), D4 (Van Tol *et al.*, 1991), and D5 receptors (Sunahara *et al.*, 1991;

Weinshank *et al.*, 1991), have been cloned. The cDNA sequences and pharmacological properties of the D3 and D4 receptors are comparable to the D2 receptors (Sokoloff *et al.*, 1990; Van Tol *et al.*, 1991), whereas the D5 receptors are similar to that of the D1 receptors (Sunahara *et al.*, 1991; Weinshank *et al.*, 1991). Based on a classification scheme newly developed by Sibley and Monsma (1992), DA receptors can be divided into the "D1" and "D2" subfamilies. The D1 and D5 receptors are subtypes of the "D1" receptor subfamily; whereas the D2 (including the $D2_A$ and $D2_B$ receptors), D3, and D4 receptors are subtypes of the "D2" receptor subfamily. Although the scheme proposed is likely to reflect a more natural way to classify DA receptors, the physiological roles of some of these DA receptor subtypes, such as the D4 and D5 receptors, are still largely unknown. (For recent reviews on the molecular biology of DA receptors, see Civelli *et al.*, 1991; Gingrich *et al.*, 1992; Sibley and Monsma, 1992).

1.2.2 Dopaminergic System and its Functions

1.2.2.1 Dopaminergic systems in the brain of mammals

In mammals, the biological actions of DA in the CNS are mediated mainly through three principle dopaminergic pathways, namely (1) the nigro-striatal pathway, (2) the mesocorticolimbic pathway, and (3) the tuberoinfundibular / tuberohypophyseal pathway (for review, see Webster, 1989). The nigro-striatal dopaminergic pathway is involved in the control of body movements (Riederer and Wuketich, 1976; Jellinger, 1986). Degeneration of this pathway is considered to be the major cause of Parkinson's disease (Jellinger, 1986; Horellou *et al.*, 1990). The mesocorticolimbic pathway is involved in the maintenance of emotional stability (Seeman, 1987a) and abnormality in this pathway contributes to schizophrenia (Seeman, 1987b; Carlsson, 1988). In mammals, especially in the rat, the tuberoinfundibular / tuberohypophyseal pathway originates from the area A12 of the hypothalamus (including the arcuate and periventricular nuclei). Nerve fibers from the tuberoinfundibular pathway project into the median eminence, whereas the neurons from the tuberohypophyseal pathway directly innervate the posterior pituitary (Moore and Demarest, 1982; Fuxe *et al.*, 1985). In the rat, release of DA from the tuberoinfundibular neurons into the hypophyseal portal blood system has been reported

(Gibbs and Neil, 1978), and DA released from this pathway regulates hormone release from the anterior pituitary (Moore and Demarest, 1982). Recent release of L-DOPA, the precursor of DA, into the portal blood of the rat has been demonstrated (Telford *et al.*, 1992), and L-DOPA may be subsequently converted into DA at the pituitary level. Moreover, cell bodies immunoreactive to L-DOPA have been identified in the preoptic area of the rat hypothalamus, and do not overlap with the DA neurons from the A12 areas (Mons *et al.*, 1990), suggesting that dopaminergic systems other than the tuberoinfundibular pathway may also participate in the regulation of hormone release from the anterior pituitary.

1.2.2.2 Dopamine regulation of pituitary hormone release

In mammals, DA released from the tuberoinfundibular pathway functions as a prolactin (PRL)-release inhibitor at the pituitary level (for review, see Ben-Jonathan, 1985). DA at nanomolar doses inhibits basal PRL levels (Delbeke *et al.*, 1986) and the PRL responses to thyrotropin-releasing hormone (Hanna and Shin, 1992) and vasoactive intestinal peptide (Lopez *et al.*, 1989). These inhibitory actions of DA are mediated through the D2 receptor subtype (Ben-Jonathan, 1985), activation of which causes a reduction of cAMP synthesis (Onali *et al.*, 1981), lowering of intracellular Ca^{2+} levels (Winiger *et al.*, 1987), and hyperpolarization of the plasma membrane of lactotrophs (Israel *et al.*, 1990) (for a recent review on the mechanisms of DA inhibition on PRL release, see de la Escalera and Weiner, 1992). DA administered at picomolar doses has been reported to stimulate PRL secretion in the rat (Denef *et al.*, 1984) and human (Boesgaard *et al.*, 1990). This stimulatory action has been proposed to be mediated by a DA receptor subtype which is similar and yet distinct from the D2 receptors (Burris *et al.*, 1991).

In contrast to that of PRL, the role of DA on GH regulation in mammals is still controversial. Stimulatory actions of L-DOPA, DA and the DA agonist apomorphine on GH release have been reported in the rat (Sonntag *et al.*, 1982; Meister and Hulting, 1987), dog (Casanueva *et al.*, 1981) and primates, including rhesus monkey (Chambers and Brown, 1976), baboon (Steiner *et al.*, 1978), and human (Vasavan Nair *et al.*, 1986; Delitala *et al.*, 1987; Lal *et al.*, 1991). These results are in agreement with the

previous studies that disruption of the catecholaminergic pathways in the brain by reserpine (Eden *et al.*, 1979) and 6-hydroxydopamine (Willoughby and Day, 1981) suppressed the plasma GH levels in the rat. However, similar L-DOPA and DA treatments in patients with acromegaly were found to be inhibitory to GH secretion (Verde *et al.*, 1976; Bell *et al.*, 1986; Hanew *et al.*, 1987). In some other studies, especially in the rat (Bluet-Pajot *et al.*, 1980) and human (Lindholm *et al.*, 1981; Jordan *et al.*, 1986), GH secretion was not affected by dopaminergic stimulation. The discrepancy amongst these studies can be attributed to the different ways of drug administration, pathological conditions of the animal model, interspecies variations of GH responses, and also, to some extent, a result of the complexity of DA actions on GH regulation.

It is generally accepted that the actions of DA on GH release are exerted at two different levels, directly at the pituitary level and indirectly at the hypothalamic level (for reviews, see Buonomo and Baile, 1990; Devesa *et al.*, 1992). The indirect actions of DA are mediated mainly by modulating the release of SRIF and GHRH from the hypothalamus. In the rat, DA stimulates SRIF release *in vitro* from hypothalamic synaptosomes (Wakabayashi *et al.*, 1977), perifused hypothalamic slices (Terry *et al.*, 1980; Kitajima *et al.*, 1989) and hypothalamic cells in primary cultures (Richardson *et al.*, 1983). These results are consistent with the *in vivo* observations that intraventricular (icv) injection of L-DOPA and DA increases SRIF levels in the hypophyseal portal blood (Chihara *et al.*, 1979) and depletes SRIF contents in the hypothalamus (Torres *et al.*, 1982). The actions of DA on SRIF release are mediated via D2 receptors (Lewis *et al.*, 1986), probably through the epoxygenase pathway of arachidonic acid metabolism (Junier *et al.*, 1990).

In mammals, DA is also stimulatory to GHRH release from the hypothalamus, both *in vivo* (Chihara *et al.*, 1986; Sopwith *et al.*, 1986) and *in vitro* (Kitajima *et al.*, 1989). In the rat, the GHRH-releasing effects of DA are more readily observed following immunoneutralization of SRIF (Kitajima *et al.*, 1989). To date, the DA receptor subtype mediating this GHRH-releasing action is still unknown. More recently, DA and GHRH have been shown to colocalize in hypothalamic neurons projecting into the median eminence of the rat (Niimi *et al.*, 1992). Whether these two neurohormones

are released at the same time during neuronal stimulation is not clear.

The direct effects of DA on GH release at the pituitary level are still open to debate, as contradicting results have been reported. DA is inhibitory to GH release from monolayer cultures of normal human pituitary cells; however, by using pituitary cells from patients with acromegaly, DA either stimulates or has no effects on GH release (Tallo and Malarkey, 1981; for a recent review on GH regulation in acromegaly, see Jaffe and Barkan, 1992). In the rat, stimulatory (Serri *et al.*, 1987; Wood *et al.*, 1987), inhibitory (Cronin *et al.*, 1984) and even no effects (Meister and Hulting, 1987; Chen *et al.*, 1989) of DA treatment on GH release from pituitary cells have been reported. In those studies in which DA has a stimulatory effect on GH release, the involvement of DA D1 receptors has been suggested (Bluet-Pajot *et al.*, 1990). These stimulatory actions of DA do not involve the modulation of GH gene expression (Wood *et al.*, 1987). To add to the confusion, recent autoradiographic studies on the distribution of DA receptors failed to identify the D1-specific binding sites (Mansour *et al.*, 1990) and the mRNA for D1 receptors in the rat pituitary (Meador-Woodruff *et al.*, 1991).

Despite these contradictory results on the direct actions of DA on basal GH release, DA consistently inhibits the GH-releasing effect of GHRH at the pituitary cell level (Meister and Hulting, 1987; Serri *et al.*, 1987; Lindstrom and Ohlsson, 1987; Arce *et al.*, 1991b). This DA action can be blocked by the D2 antagonists domperidone (Giusti *et al.*, 1984) and metoclopramide (Serri *et al.*, 1987; Arce *et al.*, 1991b), indicating that it is mediated by the DA D2 receptors.

Besides GH and PRL, the release of other pituitary hormones, including GTH, adrenocorticotropin (ACTH), thyrotropin (TSH), and melanocyte stimulating hormone (MSH), are also under the influence of hypothalamic dopaminergic pathways (for review, see Thorner and Vance, 1989). The effects of DA on GTH release in mammals are variable, as both stimulatory and inhibitory actions have been reported (for reviews, see Ramirez *et al.*, 1984; Kalra and Kalra, 1985; Weiner *et al.*, 1988). Administration of L-DOPA, DA, and the DA agonist apomorphine stimulates the release of LH and FSH in rodents, including the rat and mouse (for review, see Barraclough and Wise, 1982); however, similar treatments are inhibitory in the ewe (Deaver and Dailey, 1982; Curlewis *et al.*, 1991) and human (Matsubara *et al.*, 1987; Samuels *et al.*, 1992). In the rat, the

stimulatory actions of DA is partially mediated by the release of GnRH. Increase of GnRH levels in the hypophyseal portal blood (Kamberi *et al.*, 1970) and enhancement of GnRH mRNA contents in the hypothalamus (Li and Pelletier, 1992) have been observed in the rat following dopaminergic stimulation. Recently, DA-induced GnRH release has been demonstrated in a GnRH-releasing neuronal cell line from the mouse, and this DA action is mediated through D1 receptors coupled to the cAMP-dependent pathway (de la Escalera *et al.*, 1992).

In mammals, DA and its agonists reduce basal (Agner *et al.*, 1986; Samuels *et al.*, 1992) and TRH-stimulated TSH release (Ignar and Kuhn, 1988), abolish the nocturnal TSH surge (Sowers *et al.*, 1982), inhibit the TSH gene expression (Shupnick *et al.*, 1986; Franklyn *et al.*, 1987) and synthesis of TSH α- and β-subunits (Cooper *et al.*, 1983; Samuels *et al.*, 1992). The release of TSH is also under the inhibition of DA D2 actions (Foord *et al.*, 1984). Moreover, the D2 receptor capacity in thyrotrophs can be up-regulated by TSH stimulation (Foord *et al.*, 1985), suggesting the presence of an ultra-short loop feedback in the control of TSH release.

ACTH and α-MSH are the gene products of pro-opiomelanocortin (POMC) (for review, see Smith and Funder, 1988), and both of them are under the inhibitory control of the tuberohypophyseal DA pathway (Fuxe *et al.*, 1985). DA is known to reduce POMC mRNA contents in the neurointermediate lobe of the rat (Lundblad and Roberts, 1988). It is generally accepted that DA inhibits α-MSH release via the D2 receptors (Cote *et al.*, 1982; Lidy *et al.*, 1986), and this action is mediated through inhibition of the adenylate cyclase-cAMP pathway (Miyazaki *et al.*, 1984; Newman *et al.*, 1985). Recently, α-MSH-induced activation of the tuberoinfundibular DA neurons has been demonstrated in the rat (Lindley *et al.*, 1990). This may be involved in the control of PRL release from the rat pituitary, as α-MSH treatment (ip) is inhibitory to basal and stimulated PRL release *in vivo* (Khorram *et al.*, 1982; Newman *et al.*, 1985).

The release of oxytocin and vasopressin from the posterior pituitary is also under the influence of the tuberohypophyseal DA pathway (Moos *et al.*, 1982; Mason, 1983). DA stimulates and inhibits the release of oxytocin via the D1 and D2 receptors, respectively (Crowley *et al.*, 1991). In the rat, the D1-stimulated oxytocin release is

mediated via the central actions of DA at the paraventricular and supraoptic nuclei of the hypothalamus (Parker and Crowley, 1992); however, the site of action for DA D2 receptors on oxytocin release is still unknown.

1.3 Growth Hormone Release and Its Signal Transduction Pathways

1.3.1 Regulation of Growth Hormone Release

1.3.1.1 GHRH and SRIF as the major GH-regulators from the hypothalamus

In mammals, GH release is mainly regulated by the interplay of two hypothalamic peptides, the stimulatory GHRH and the inhibitory SRIF (for review, see Frohman and Jansson, 1986). In the rat, GHRH is known to stimulate GH release directly from purified somatotrophs (Kraicer *et al.*, 1988; Lussier *et al.*, 1991a), increase the pituitary GH mRNA contents (Gick *et al.*, 1984), and enhance GH gene transcription in cultured pituitary cells (Barinaga *et al.*, 1983; Barinaga *et al.*, 1985). On the contrary, SRIF inhibits GH release without affecting GH synthesis in the pituitary (Gick *et al.*, 1984; Gick and Bancroft, 1987). Similar involvement of GHRH and SRIF in GH regulation has been reported in the dog (Arce *et al.*, 1991a), sheep (Blanchard *et al.*, 1988; Rosenthal *et al.*, 1991), pig (Rosenthal *et al.*, 1991), cattle (Silverman *et al.*, 1988) and primates, including the monkey (Malozowski *et al.*, 1990) and human (Losa *et al.*, 1985; Namba *et al.*, 1989).

GHRH and SRIF are secreted into the hypophyseal portal blood in an asynchronous manner (Plotsky and Vale, 1985), approximately 180° out of phase (Tannenbaum and Ling, 1984). This asynchrony of GHRH and SRIF release is responsible for the observed pulsatility of GH release *in vivo* (for reviews, see Tannenbaum 1990; Tannenbaum *et al.*, 1990a). It is generally accepted that SRIF is secreted during the trough period to maintain the low GH levels (Tannenbaum, 1988; Tannenbaum *et al.*, 1990b), whereas GHRH is released during the peak period to induce the GH surge (Wehrenberg *et al.*, 1982; Lumpkin *et al*, 1989; Lumpkin and McDonald, 1989). This temporal pattern of GHRH and SRIF signalling can be attributed in part to their interactions at the hypothalamus level. GHRH release from the hypothalamus is known to be under the direct inhibition by SRIF (Katakami *et al.*, 1988; Ieiri *et al.*,

1988). In the rat, the primary source of SRIF nerve terminals in the median eminence is from the periventricular nucleus (Johansson *et al.*, 1984), whereas the GHRH nerve fibers are from the arcuate nucleus (Sawchenko *et al.*, 1983). Synaptic contacts between SRIF and GHRH neurons in the hypothalamus have been previously described (Liposits *et al.*, 1988; Horvath *et al.*, 1989). More recently, SRIF receptors have been identified on GHRH neurons in the arcuate nucleus (McCarthy *et al.*, 1992; Bertherat *et al.*, 1992), and these SRIF receptors can be up-regulated by estradiol (Slama *et al.*, 1992). It is likely that this modulatory action of sex steroids on SRIF receptor capacity partially contributes to the sexual dimorphism of GH release observed in the rat (for a review on sexual dimorphism of GH regulation, see Jansson *et al.*, 1985).

On the other hand, the release of SRIF from the hypothalamus is also under the influence of GHRH. GHRH administration (icv) is known to increase SRIF release into the hypophyseal portal blood of the rat (Mitsugi *et al.*, 1990). In the same animal model, similar stimulatory effects of GHRH have also been demonstrated using *in vitro* cultures of median eminence explants (Aguila *et al.*, 1990; Aguila *et al.*, 1991) and dispersed hypothalamic cells (de los Frailes *et al.*, 1992). These stimulatory actions of GHRH are calmodulin-dependent (Aguila and McCann, 1988), and involve phosphatidyl inositol turnover (Aguila *et al.*, 1991) and the phospholipase A_2-arachidonic acid pathways (Aguila *et al.*, 1990). Although the cAMP- and protein kinase C-dependent pathways have been reported to mediate the release of SRIF *in vitro* (Patel *et al.*, 1991), their functional roles in the SRIF response to GHRH stimulation are still not known.

Recently, an asynchronous expression of SRIF mRNA (in the periventricular nucleus) and GHRH mRNA (in the arcuate nucleus) has been reported in the rat (Zeitler *et al.*, 1990a; Zeitler *et al.*, 1991), suggesting that GH pulsatility may be in part regulated by a "transcriptional oscillator" in the hypothalamus. Modulation of GHRH and SRIF mRNA levels by sex steroids has been described in the rat hypothalamus (Zeitler *et al.*, 1990b; Argente *et al.*, 1990; Argente *et al.*, 1991), and it is likely that the operation of this "transcriptional oscillator" is also under the influence of sex steroids.

The reciprocal relationship of GHRH and SRIF signalling at the pituitary level is essential to optimize the responsiveness of somatotrophs to GHRH stimulation. The GH-releasing effect of GHRH is known to undergo rapid desensitization during the

prolonged treatment with GHRH (Wehrenberg *et al.*, 1986; Rittmaster *et al.*, 1987). However, this desensitization response can be reversed, or even prevented, by previous exposure to SRIF (Clayton and Bailey, 1987; Soya and Suzuki, 1988; Soya and Suzuki, 1990). Recently, SRIF pretreatment has been shown to potentiate the GH responses to subsequent GHRH stimulation (Tannenbaum *et al.*, 1989; Richardson and Twente, 1991). This phenomenon may in part contribute to the rebound increase of GH release which is commonly observed after the termination of SRIF treatment (Login and Judd, 1986; Weiss *et al.*, 1987; Rittmaster *et al.*, 1987). In general, it is accepted that the gradual withdrawal of SRIF acts in concert with GHRH stimulation to determine the timing, duration, and magnitude of the GH pulses in circulation (Rittmaster *et al.*, 1987; Kraicer *et al.*, 1988; Stachura *et al.*, 1988). The pulsatile release of GH is essential to maintain the functional levels of hepatic GH receptors (Bass *et al.*, 1991; Bick *et al.*, 1992), and regulate the serum levels of insulin-like growth factors (IGF) and their binding proteins (Isgaard *et al.*, 1988; Bick *et al.*, 1992). The pulsatility of GH release is, therefore, indispensable for the normal functioning of GH to induce body growth (Vasilatos-Younken *et al.*, 1988; Byatt *et al.*, 1991)

1.3.1.2 Other GH-regulators from the hypothalamus

Besides being regulated by GHRH and SRIF, GH release in mammals is also under the influences of (1) neurotransmitters, e.g., norepinephrine (Malozowski *et al.*, 1990; Devesa *et al.*, 1991), dopamine (Bluet-Pajot *et al.*, 1990; Arce *et al.*, 1991b; see previous section on DA), serotonin (Lopez *et al.*, 1986; Willoughby *et al.*, 1987) and acetylcholine (Tamai *et al.*, 1990; Arce *et al.*, 1991a), (2) neuropeptides, e.g., neuropeptide Y (Rettori *et al.*, 1990), endogenous opioid peptides (Kiem *et al.*, 1991), and galanin (Murakami *et al.*, 1989; Tanoh *et al.*, 1991), and (3) excitatory amino acids, e.g., glutamate (Acs *et al.*, 1990; Cocilovo *et al.*, 1992) and γ-aminobutyric acid (GABA) (Acs *et al.*, 1990; Gillies and Davidson, 1992). In most of the cases, the actions of these neuroendocrine factors on GH release are mediated indirectly through the release of GHRH and SRIF from the hypothalamus.

Stimulatory actions of acetylcholine on GH release have been reported in the rat (Torsello *et al.*, 1988), dog (Arce *et al.*, 1991a), and human (Tamai *et al.*, 1990;

Devesa *et al.*, 1991). Evidence accumulated suggests that this stimulatory action of acetylcholine is mediated via a suppression of SRIF release from the hypothalamus (Wehrenberg *et al.*, 1992). The actions of norepinephrine on GH release in mammals are more variable. In the rat, norepinephrine directly stimulates GH release from the pituitary via $\alpha 1$ receptors (Pandiella *et al.*, 1988); however, norepinephrine also acts centrally to inhibit GH release via β receptors, probably by inducing the release of SRIF (Krieg *et al.*, 1988). In humans, the actions of norepinephrine on GH release are dose-related, being inhibitory at nanomolar doses and stimulatory at micromolar concentrations. Low doses of norepinephrine activate SRIF release from the hypothalamus via $\beta 2$ receptors, whereas high doses of norepinephrine induce the opposite effect via $\alpha 2$ receptors (for a recent review on norepinephrine regulation of GH release in humans, see Devesa *et al.*, 1992). Serotonin stimulates GH secretion in the rat (Lopez *et al.*, 1986), and its GH-releasing action is in part mediated by GHRH release from the hypothalamus (Murakami *et al.*, 1986; Willoughby *et al.*, 1987).

The action of neuropeptide Y (NPY) on GH release is mainly inhibitory. In the rat, immunoneutralization of NPY induces a rise in plasma GH levels (Rettori *et al.*, 1990). Results from *in vitro* studies using the same animal model also demonstrated that NPY stimulates SRIF release from the median eminence (Rettori *et al.*, 1990), suggesting that the inhibitory effect of NPY on GH release probably is an indirect action. In addition, colocalization of NPY and GHRH immunoreactivity has been demonstrated in the arcuate nucleus of the rat (Ciofi *et al.*, 1987) and human (Ciofi *et al.*, 1988). These anatomical observations also raise the possibility that GHRH release from the hypothalamus may be under the modulation of NPY.

In mammals, the physiological role of excitatory amino acids in GH regulation is not well understood. In the rat, plasma GH levels can be increased by subcutaneous injection of GABA (Acs *et al.*, 1990). Recently, it has been demonstrated that activation of the $GABA_A$ receptors inhibits SRIF release from the rat hypothalamus neurons in primary cultures (Gillies and Davidson, 1992), indicating that the GH-releasing effect of GABA probably is an indirect action. In the same animal model, high doses of glutamate are known to deplete GHRH contents in the arcuate nucleus (Corder *et al.*, 1990).

Pharmacological blockade of the glutamate receptors, in particular the N-methyl-D-aspartic acid subtype, also elevates GHRH mRNA contents in the hypothalamus, as well as the GH and IGF levels in circulation (Cocilovo *et al.*, 1992).

1.3.2 Signal transduction pathways for GH release

It is generally accepted that the cAMP-dependent pathway is a major cellular mechanism mediating GH release from the somatotrophs (for review, see Frohman and Jansson, 1986). In mammals, especially in the rat, an increase in cAMP synthesis preceding the GH release induced by GHRH stimulation has been reported in mixed populations of pituitary cells (Bilezikjian and Vale, 1983; Cronin *et al.*, 1984), purified somatotrophs (Spence *et al.*, 1980; Sheppard *et al.*, 1985), and GH-secreting tumor cell lines (Reyl-Desmars and Zeytin, 1985). It is also known that elevating the functional levels of cAMP by using (1) the adenylate cyclase activator forskolin (Cronin *et al.*, 1984), (2) the phosphodiesterase inhibitor IBMX (Kraicer and Chow, 1982; Sheppard *et al.*, 1985), and (3) the membrane permeant cAMP analogs, such as 8Br.cAMP (Kraicer and Chow, 1982), are all stimulatory to GH secretion. Moreover, activation of adenylate cyclase (Robberecht *et al.*, 1986; Narayanan *et al.*, 1989) and protein kinase A (both type I and II) as a result of GHRH stimulation (Bilezikjian *et al.*, 1987) has been reported in the rat pituitary. This stimulatory action of GHRH appears to be mediated through a membrane-bound stimulatory G protein (Gs) (Narayanan *et al.*, 1989).

The cAMP-dependent pathway is also involved in the post-receptor mechanisms of SRIF (for review, see Toro *et al.*, 1988). SRIF inhibits the GH release and cAMP accumulation induced by GHRH (Bilezikjian and Vale, 1983; Cronin *et al.*, 1984). The inhibitory action of SRIF on cAMP accumulation is mediated mainly by stimulating the cAMP-degrading enzyme phosphodiesterase (Rendon *et al.*,1988). However, SRIF has no effects on the basal and GHRH-stimulated adenylate cyclase activity (Narayanan *et al.*, 1989). The cAMP-dependent pathway is also involved in the regulation of GH gene expression. This is supported by the findings that a cAMP-responsive element (CRE) is present in the 5'-flanking region of the GH gene (Copp and Samuels, 1989; Dana and Karin, 1989) and Pit-1 gene of the rat (Theill *et al.*, 1989; Kapiloff *et al.*, 1991). Pit-1 (sometimes also referred to as GHF-1) is a transcription factor specific for GH and PRL

gene expression (Mangalam *et al.*, 1989). Recently, involvement of cAMP in the expression of other transcription factors, such as c-*fos* and c-*jun*, has also been reported (Lavergne *et al.*, 1992).

Evidence accumulated also indicates the involvement of Ca^{2+}-dependent pathways in GH secretion. The GH responses to GHRH stimulation *in vitro* are sensitive to the availability of extracellular Ca^{2+} (Spence *et al.*, 1980; Kraicer and Chow, 1982; Lussier *et al.*, 1991a), the presence of Ca^{2+} competitors, such as Co^{2+} (Holl *et al.*, 1988a,b), and the inhibition by voltage-sensititve Ca^{2+} channel (VSCC) blockers, such as nifedipine and verapamil (Drouva *et al.*, 1988; Sheppard *et al.*, 1987). Moreover, the stimulatory actions of GHRH can be mimicked by the dihydropyridine VSCC agonist Bay K8644 (Drouva *et al.*, 1988; Stojilkovic *et al.*, 1988). These findings strongly indicate that Ca^{2+} entry via the VSCC is involved in GHRH-stimulated GH release. With the recent advancements in spectrofluorometry using Ca^{2+}-sensitive probes (for reviews, see Leong, 1989; Gurney, 1990), an increase in intracellular Ca^{2+} levels ($[Ca^{2+}]i$) immediately after GHRH stimulation has been demonstrated in human GH-secreting adenoma cells (Dufy-Barbe *et al.*, 1992), purified rat somatotrophs (Lussier *et al.*, 1991a,b,c), and rat somatotrophs identified by the reverse hemolytic plaque assay (Holl *et al.*, 1988a,b). This increase in $[Ca^{2+}]i$ is localized in the subplasmalemmal region of individual somatotrophs (Kato *et al.*, 1992), and can be blocked by the VSCC blocker nifedipine (Lussier *et al.*, 1991a,c) and the GH-release inhibitor SRIF (Holl *et al.*, 1988a,b; Lussier *et al.*, 1991b). Recently, oscillations of cytosolic free Ca^{2+} in the regulation of pituitary hormone release have been receiving a lot of attention (for a recent review, see Stojilkovic and Catt, 1992). It is generally accepted that a part of the intracellular signals mediating GHRH and SRIF regulation of GH release is encoded in the frequency and amplitude of these $[Ca^{2+}]i$ transients, which subsequently control the process of GH exocytosis (Holl *et al.*, 1988a,b; Ohlsson and Lindstrom, 1990; Tornquist and Tashjian, 1991; Cuttler *et al.*, 1992).

In mammals, the involvement of phosphatidylinositol-protein kinase C (PKC) pathway in the mediation of GH release has also been suggested. This is supported by the *in vitro* findings that activation of PKC by phorbol esters and diacylglycerol analogs stimulates GH release from primary cultures of rat pituitary cells (Negro-Vilar and

Lapetina, 1985). However, the physiological role of PKC in GHRH-stimulated GH release has been questioned. Using purified somatotrophs of the rat, previous exposure to the phorbol ester TPA has no effects on the GH responses to subsequent GHRH stimulation (French *et al.*, 1989), although a similar TPA pretreatment is known to down-regulate PKC activity in the rat pituitary cells (McArdle and Conn, 1989). Translocation of PKC from the cytosolic to the membrane fraction has been used as an index for PKC activation (for review, see Huang, 1989). Similar PKC translocation is observed in the rat somatotrophs after stimulation with phorbol esters and diacylglycerol analogs; in contrast, GHRH is not effective in this respect (French *et al.*, 1991). These results indicate that PKC is not a second messenger for GHRH-stimulated GH release. Probably, PKC is involved in the GH-releasing actions of another neuroendocrine factor(s).

Involvement of the phosphatidylinositol system in GHRH-stimulated GH release is still controversial. Using mixed populations of rat pituitary cells, it has been shown that GHRH either stimulates (Canonico *et al.*, 1983) or has no effects (Raymond *et al.*, 1984; Escobar *et al.*, 1986) on the metabolism of phosphatidylinositol bisphosphate (PIP2). To add to the confusion, a recent report using purified somatotrophs of the rat has demonstrated that GHRH inhibits the synthesis of inositol trisphosphate (IP3) and bisphosphate (IP2) (French *et al.*, 1990). The cause of these variations may be partly due to the differential use of lithium ion in their respective assay systems. Lithium ion is known to inhibit the degradation of IP metabolites and is essential for the accurate measurment of PIP2 turnover (for a recent review, see Nahorski *et al.*, 1991).

In contrast to GHRH, the involvement of the phosphatidylinositol system in the signal transduction pathway of thyrotropin-releasing hormone (TRH) is well documented. In mammals, TRH is known to stimulate GH release from various GH-secreting tumor cell lines, including human adenomas (Simard *et al.*, 1988), GH3 cells (Boc ckfor *et al.*, 1985; Boockfor and Schwartz, 1988) and GH4C1 cells (Aizawa and Hinkle, 1985) (for a review on the role of TRH as a GH-releasing factor, see Harvey, 1990). TRH receptors are positively coupled to phospholipase C through a stimulatory G protein (Gs) (Aub *et al.*, 1986), activation of which stimulates the production of IP3, IP2 and IP1 (Smallridge *et al.*, 1992). Recently, arachidonate release as a response to

TRH stimulation has also been demonstrated in primary cultures of rat pituitary cells (Judd and MacLeod, 1992), suggesting that arachidonic acid and its metabolites may be a part of the post-receptor mechanisms mediating TRH-stimulated GH release.

1.4 Growth Hormone Regulation in Teleosts

Similar to mammals, GH release in teleosts is under the control of multiple neuroendocrine factors from the hypothalamus (for review, see Nishioka *et al.*, 1988). Immunoreactivity of GHRH has been demonstrated in the hypothalamic-pituitary axis of the cod (Pan *et al.*, 1985), sea bass (Marivoet *et al.*, 1988; Moons *et al.*, 1988), rainbow trout (Luo and McKeown, 1989a), chum salmon and coho salmon (Parker and Sherwood, 1990). In the goldfish, the GH-releasing actions of a human pancreatic GHRH have been previously described (Peter *et al.*, 1984). Recently, a GHRH-like peptide has been isolated from the common carp (Vaughan *et al.*, 1993), and this carp GHRH stimulates GH release from pituitary cells of the goldfish (Peng *et al.*, 1990; Vaughan *et al.*, 1992) and rainbow trout (Luo and McKeown, 1989b; Luo *et al.*, 1990). These results strongly suggest that GHRH is involved in GH regulation in teleost fishes.

Besides GHRH, other neuropeptides are also known to have GH-releasing activity in teleosts. GnRH is a hypothalamic releasing factor well known for its action to stimulate GTH release in fishes (for review, see Peter *et al.*, 1986). In the goldfish, two forms of GnRH, salmon GnRH (sGnRH) and chicken-II GnRH (cGnRH-II), have been identified (Yu *et al.*, 1987; Yu *et al.*, 1988). Both of them stimulate GH release from goldfish pituitary fragments (Marchant *et al.*, 1989b; Habibi *et al.*, 1992) and pituitary cells (Chang *et al.*, 1990; Jobin and Chang, 1992). In general, sGnRH is more potent than cGnRH-II in stimulating GH release (Habibi *et al.*, 1992). The GH-releasing actions of these two GnRHs are Ca^{2+}- (Chang and de Leeuw, 1990; Jobin and Chang, 1992) and PKC-dependent (Chang *et al.*, 1991), but do not involve cAMP as a second messenger (Chang *et al.*, 1992; for a recent review on second messengers for GnRH actions in the goldfish, see Chang *et al.*, 1993). Furthermore, GnRH receptors have been recently demonstrated on the somatotrophs of goldfish (Cook *et al.*, 1991).

Neuropeptide Y (NPY) is highly conserved during vertebrate evolution (Larhammar *et al.*, 1993), and has been identified in the brain of teleosts (for review, see Danger *et al.*, 1990). In the goldfish, this peptide is colocalized with SRIF in neurons within the hypothalamus (Pickavance *et al.*, 1992) and directly innervates both the somatotrophs and gonadotrophs in the anterior pituitiary (Pontet *et al.*, 1989). More recently, GH-releasing actions of NPY have been demonstrated in the goldfish using *in vitro* perifusion of pituitary fragments (Peng *et al.*, 1990) and pituitary cells (Peng *et al.*, 1993). In addition to its direct effects at the pituitary cell level, NPY also exerts its stimulatory actions on GH release indirectly by inducing GnRH release from the hypothalamus via Y_2 receptors (Peng *et al.*, 1993).

The tripeptide TRH is also known to be a GH-releasing factor in vertebrates, especially in birds (for review, see Harvey, 1990). However, very little is known in teleosts concerning its physiological role in GH regulation. In the goldfish, TRH injected intraperitoneally (ip) elevates serum GH levels (Cook and Peter, 1984). The GH-releasing actions of TRH have been recently confirmed by *in vitro* studies using perifused goldfish pituitary fragments (Trudeau *et al.*, 1992). Furthermore, the goldfish pituitary appears to be more sensitive to TRH stimulation when the fish are sexually mature (i.e., in the prespawning stages) than when the fish are sexually regressed (Trudeau *et al.*, 1992). Recently, stimulatory effects of TRH on GH release have also been reported in the common carp (Lin *et al.*, 1993).

Similar to GHRH, the GH-release inhibitor SRIF is also widely distributed among the teleosts. SRIF has been identified in the anglerfish (Hobert *et al.*, 1980), catfish (Oyama *et al.*, 1980), coho salmon (Plisetskaya *et al.*, 1986; Nozaki *et al.*, 1988) and rainbow trout (Nozaki *et al.*, 1988). Its inhibitory actions on GH release have been previously described in the tilapia (Rivas *et al.*, 1986; Helms *et al.*, 1987), coho salmon (Sweeting and MacKeown, 1986), and goldfish (Cook and Peter, 1984; Marchant *et al.*, 1987). In the goldfish, cell bodies of SRIF neurons are located in the periventricular nucleus of the hypothalamus (Kah *et al.*, 1982), and nerve terminals of SRIF fibers are frequently identified close to the somatotrophs (Olivereau *et al.*, 1984). These anatomical observations are in agreement with the *in vivo* findings that hypothalamic lesions in the periventricular area significantly elevate serum GH levels in the goldfish

(Fryer, 1981; Cook and Peter, 1983).

Seasonal changes in SRIF levels have also been reported in the goldfish; the hypothalamic and pituitary SRIF contents are the highest during gonadal regression, and the lowest during the recrudescence and prespawning stages (Marchant *et al.*, 1989a). The seasonality of SRIF is opposite to the seasonal changes of serum GH levels in the goldfish, in which the highest GH levels are observed during the spawning and post-spawning season (Marchant and Peter, 1986). In the goldfish, SRIF inhibits basal GH levels (Marchant *et al.*, 1987) and the GH responses to various GH-releasing factors, including sGnRH (Marchant *et al.*, 1989b), NPY (Peng *et al.*, 1993), TRH (Cook and Peter, 1984), and carp GHRH (Peng *et al.*, 1990).

In addition to neuropeptides, neurotransmitters are also involved in GH regulation in teleosts. In the goldfish, administration of DA (icv) and the DA agonist apomorphine (ip) has been shown to increase serum GH levels (Chang *et al.*, 1985). This stimulatory effect of DA appears to act directly at the pituitary level, as DA can stimulate GH release from perifused goldfish pituitary fragments (Peter *et al.*, 1990). In contrast to the stimulatory actions of DA, norepinephrine (Chang *et al.*, 1985) and serotonin (Somoza and Peter, 1991) are inhibitory to GH release in the goldfish.

Involvement of sex steroids in GH regulation has been suggested in the goldfish. This is supported by the findings that the seasonal changes in serum GH levels are closely associated with the reproductive cycle (see above). Moreover, a preovulatory GH surge, which occurs simultaneously with the GTH surge, is observed in both male and female goldfish during spontaneous spawning (Yu *et al.*, 1991). In addition, in female goldfish, serum estradiol and testosterone levels are known to increase prior to the preovulatory GTH surge (Kobayashi *et al.*, 1986; Kobayashi *et al.*, 1987). Recently, estradiol implantation in the goldfish has been shown to increase basal GH levels and potentiate the GH responses to sGnRH and TRH; however, testosterone implantation is not effective in these respects (Trudeau *et al.*, 1992). These findings indicate that sex steroids, such as estradiol, can exert their actions on GH release indirectly by modulating the responsiveness of pituitary cells to GH-releasing factors. Although immunoreactivity of inhibin βA and βB subunits has been recently identified in the ovary of goldfish (Ge *et al.*, 1993), the physiological role of these gonadal peptides in GH regulation in

teleosts is still unknown.

1.5 Objective of the Research Project

The objective of the present study is to investigate the functional role of DA as a GH-releasing factor in the goldfish and the mechanisms by which it stimulates GH release from goldfish pituitary cells. The goldfish (*Carassius auratus*) was chosen as the animal model for the following reasons : Firstly, the goldfish is a representative of the family Cyprinidae (i.e. the carp family). Cyprinids are fresh water fishes having a high commerical value in Asian countries, especially in China, Taiwan, India, and Malaysia (Rainboth, 1991). A better understanding of GH regulation in the goldfish may have important implications in the farming of cyprinid species. Secondly, the goldfish is an example of the teleost fishes. Teleosts are unique among the vertebrates in that they lack a functional hypothalamo-hypophyseal portal blood system. In contrast to that of the mammals, the anterior pituitary of teleost fishes is under direct innervation from the hypothalamus (for review, see Peter *et al.*, 1990). Thirdly, the goldfish is also a well-studied species among the teleosts. The background information available on the physiology and endocrinology of the goldfish greatly facilitates the present research.

This thesis is composed of 9 chapters, summarizing the results of my research in the past four years. The first chapter provides the background information for the present study. In chapter 2, pharmacological evidence demonstrating the involvement of D1 receptors in the GH-releasing actions of DA at the pituitary level is presented. This is a novel finding as DA D1 receptors have not been identified in the pituitary of vertebrates, and yet our studies indicate that this DA receptor subtype is present in the pituitary of the goldfish. In chapter 3, the physiological role of this DA D1 action on GH release was investigated using different approaches, in particular by (1) the demonstration of a seasonality in DA-stimulated GH release, (2) the body growth-promoting effects of the DA D1 actions, and (3) the ability of a DA D1 antagonist to inhibit serum GH levels *in vivo*. In chapter 4, the physiological role of DA as a GH-releasing factor was further substantiated by investigating its interactions with other known GH regulators in the goldfish, including sGnRH, SRIF, and possible influences from the gonad (data for the interactions of DA D1 actions with serotonin and norepinephrine on GH release are

presented in Appendix II). The results from chapters 3 and 4 provided substantial evidence to support the idea that DA D1-stimulated GH release is an integral part of the complex GH regulatory system in the goldfish.

In chapter 5, by using a newly developed DA D1 radioreceptor assay, the D1 receptors mediating the GH-responses to DA were characterized using goldfish pituitary cells. This study represents the first direct demonstration of D1 receptors in the pituitary of vertebrates. To date, the post-receptor mechanisms of DA D1 actions are virtually unknown in the non-mammalian species. Therefore, the last three data chapters are devoted to an examination of the signal transduction pathways involved in DA D1-stimulated GH release in the goldfish. In chapter 6, *in vitro* evidence is presented to support the involvement of the adenylate cyclase-cAMP-PKA pathway in the GH responses to DA stimulation. The presence of a Ca^{2+}-dependent component, in particular the entry of extracellular Ca^{2+} through VSCC, is described in chapter 7. Finally, *in vitro* data demonstrating the absence of PKC involvement in DA D1-actions, and the independence of cAMP and PKC pathways on GH release are included in chapter 8.

In the last chapter, the major findings of the present study are summarized into models describing (1) the functional role of DA as a GH-releasing factor in the goldfish, and (2) the post-receptor mechanism by which DA stimulates GH release from goldfish pituitary cells. In addition, the limitations, implications, and future directions of the current studies have also been addressed.

1.6 References

Acs Z, Lonart G, Makara GB 1990 Role of hypothalamic factors (growth-hormone-releasing hormone and gamma-aminobutyric acid) in the regulation of growth hormone secretion in the neonatal and adult rat. Neuroendocrinology 52:156-160

Agner T, Hagen C, Andersen AN, Djursing H 1986 Increased dopaminergic activity inhibits basal and metoclopramide-stimulated prolactin and thyrotropin secretion. J Clin Endocrinol Metab 62:778-782

Aguila MC, McCann SM 1988 Calmodulin dependence of somatostatin release stimulated by growth hormone-releasing factor. Endocrinology 123:305-309

Aguila MC, Milenkovic L, McCann SM, Snyder GD 1990 Role of arachidonic acid or its metabolites in growth hormone-releasing factor-induced release of somatostatin from the median eminence. Neuroendocrinology 52:238-242

Aguila MC, Snyder GD, McCann SM 1991 Rat growth hormone-releasing factor stimulates cyclic GMP formation and phosphatidylinositol metabolism in the median eminence. Life Sci 49:67-74

Aizawa T, Hinkle PM 1985 Thyrotropin-releasing hormone rapidly stimulates a biphasic secretion of prolactin and growth hormone in GH4C1 rat pituitary tumor cells. Endocrinology 116:73-82

Andersen PH, Gingrich JA, Bates MD, Dearry A, Falardeau P, Senogles SE, Caron MG 1990 Dopamine receptor subtypes : Beyond the D1/D2 classification. Trends Pharmacol Sci 11:231-236

Arce VM, Cella SG, Locatelli V, Muller EE 1991a Studies of growth hormone secretion in calorically restricted dogs: effect of cholinergic agonists and antagonists, glucose and thyrotropin-releasing hormone. Neuroendocrinology 53:467-472

Arce V, Lima L, Lois N, Rodriguez A, Diaz MJ, Tresguerres JAF, Devesa J 1991b Role of central dopaminergic pathways in the neural control of growth hormone secretion in normal men : studies with metoclopramide. Neuroendocrinology 53:143-149

Argente J, Chowen-Breed JA, Steiner RA, Clifton DK 1990 Somatostatin messenger RNA in hypothalamic neurons is increased by testosterone through activation of

androgen receptors and not by aromatization to estradiol. Neuroendocrinology 52:342-349

Argente J, Chowen JA, Zeitler P, Clifton DK, Steiner RA 1991 Sexual dimorphism of growth hormone-releasing hormone and somatostatin gene expression in the hypothalamus of the rat during development. Endocrinology 128:2369-2375

Aub DL, Frey EA, Sekura RD, Cote TE 1986 Coupling of the thyrotropin-releasing hormone receptor to phospholipase C by a GTP-binding protein distinct from the inhibitory or stimulatory GTP-binding protein. J Biol Chem 261:9333-9340

Bass JJ, Oldham JM, Hodgkinson SC, Fowke PJ, Sauerwein H, Molan P, Breier BH, Gluckman PD 1991 Influence of nutrition and bovine growth hormone (GH) on hepatic GH binding, insulin-like growth factor-I and growth of lambs. J Endocrinol 128:181-186

Barinaga M, Bilezikjian LM, Vale WW, Rosenfeld MG, Evans RM 1985 Independent effects of growth-hormone releasing factor on growth hormone release and gene transcription. Nature 314:279-281

Barinaga M, Yamonoto G, Rivier C, Vale W, Evans R, Rosenfeld MG 1983 Transcriptional regulation of growth hormone gene expression by growth hormone-releasing factor. Nature 306:84-85

Barnard R, Ng KW, Martin TJ, Waters MJ 1991 Growth hormone (GH) receptors in clonal osteoblast-like cells mediate a mitogenic response to GH. Endocrinology 128:1459-1464

Barraclough CA, Wise PM 1982 The role of catecholamines in the regulation of pituitary luteinizing hormone and follicle-stimulating hormone secretion. Endocr Rev 3:91-119

Bates MD, Caron MG, Raymond JR 1991 Desensitization of DA1 dopamine receptors coupled to adenylyl cyclase in opossum kidney cells. Am J Physiol 260:F937-F945

Battaglia G, Norman AB, Hess EJ, Creese I 1985 D2 dopamine receptor-mediated inhibition of forskolin-stimulated adenylate cyclase activity in rat striatum. Neurosci Let 59:177-182

Battaglia G, Norman AB, Hess EJ, Creese I 1986a Functional recovery of D1

dopamine receptor-mediated stimulation of rat striatal adenylate cyclase activity following irreversible receptor modification by N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) : Evidence for spare receptors. Neurosci Let 69:290-295

Battaglia G, Norman AB, Hess EJ, Creese I 1986b Forskolin potentiates the stimulation of rat striatal adenylate cyclase mediated by D1 dopamine receptors, guanine nucleotides, and sodium fluoride. J Neurochem 46:1180-1185

Batten TFC, Ingleton PM 1987 The structure and function of the hypothalamus and pituitary gland. In : Chester-Jones I, Ingleton PM, Phillip JG (eds) Fundamentals of Comparative Vertebrate Endocrinology. Plenum Press, NY, pp 285-409

Bell PM, Atkinson AB, Hadden DR 1986 Bromocriptine reduces growth hormone in acromegaly. Arch Intern Med 146:1145-1149

Ben-Jonathan N 1985 Dopamine: A prolactin-inhibiting hormone. Endocr Rev 6:564-589

Bertherat J, Dournaud P, Berod A, Normand E, Bloch B, Rostene W, Kordon C, Epelbaum J 1992 Growth hormone-releasing hormone synthesizing neurons are a subpopulation of somatostatin receptor-labelled cells in the rat arcuate nucleus: a combined *in situ* hybridization and receptor light-microscopic radioautographic study. Neuroendocrinology 56:25-31

Bick T, Hochberg Z, Amit T, Isaksson OGP, Jansson JO 1992 Roles of pulsatility and continuity of growth hormone (GH) administration in the regulation of hepatic GH-receptors, and circulating GH-binding protein and insulin-like growth factor-I. Endocrinology 131:423-429

Bigornia L, Allen CN, Jan CR, Lyon RA, Titeler M, Schneider AS 1990 D2 dopamine receptors modulate calcium channel currents and catecholamine secretion in bovine adrenal chromaffin cells. J Pharmacol Exp Ther 252:586-592

Bilezikjian LM, Erlichman J, Fleisher N, Vale WW 1987 Differential activation of type I and type II 3',5'-cyclic adenosine monophosphate-dependent protein kinases by growth hormone-releasing factor. Mol Endocrinol 1:137-146

Bilezikjian LM, Vale WW 1983 Stimulation of adenosine 3',5'-monophosphate production by growth hormone-releasing factor and its inhibition by somatostatin in

anterior pituitary cells *in vitro.* Endocrinology 113:1726-1731

Blanchard MM, Goodyer CG, Charrier J, and Barenton B 1988 *In vitro* regulation of growth hormone (GH) release from ovine pituitary cells during fetal and neonatal development : Effects of GH-releasing factor, somatostatin, and insulin-like growth factor I. Endocrinology 122:2114-2120

Bluet-Pajot MT, Mounier F, Durand D, Kordon C 1990 Involvement of dopamine D1 receptors in the control of growth hormone in the rat. J Endocrinol 127:191-196

Bluet-Pajot MT, Schaub C, Mounier F, Segalen A, Duhault J, Kordon C 1980 Monoaminergic regulation of growth hormone in the rat. J Endocrinol. 86:387-396

Boesgaard S, Hagen C, Andersen AN, Fenger M, Eldrup E 1990 Pulsatile gonadotropin secretion and basal prolactin levels during dopamine D-1 receptor stimulation in normal women. Fertil Steril 55:281-286

Boockfor FR, Hoeffler, Frawley LS 1985 Cultures of GH3 cells are functionally homogeneous. Thyrotropin-releasing hormone, estradiol and cortisol cause reciprocal shifts in the proportion of growth hormone and prolactin secretors. Endocrinology 117:418-420

Boockfor FR, Schwartz LK 1988 Cultures of GH3 cells contain both single and dual hormone secretors. Endocrinology 122:762-764

Borgundvaag B, George SR 1985 Dopamine inhibition of anterior pituitary adenylate cyclase is mediated through the high-affinity state of the D2 receptor. Life Sci 37:379-386

Bunzow JR, Van Tol HHM, Grandy D, Albert P, Salon J, Christie M, Machida C, Neve K, Civelli O 1988 Cloning and expression of a rat D2 dopamine receptor cDNA. Nature 336:783-787

Buonomo FC, Baile CA 1990 The neurophysiological regulation of growth hormone secretion. Domest Anim Endocrinol 7:435-450

Burris TP, Stringer LC Freeman ME 1991 Pharmacologic evidence that a D2 receptor subtype mediates dopaminergic stimulation of prolactin secretion from the anterior pituitary gland. Neuroendocrinology 54:175-183

Byatt JC, Staten NR, Schmuke JJ, Buonomo FC, Galosy SS, Curran DF, Krivi GG, Collier RJ 1991 Stimulation of body weight gain of the mature female rat by

bovine GH and bovine placental lactogen. J Endocrinol 130:11-19

Canonico PL 1989 D-2 dopamine receptor activation reduces free [^{3}H]arachidonate release induced by hypophysiotropic peptides in anterior pituitary cells. Endocrinology 125:1180-1186

Canonico PL, Cronin MJ, Thorner MO, MacLeod RM 1983 Human pancreatic GRF stimulates phosphatidylinositol labeling in cultured anterior pituitary cells. Am J Physiol 245:E587-E590

Carlsson A 1988 The current status of the dopamine hypothesis of schizophrenia. Neuropsychopharmacology 1:179-191

Casanueva F, Betti R, Cocchi D, Zanardi P, Motta T, Muller EE 1981 A role for dopamine in growth hormone regulation in the dog. Endocrinology 108:1469-1475

Chambers JW, Brown GM 1976 Neurotransmitter regulation of growth hormone and ACTH in the rhesus monkey: Effects of biogenic amines. Endocrinology 98:420-428

Chang JP, de Leeuw R 1990 *In vitro* goldfish growth hormone responses to gonadotropin-releasing hormone. Possible roles of extracellular calcium and arachidonic acid metabolism ? Gen Comp Endocrinol 80:155-164

Chang JP, Jobin RM, de Leeuw R 1991 Possible involvement of protein kinase C in gonadotropin and growth hormone release from dispersed goldfish pituitary cells. Gen Comp Endocrinol 81:447-463

Chang JP, Jobin RM, Wong AOL 1993 Intracellular mechanisms mediating gonadotropin and growth hormone release in the goldfish, *Carassius auratus.* Fish Physiol Biochem (in press)

Chang JP, MacKenzie DS, Gould DR, Peter RE 1984 Effects of dopamine and norepinephrine on *in vitro* spontaneous and gonadotropin hormone-releasing hormone-induced gonadotropin release by dispersed cells or fragments of the goldfish pituitary. Life Sci 35:2027-2033

Chang JP, Marchant TA, [illegible]ok AF, Nahorniak CS, Peter RE 1985 Influences of catecholamines on growth hormone release in female goldfish, *Carassius auratus.* Neuroendocrinology 40:463-470

Chang JP, Peter RE 1983 Effects of dopamine on gonadotropin release in female goldfish, *Carassius auratus*. Neuroendocrinology 36:351-357

Chatelain PG, Sanchez P, Saez JM 1991 Growth hormone and insulin-like growth factor I treatment increase testicular luteinizing hormone receptors and steroidogenic responsiveness of growth hormone deficient dwarf mice. Endocrinology 128:1857-1862

Chen C, Israel JM, Vincent JD 1989 Electrophysiological responses to somatostatin of rat hypophysial cells in somatotroph-enriched primary culture. J Physiol 408:493-510

Chihara K, Arimura A, Schally AV 1979 Effect of intraventricular injection of dopamine, norepinephrine, acetylcholine, and 5-hydroxytryptamine on immunoreactive somatostatin release into rat hypophyseal portal blood. Endocrinology 104:1656-1662

Chihara K, Kashio Y, Kita T, Okimura Y, Kaji H, Abe H, Fujita T 1986 L-Dopa stimulates release of hypothalamic growth hormone-releasing hormone in humans. J Clin Endocrinol Metab 62:466-473

Ciofi P, Croix D, Tramu G 1987 Coexistence of hGHRF and NPY immunoreactivities in neurons of the arcuate nucleus of the rat. Neuroendocrinology 45:425-428

Ciofi P, Croix D, Tramu G 1988 Coexistence of hGHRF and NPY immunoreactivities in neurons of the infundibular area of human brain. Neuroendocrinology 47:469-472

Civelli O, Bunzow JR, Crandy DK, Zhou QY, Van Tol HHM 1991 Molecular biology of the dopamine receptors. Eur J Pharmacol 207:277-286

Clayton RN, Bailey LC 1987 Somatostatin partially reverses desensitization of somatotrophs induced by growth hormone-releasing factor. J Endocrinol 112:69-76

Cocilovo L, Colonna VDG, Zoli M, Biagini G, Settembrini BP, Muller EE, Cocchi D 1992 Central mechanisms subserving the impaired growth hormone secretion induced by persistent blockade of NMDA receptors in immature male rat. Neuroendocrinology 55:416-421

Cook H, Berkenbosch JW, Fernhout MJ, Yu KL, Peter RE, Chang JP, Rivier JE 1991

Demonstration of gonadotropin-releasing hormone receptors on gonadotrophs and somatotrophs of the goldfish : An electron microscope study. Regul Pept 36:369-378

Cook AL, Peter RE 1983 Effects of hypothalamic lesions on serum growth hormone levels and growth rates in goldfish, *Carassius auratus.* Gen Comp Endocrinol 51:175-182

Cook AF, Peter RE 1984 The effects of somatostatin on serum growth hormone levels in the goldfish, *Carassius auratus.* Gen Comp Endocrinol 54:109-113

Cooper DS, Klibanski A, Ridgway EC 1983 Dopaminergic modulation of TSH and its subunits : *In vivo* and *in vitro* studies. Clin Endocrinol (Oxf) 18:265-276

Copp RP, Samuels HH 1989 Identification of an adenosine 3',5'-monophosphate (cAMP)-responsive region in the rat growth hormone gene : Evidence for independent and synergistic effects of cAMP and thyroid hormone on gene expression. Mol Endocrinol 3:790-796

Corder R, Saudan P, Mazlan M, McLean C, Gaillard RC 1990 Depletion of hypothalamic growth hormone-releasing hormone by neonatal monosodium glutamate treatment reveals an inhibitory effect of betamethasone on growth hormone secretion in adult rats. Neuroendocrinology 51:85-92

Cote TE, Grewe CW, Tsuruta K, Stoof JC, Eskay RL, Kebabian 1982 D-2 dopamine receptor-mediated inhibition of adenylate cyclase activity in the intermediate lobe of the rat pituitary gland requires guanosine 5'-triphosphate. Endocrinology 110:812819

Cronin MJ, Hewlett EL, Evans WS, Thorner MO, Rogol AD 1984 Human pancreatic tumor growth hormone (GH)-releasing factor and cyclic adenosine 3',5'-monophosphate evoke GH release from anterior pituitary cells : The effects of pertussis toxin, cholera toxin, forskolin, and cycloheximide. Endocrinology 114:904-913

Cronin MJ, Thorner MO, Hellmann P, Rogol AD 1984 Bromocriptine inhibits growth hormone release from rat pituitary cells in primary culture. Proc Soc Exp Biol Med 175:191-195

Crowley WR, Parker SL, Armstrong WE, Wang W, Grosvenor CE 1991 Excitatory

and inhibitory dopaminergic regulation of oxytocin secretion in the lactating rat: evidence for respective mediation by D-1 and D-2 dopamine receptor subtypes. Neuroendocrinology 53:493-502

Curlewis JD, Naylor AM, McNeilly AS 1991 Evaluation of a possible role for the dopamine D1 and D2 receptors in the steroid-dependent suppression of luteinizing hormone secretion in the seasonally anoestrous ewe. J Neuroendocrinol 3:387-391

Cuttler L, Glaum SR, Coolins BA, Miller RJ 1992 Calcium signalling in single growth hormone-releasing factor-responsive pituitary cells. Endocrinology 130:945-953

Dal Toso R, Sommer B, Ewert M, Herb A, Prichett DB, Bach A, Shivers BD, Seeburg PH 1989 The dopamine D2 receptor : Two molecular forms generated by alternative splicing. EMBO J 8:4025-4034

Dana S, Karin M 1989 Induction of human growth hormone promoter activity by the adenosine 3',5'-monophosphate pathway involves a novel responsive element. Mol Endocrinol 3:815-821

Danger JM, Tonon MC, Jenks BG, Sain-Pierre S, Martel JC, Fasolo A, Breton B, Quirion R, Pelletier G, Vaudry H 1990 Neuropeptide Y : Localization in the central nervous system and neuroendocrine functions. Fundam Clin Pharmacol 23:307-340

de la Escalera GM, Gallo F, Choi ALH, Weiner RI 1992 Dopaminergic regulation of the GT1 gonadotropin-releasing hormone (GnRH) neuronal cell lines: stimulation of GnRH release via D1-receptors positively coupled to adenylate cyclase. Endocrinology 131:2965-2971

de la Escalera GM, Weiner RI 1992 Dissociation of dopamine from its receptor as a signal in the pleiotropic hypothalamic regulation of prolactin secretion. Endocr Rev 241-255

de Leeuw R, Goos HJT, Van Oordt PGWJ 1986 The dopaminergic inhibition of the gonadotropin-releasing hormone-induced gonadotropin release: an *in vitro* study with fragments and cell suspension from pituitaries of the African catfish, *Clarias gariepinus* (Burchell). Gen Comp Endocrinol 63:171-177

de los Frailes MT, Cacicedo L, Fernandez G, Tolon RM, Lorenzo MJ, Aguado F, Franco FS 1992 Role of locally produced growth hormone-releasing factor in

somatostatin regulation by fetal rat brain cells in culture. Neuroendocrinology 55:221-229

Deaver DR, Dailey RA 1982 Effects of dopamine, norepinephrine and serotonin on plasma concentrations of luteinizing hormone and prolactin in ovariectomized and anestrous ewes. Biol Reprod 27:624-632

Delbeke D, Scammell JG, Martinez-Campos A, Dannies PS 1986 Dopamine inhibits prolactin release when cyclic adenosine 3',5'-monophosphate levels are elevated. Endocrinology 118:1271-1277

Delitala G, Palermo M, Ross R, Coy D, Besser M, Grossman A 1987 Dopaminergic and cholinergic influences on the growth hormone response to growth hormone-releasing hormone in man. Neuroendocrinology 45:243-247

Denef C, Baes M, Schramme C 1984 Stimulation of prolactin secretion after short term or pulsatile exposure to dopamine in superfused anterior pituitary cell aggregates. Endocrinology 114:1371-1378

Devesa J, Diaz MJ, Tresguerres JAF, Arce V, Lima L 1991 Evidence that α2-adrenergic pathways play a major role in growth hormone (GH) neuroregulation : α2-adrenergic agonism counteracts the inhibitory effect of muscarinic cholinergic receptor blockade on the GH response to GH-releasing hormone, while α2-adrenergic blockade diminishes the potentiating effect of increased cholinergic tone on such stimulation in normal men. J Clin Endocrinol Metab 73:251-256

Devesa J, Lima L, Tresguerres JAF 1992 Neuroendocrine control of growth hormone secretion in humans. Trends Endocrinol Metab 3:175-183

Drouva SV, Rerat E, Bihoreau C, Laplante E, Rasolonjanahary R, Clauser H, Kordon C 1988 Dihydropyridine-sensitive calcium channel activity related to prolactin, growth hormone, and luteinizing hormone release from anterior pituitary cells in cultures : Interactions with somatostatin, dopamine, and estrogens. Endocrinology 123:2762-2773

Dufy-Barbe L, Bresson L, Sartor P, Odessa MF, Dufy B 1992 Calcium homeostasis in growth hormone (GH)-secreting adenoma cells : Effect of GH-releasing factor. Endocrinology 131:1436-1444

Eden S, Bolle P, Modigh K 1979 Monaminergic control of episodic growth hormone secretion in the rat : Effects of reserpine, α-methyl-*p*-tyrosine, *p*-chlorophenyl alanine, and haloperidol. Endocrinology 105:523-529

Elazar Z, Siegel G, Fuchs S 1989 Association of two pertussis toxin-sensitive G-proteins with the D2-dopamine receptor from bovine striatum. EMBO J 8:2353-2357

Enjalbert A, Bockaert J 1983 Pharmacological characterization of the D2 dopamine receptor negatively coupled with adenylate cyclase in rat anterior pituitary. Mol Pharmacol 23:263-264

Escobar DC, Vicentini LM, Ghigo E, Ciccarelli E, Uselini L, Capella C, Cocchi D 1986 Growth hormone-releasing factor does not stimulate phosphoinositides breakdown in primary cultures of rat and human pituitary cells. Acta Endocrinol 112:345-350

Felder CC, Blecher M, Jose PA 1989 Dopamine-1-mediated stimulation of phospholipase C activity in rat renal cortical membranes. J Biol Chem 264:8739-8745

Foord SM, Peters JR, Dieguez C 1984 Hypothyroid pituitary cells in culture : An analysis of TSH and PRL responses to dopamine and dopamine receptor binding. Endocrinology 115:407-415

Foord SM, Peters JR, Dieguez C 1985 TSH regulates thyrotroph responsiveness to dopamine *in vitro*. Endocrinology 118:1319-1326

Franklyn JA, Wood DF, Balfour NJ, Ramsden DB, Docherty K, Chin WW, Sheppard MC 1987 Effect of hypothyroidism and thyroid hormone replacement *in vivo* on pituitary cytoplasmic concentrations of thyrotropin-β and α-subunit messenger ribonucleic acids. Endocrinology 120:2279-2288

French MB, Lussier BT, Moor BC, Kraicer J 1990 Effect of growth hormone-releasing factor on phosphoinositide hydrolysis in somatotrophs. Mol Cell Endocrinol 72:221-226

French MB, Moor BC, Lussier BT, Kraicer J 1989 Protein kinase C is not essential for growth hormone (GH)-releasing factor-induced GH release from rat somatotrophs. Endocrinology 124:2235-2244

French MB, Moor BC, Lussier BT, Kraicer J 1991 Growth hormone-releasing factor

does not activate protein kinase C in somatotrophs. Mol Cell Endocrinol 79:139-146

Frohman LA, Jansson JO 1986 Growth hormone-releasing hormone. Endocr Rev 7:223-253

Fryer JN 1981 Hypothalamus lesions stimulating growth hormone cell activity in the goldfish. Cell Tissue Res 214:387-395

Fryer JN, Boudreault-Chateauvert C, Kirby RP 1985 Pituitary afferents originating in the paraventricular organ (PVO) of the goldfish hypothalamus. J Comp Neurol 242:475-484

Fuxe K, Agnati LF, Kalia M, Goldstein M, Andersson K, Harfstrand A 1985 Dopaminergic systems in the brain and pituitary. In : Fluckiger E, Muller EE, Thorner MO (eds) The Dopaminergic System. Basic and Clinical Aspects of Neuroscience (Springer-Verlag, New York) 1:11-25

Ge W, Cook H, Peter RE, Vaughan J, Vale 1993 Immunocytochemical evidence for the presence of inhibin and activin-like proteins and their localization in goldfish gonads. Gen Comp Endocrinol (in press)

Gibbs DM, Neil JD 1978 Dopamine levels in hypophyseal stalk blood in the rat are sufficient to inhibit prolactin secretion *in vivo*. Endocrinology 102:1895-1902

Gick GG, Bancroft 1987 Glucocorticoid stimulation of growth hormone messenger ribonucleic acid levels in GH3 cells is inhibited by calcium but not by somatostatin. Endocrinology 120:1986-1990

Gick GG, Zeytin FN, Brazeau P, Ling NC, Esch FS, Bancroft C 1984 Growth hormone-releasing factor regulates growth hormone mRNA in primary cultures of rat pituitary cells. Proc Nat Acad Sci USA 81:1553-1555

Gillies G, Davidson K 1992 GABAergic influences on somatostatin secretion from hypothalamic neurons cultured in defined medium. Neuroendocrinology 55:248-256

Gingrich JA, Dearry A, Falardeau P, Fremeau RT Jr,Bales MD, Caron MG 1991 Molecular characterization of G-protein coupled receptors : Isolation and cloning of a D1 dopamine receptor. J Receptor Res 11:521-534

Gingrich JA, Jarvie K, Tiberi M, Fremeau RT Jr, Caron MG 1992 The family of

receptors for dopamine cloning, structures and properties. Du Pont Biotech Update 7:105-118

Giusti M, Mazzocchi G, Sessarego P, Mignone D, Lomeo A, Monachesi M, Giordano G 1984 Effects of GRF(1-40) and domperidone on GH secretion in normal man. Clin Endocrinol 21:339-343

Grenader A, Healy DP 1991 Locally formed dopamine stimulates cAMP accumulation in LLC-PK1 cells via a DA1 dopamine receptor. Am J Physiol 260:F906-F912

Gurney AM 1990 Measurement and control of intracellular calcium. In: Hulme EC (ed) Receptor-Effector Coupling: A Practical Approach. IPL Press, Oxford, pp 117-153.

Habibi HR, Peter RE, Nahorniak CS, de L. Milton RC, Millar RP 1992 Activity of vertebrate gonadotropin-releasing hormones and analogs with variant amino acid residues in positions 5,7 and 8 in the goldfish pituitary. Regul Pept 37:271-284

Hahn RA, MacDonald BR 1984 Primate cardiovascular responses mediated by dopamine receptors : Effects of N,N-di-n-propyldopamine and LY17155. J Pharmacol Exp Ther 229:132-139

Hanew K, Sasaki A, Sato S, Goh M, Yoshinaga K 1987 Growth hormone inhibitory and stimulatory actions of L-Dopa in patients with acromegaly. J Clin Endocrinol Metab 64:255-260

Hanna S, Shin SH 1992 Differential inhibition of dopamine and bromocriptine on induced prolactin release : Multiple sites for the inhibition of dopamine. Neuroendocrinology 55: 591-599

Harvey S 1983 Neuroendocrine control of growth hormone secretion in birds. In : Nistico G, Bolis L (eds) Progress in Non-Mammalian Brain Research, Boca Raton, CRC Press, vol.3, pp 307-327

Harvey S 1990 Thyrotrophin-releasing hormone : A growth hormone-releasing factor. J Endocrinol 125:345-358

Hayes G, Bident TJ, Selbie LA, Shine J 1992 Structural subtypes of the dopamine D2 receptor are functionally distinct: expression of the cloned D2A and D2B subtypes in a heterologous cell line. Mol Endocrinol 6:920-925

Helms LMH, Grau EG, Shimoda SK, Nishioka RS, Bern HA 1987 Studies on the

regulation of growth hormone release from the proximal pars distalis of male tilapia, *Oreochromis mossambicus, in vitro.* Gen Comp Endocrinol 65:48-55

Hobert P, Crawford R, Shen LP, Pictet R, Rutter WJ 1980 Cloning and sequence analysis of cDNAs encoding two distinct somatostatin precursors found in the endocrine pancreas of anglerfish. Nature 288:137-141

Holder AT, Spencer EM, Preece MA 1981 Effects of bovine growth hormone and a partially pure preparation of somatomedin on various growth parameters in hypopituitary dwarf mice. J Endocrinol 89:275-282

Holl RW, Thorner MO, Leong DA 1988b Intracellular calcium concentration and growth hormone secretion in individual somatotropes : Effects of growth hormone-releasing factor and somatostatin. Endocrinology 122:2927-2932

Holl RW, Thorner MO, Mandell GL, Sullivan JA, Sinha YN, Leong DA 1988a Spontaneous oscillations of intracellular calcium and growth hormone secretion. J Biol Chem 263:9682-9685

Horellou P, Brundin P, Kalen P, Mallet J, Bjorklund A 1990 *In vivo* release of DOPA and dopamine from genetically engineered cells grafted to the denervated rat striatum. Neuron 5:393-402

Horvath S, Palkovits M, Gorcs T, Arimura A 1989 Electron microscopic immunocytochemical evidence for the existence of bidirectional synaptic connections between growth hormone-releasing hormone- and somatostatin-containing neurons in the hypothalamus of the rat. Brain Res 48:8-15

Hoshino Y, Obara H, Iwai S 1986 Relaxant effect of dopamine on isolated rabbit pulmonary artery. Life Sci 39:2525-2531

Huang KP 1989 The mechanism of protein kinase C activation. Trends Neurosci 12:425-432

Ieiri T, Ishikawa K, Suzuki M, Shimoda SI 1988 Growth hormone synthesis decreased after anterolateral deafferentation of the medial basal hypothalamus in the rat. Neuroendocrinology 47:431-436

Ignar DM, Kuhn CM 1988 Relative ontogeny of opioid and catecholaminergic regulation of thyrotropin secretion in the rat. Endocrinology 123:567-571

Iorio LC, Barnett A, Billard W, Gold EH 1986 Benzazepines: Structure-activity relationships between D1 receptor blockade and selected pharmacological effects. In : Breese GR, Creese I (eds) Neurobiology of Central D1-Dopamine Receptors, Plenum Press, New York pp 1-14

Isgaard J, Carlsson L, Isaksson OGP, Jansson JO 1988 Pulsatile intravenous growth hormone (GH) infusion to hypophysectomized rats increases insulin-like growth factor I messenger ribonucleic acid in skeletal tissues more effectively than continuous GH infusion. Endocrinology 123:2605-2610

Israel JM, Kukstas LA, Vincent JD 1990 Plateau potentials recorded from lactating rat enriched lactotroph cells are triggered by thyrotropin releasing hormone and shortened by dopamine. Neuroendocrinology 51:113-122

Jaffe CA, Barkan AL 1992 Treatment of acromegaly with dopamine agonists. Endocrinol Metab Clinics N Amer 21:713-735

Jansson JO, Eden S, Isaksson O 1985 Sexual dimorphism in the control of growth hormone secretion. Endocr Rev 6:128-150

Jellinger K 1986 The pathology of Parkinson's disease. In : Fahn ST, Marden CD (eds) The Pathology of Parkinson's Disease. Butterworths, London, pp 107-125

Jia XC, Kalmyn J, Hseuh AJW 1986 Growth hormone enhances follicle-stimulating hormone-induced differentiation of cultured rat granulosa cells. Endocrinology 118:1401-1409

Jobin RM, Chang JP 1992 Differences in extracellular calcium involvement mediating the secretion of gonadotropin and growth hormone stimulated by two closely related endogenous GnRH peptides in goldfish pituitary cells. Neuroendocrinology 55:156-166

Johansson O, Hokfelt T, Elde RP 1984 Immunohistochemical distribution of somatostatin-like immunoreactivity in the central nervous system of the adult rat. Neuroscience 13:265-339

Jordan V, Dieguez C, Lafaffian I, Rodriguezarnao MD, Gomez-Pan A, Hall R, Scanlon MF 1986 Influence of dopaminergic, adrenergic and cholinergic blockade and TRH administration on GH responses to GRF 1-29. Clin Endocrinol 24:291-298

Judd AM, MacLeod RM 1992 Thyrotropin-releasing hormone and lysine-bradykinin

stimulate arachidonate liberation from rat anterior pituitary cells through different mechanisms. Endocrinology 131:1251-1260

Junier MP, Dray F, Blair I, Capdevila J, Dishman E, Falck JR, Ojeda SR 1990 Epoxygenase products of arachidonic acid are endogenous constituents of the hypothalamus involved in D2 receptor-mediated, dopamine-induced release of somatostatin. Endocrinology 126:1534-1540

Kah O, Chambolle P, Dubourg P, Dubois MP 1982 Localisation immunocytochimique de la somatostatine dans le cerveau anterieur et l'hypophyse de deux Teleosteens, le Cyprin (*Carassius auratus*) et *Gambusia* sp. C R Acad Sci 294:519-524

Kah O, Dulka JG, Dubourg P, Thibault J, Peter RE 1987 Neuroanatomical substrate for the inhibition of gonadotropin secretion in goldfish : Existence of a dopaminergic preoptico-hypophyseal pathway. Neuroendocrinology 45:451-458

Kaiser C 1983 Stereoisomeric probes of dopamine receptor. In: Kaiser C, Kebabian JW (eds) Dopamine Receptors. American Chemical Society, Washington DC, ACS Symposium Series 22: 223-246

Kalra PS, Kalra SP 1985 Control of gonadotropin secretion. In : Imura H (ed) The Pituitary Gland. Raven Press, New York, pp 189-219

Kamberi IA, Nicol RS, Porter JC 1970 Effect of anterior pituitary perfusion and intraventricular injection of catecholamines and indoleamines on LH release. Endocrinology 87:1-5

Kapiloff MS, Farkash Y, Wegner M, Rosenfeld MG 1991 Variable effects of phosphorylation of Pit-1 dictated by the DNA response elements. Science 253:786-789

Katakami H, Downs TR, Frohman LA 1988 Inhibitory effect of hypothalamic medial preoptic area somatostatin on growth hormone-releasing factor in the rat. Endocrinology 123:1103-1109

Kato M, Hoyland J, Sikdar SK, Mason WT 1992 Imaging of intracellular calcium in rat anterior pituitary cells in response to growth hormone releasing factor. J Physiol 447:171-189

Kebabian JW, Beaulieu M, Itoh Y 1984 Pharmacological and biochemical evidence for the existence of two categories of dopamine receptor. Can J Neuro Sci 11:114-117

Kelley KW 1990 The role of growth hormone in modulation of the immune response. In : O'Dorsio MS, Panerai A (eds) Neuropeptides and Immunopeptides : Messengers in a Neuroimmune Axis. The New York Academy of Sciences, NY, pp 95-103

Khorram O, Mizunuma H, McCann SM 1982 Effect of α-melanocyte stimulating hormone on basal and stimulated release of prolactin : Evidence for dopaminergic mediation. Neuroendocrinology 34:433-437

Kiem DT, Bartha L, Harsing LG, Makara GB 1991 Reevaluation of the role of alpha2-adrenoreceptors in morphine-stimulated release of growth hormone. Neuroendocrinology 53:516-522

Kitajima N, Chihara K, Abe H, Okimura Y, Fujii Y, Sato M, Shakutsui S, Watanabe M, Fujita T 1989 Effects of dopamine on immunoreactive growth hormone-releasing factor and somatostatin secretion from rat hypothalamic slices perifused *in vitro*. Endocrinology 124:69-76

Kobayashi M, Hanyu I 1986 Annual change in plasma levels of gonadotropin and steroid hormones in goldfish. Bull Japan Soc Sci Fish 52:1153-1158

Kobayashi M, Aida K, Hanyu I 1987 Hormone changes during ovulation and effects of steroid hormones on plasma gonadotropin levels and ovulation in goldfish. Gen Comp Endocrinol 67:24-32

Kraicer J, Chow AEH 1982 Release of growth hormone from purified somatotrophs : Use of perifusion system to elucidate interrelations among Ca^{++}, adenosine 3',5'-monophosphate, and somatostatin. Endocrinology 111:1173-1180

Kraicer J, Sheppard MS, Luke J, Lussier B, Moor BC, Cowan JS 1988 Effect of withdrawal of somatostatin and growth hormone (GH)-releasing factor on GH release *in vitro*. Endocrinology 122:1810-1815

Krieg RJ, Perkins SN, Johnson JH, Rogers JP, Arimura A, Cronin MJ 1988 β-adrenergic stimulation of growth hormone (GH) release *in vivo*, and subsequent inhibition of GH-releasing factor-induced GH secretion. Endocrinology 122:531-537

Kukstas LA, Domec C, Bascles L, Bonnet J, Verrier D, Israel JM, Vincent JD 1991 Different expression of the two dopaminergic D2 receptors, $D2_{415}$ and $D2_{444}$, in

two types of lactotroph each characterized by their response to dopamine, and modification of expression by sex steroids. Endocrinology 129:1101-1103

Lal S, Vasava Nair NP, Thavundayil JX, Tawar V, Quirion R, Guyda H 1991 Stereospecificity of the dopamine receptor mediating the growth hormone response to apomorphine in man. J Neural Transm [GenSect] 85:157-164

Larhammar D, Soderberg C, Blomqvist AG 1993 Evolution of neuropeptide Y family of peptides. In: Wahlestedt C, Colmers WF (eds) The Neurobiology of Neuropeptide Y and Related Peptides. Humana Press, Cuffon, NJ. (in press)

Lavergne C, Breant B, Rosselin G 1992 Modulation of growth-related gene expression and growth inhibition by cyclic adenosine 3',5'-monophosphate-elevating agents in the insulin-producing cell line βTC1. Endocrinology 131:2351-2356

Leong DA 1989 Intracellular calcium levels in rat anterior pituitary cells : Single-cell techniques. In: Conn PM (ed) Methods in Enzymology, Academic Press, Inc., pp 263-284

Lewis BM, Dieguez C, Lewis M, Hall R, Scanlon MF 1986 Hypothalamic D2 receptors mediate the preferential release of somatostatin-28 in response to dopaminergic stimulation. Endocrinology 119:1712-1720

Li S, Pelletier G 1992 Role of dopamine in the regulation of gonadotropin-releasing hormone in the male rat brain as studied by *in situ* hybridization. Endocrinology 131:395-339

Lidy BM, Kemenade VV, Tonon MC, Jenks BG, Vaudry H 1986 Characteristics of receptors for dopamine in the pars intermedia of the amphibian *Xenopus laevis*. Neuroendocrinology 44:446-456

Lin XW, Lin HR, Peter RE 1993 The regulatory effects of thyrotropin-releasing hormone on growth hormone release from perifused pituitary of common carp *in vitro*. Fish Physiol Biochem (in press)

Lindholm J, Riishede J, Vestergaard S 1981 No effect of bromocriptine in acromegaly: A controlled trial. N Engl J Med 304:1450-1454

Lindley SE, Lookingland KJ, Moore KE 1990 Activation of tuberoinfundibular but not tuberohypophysial dopaminergic neurons following intracerebroventricular

administration of alpha-melanocyto-stimulating hormone. Neuroendocrinology 51:394-399

Lindstrom P, Ohlsson L 1987 Effects of 5-hydroxytryptamine, dopamine, and aromatic L-amino acids on growth hormone(GH)-releasing factor-stimulated GH release in rat anterior pituitaries. Endocrinology 120:780-784

Liposits ZS, Merchenthaler I, Paull WK, Flerko B 1988 Synaptic communication between somatostatinergic axons and growth hormone-releasing factor (GRF) synthesizing neurons in the arcuate nucleus of the rat. Histochemistry 89:247-252

Lledo PM, Legendre P, Israel JM, Vincent JD 1990b Dopamine inhibits two characterized voltage-dependent calcium currents in identified rat lactotroph cells. Endocrinology 127:990-1001

Lledo PM, Legendre P, Zhang J, Israel JM, Vincent JD 1990a Effects of dopamine on voltage-dependent potassium currents in identified rat lactotroph cells. Neuroendocrinology 52: 545-555

Lledo PM, Israel JM, Vincent JD 1991 Chronic stimulation of D2 dopamine receptors specifically inhibits calcium but not potassium currents in rat lactotrophs. Brain Res 558:231-238

Loginn IS, Kuan SI, Judd AM, MacLeod RM 1990 Interactions of dopamine and neurotensin on calcium fluxes and prolactin release in normal rat pituitary cells. Endocrinology 127:1948-1955

Loginn IS, Judd AM 1986 Trophic effects of somatostatin and calcium flux : Dynamic analysis and correlation with pituitary hormone release. Endocrinology 119:1703-1707

Lopez FJ, Dominguez JR, Sanchez-Franco F, Negro-Vilar A 1989 Role of dopamine and vasoactive intestinal peptide in the control of pulsatile prolactin secretion. Endocrinology 124:527-535

Lopez F, Gonzalez D, Aguilar E 1986 Serotonin stimulates GH secretion through a direct pituitary action : Studies in hypophysectomized autografted animals and in perifused pituitaries. Acta Endocrinol (Copenhagen) 113:317-322

Losa M, Muller OA, Sobieszczyk S, Von Werder K 1985 Interaction between growth hormone releasing factor (GRF) and somatostatin analogue (SMS 201-995) in

normal subjects. Clin Endoncrinol 23:715-721

Lumpkin MD, McDonald JK 1989a Blockade of growth hormone-releasing factor (GRF) activity in the pituitary and hypothalamus of the conscious rat with a peptidic GRF antagonist. Endocrinology 124:1522-1531

Lumpkin MD, Mulroney SE, Haramati A 1989b Inhibition of pulsatile growth hormone (GH) secretion and somatic growth in immature rats with a synthetic GH-releasing factor antagonist. Endocrinology 124:1154-1159

Lundblad JR, Roberts JL 1988 Regulation of proopiomelanocortin gene expression in pituitary. Endocr Rev 9:135-157

Luo D, McKeown BA 1989a Immunohistochemical detection of a substance resembling growth hormone-releasing factor in the brain of the rainbow trout (*Salmo gairdneri*). Experientia 45:577-580

Luo D, McKeown BA 1989b An antioxidant dependent *in vitro* response of rainbow trout (*Salmo gairdneri*) somatotrophs to carp growth hormone-releasing factor (GRF). Horm Metab Res 21:690-692

Luo D, McKeown BA, Rivier J, Vale W 1990 *In vitro* responses of rainbow trout (*Oncorhynchus mykiss*) somatotrophs to carp growth hormone-releasing factor (GRF) and somatostatin. Gen Comp Endocrinol 80:288-298

Lussier BT, French MB, Moor BC, Kraicer J 1991a Free intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) and growth hormone release from purified rat somatotrophs. I. GH-releasing factor-induced Ca^{2+} influx raises $[Ca^{2+}]i$. Endocrinology 128:570-582

Lus BT, French MB, Moor BC, Kraicer J 1991b Free intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) and growth hormone release from purified rat somatotropns. II. Somatostatin lowers $[Ca^{2+}]i$ by inhibiting Ca^{2+} influx. Endocrinology 128:583-591

Lussier BT, French MB, Moor BC, Kraicer J 1991c Free intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) and growth hormone release from purified rat somatotrophs. III. Mechanism of action of GH-releasing factor and somatostatin. Endocrinology 128:592-603

MacKenzie RG, Zigmond MJ 1984 High- and low-affinity states of striatal D2 receptors

are not affected by 6-hydroxydopamine or chronic haloperidol treatment. J Neurochem 43:1310-1318

Malozowski S, Hao EH, Ren SG, Genazzani AD, Kalogeras, Merriam GR 1990 Effects of inhibition of norepinephrine synthesis on spontaneous and growth hormone-releasing hormone-induced GH secretion in cynomogus macagues : Evidence for increase in hypothalamic somatostatin tone. Neuroendocrinology 51:455-458

Mangalam HJ, Albert VR, Ingraham HA, Kapiloff M, Wilson L, Nelson C, Elsholtz H, Rosenfeld MG 1989 A pituitary POU domain protein, Pit-1, activates both growth hormone and prolactin promoters transcriptionally. Genes Dev 3:946-958

Mansour A, [illegible] Woodruff JH, Bunzow JR, Civelli O, Akil H, Watson SJ 1990 Localization of dopamine D2 receptor mRNA and D1 and D2 receptor binding in the rat brain and pituitary: an *in situ* hybridization-receptor autoradiographic analysis. J Neurosci 10:2587-2600

Marchant TA, Chang JP, Nahorniak CS, Peter RE 1989b Evidence that gonadotropin-releasing hormone also functions as a growth hormone-releasing factor in the goldfish. Endocrinology 124:2509-2518

Marchant TA, Dulka JG, Peter RE 1989a Relationship between serum growth hormone levels and the brain and pituitary content of immunoreactive somatostatin in the goldfish, *Carassius auratus L.* Gen Comp Endocrinol 73:458-468

Marchant TA, Fraser RA, Andrews PC, Peter RE 1987 The influence of mammalian and teleost somatostatins on the secretion of growth hormone from goldfish (*Carassius auratus L.*) pituitary fragments *in vitro.* Regul Pept 17:41-52

Marchant TA, Peter RE 1986 Seasonal variations in body growth rates and circulating levels of growth hormone in the goldfish, *Carassius auratus.* J Expt Zool 237:231-239

Marivoet S, Moons L, Vandesande F 1988 Localization of growth hormone releasing factor-like immunoreactivity in the hypothalmo-hypophyseal system of the frog (*Rana temporaria*) and the sea bass (*Dicentrarchus labrax*). Gen Comp Endocrinol 72:72-79

Mason WT 1983 Excitation by dopamine of putative oxytocinergic neurons in the rat

supraoptic nucleus *in vitro* : Evidence for two classes of continuously firing neurons. Brain Res 267:113-121

Matsubara M, Tango M, Nakagawa K 1987 Effects of dopaminergic agonists on plasma luteinizing hormone-releasing hormone (LRH) and gonadotropins in man. Horm Metab Res 19: 31-34

McArdle CA, Conn PM 1989 Use of protein kinase C-depleted cells for investigation of the role of protein kinase C in stimulus-response coupling in the pituitary. In: Conn PM (ed) Methods in Enzymology, Academic Press, Inc., pp 287-301

McCarthy GF, Beaudet A, Tannenbaum GS 1992 Colocalization of somatostatin receptors and growth hormone-releasing factor immunoreactivity in neurons of the rat arcuate nucleus. Neuroendocrinology 56:18-24

McMillian MK, He XP, Hong JS, Pennypacker KR 1992 Dopamine stimulates [^{3}H] phorbol 12,13-dibutyrate binding in cultured striatal cells. J Neurochem. 58:1308-1312

Meador-Woodruff JH, Mansour A, Healy D, Kuehn R, Zhou QY, Bunzow JR, Akil H, Civelli O, Watson SJ 1991 Comparison of the distributions of D1 and D2 dopamine receptor mRNAs in rat brain. Neuropsychopharmacology 5:231-242

Meister B, Hulting AL 1987 Influence of coexisting hypothalamic messengers on growth hormone secretion from rat anterior pituitary cells *in vitro*. Neuroendocrinology 46:387-394

Meunier H, Labrie F 1982 The dopamine receptor in the intermediate lobe of the rat pituitary gland is negatively coupled to adenylate cyclase. Life Sci 30:963-968

Mitsugi N, Arita J, Kimura F 1990 Effects of intracerebroventricular administration of growth hormone-releasing factor and corticotropin-releasing factor on somatostatin secretion into rat hypophysial portal blood. Neuroendocrinology 51:93-96

Miyazaki K, Goldman ME, Kebabian JW 1984 Forskolin stimulates adenylate cyclase activity, adenosine 3',5'-monophosphate production and peptide release from the intermediate lobe of the rat pituitary gland. Endocrinology 114:761-966

Mons N, Tison F, Geffard M 1990 Existence of L-DOPA immunoreactive neurons in the rat preoptic area and anterior hypothalamus. Neuroendocrinology 51:425-428

Monsma FJ, Mahan LC, McVittie LD, Gerfen CR, Sibley DR 1990 Molecular cloning and expression of a D1 dopamine receptor linked to adenylyl cyclase activation. Proc Natl Acad Sci USA 87:6723-6727

Moons L, Cambre M, Marivoet S, Batten TFC, Vanderhaeghen JJ, Ollevier F, Vandesande F 1988 Peptidergic innervation of the adrenocorticotropic hormone (ACTH)- growth hormone (GH)-producing cells in the pars distalis of the sea bass (*Dicentrarchus labrax*). Gen Comp Endocrinol 72:171-180

Moore KE, Demarest KT 1982 Tuberoinfundibular and tuberohypophyseal dopaminergic neurons. Front Neuroendocrinol 7:161-190

Moos F, Richard P 1982 Excitatory effect of dopamine on oxytocin and vasopressin reflex releases in the rat. Brain Res 241:249-260

Morra M, Leboulenger F, Desrues L, Tonon MC, Vaudry H 1991 Dopamine inhibits inositol phosphate production, arachidonic acid formation, and corticosteroid release by frog adrenal gland through a pertussis toxin-sensitive G-protein. Endocrinology 128:2625-2632

Muller EE 1987 Neural control of somatotropic function. Physiol Rev 67:962-1053

Murakami Y, Kato Y, Kabayama Y, Tojo K, Inoue T, and Imura H 1986 Involvement of growth hormone (GH)-releasing factor in GH secretion induced by serotonergic mechanisms in conscious rats. Endocrinology 119:1089-1092

Murakami Y, Kato Y, Shimatsu A, Koshiyama H, Hattori N, Yanaihara N, Imura H 1989 Possible mechanisms involved in growth hormone secretion induced by galanin in the rat. Endocrinology 124:1224-1229

Nahorski SR, Ragan CI, Challiss RAJ 1991 Lithium and the phosphoinositide cycle : An example of uncompetitive inhibition and its pharmacological consequences. Trends Pharmacol Sci 12:297-303

Namba H, Morita S, Melmed S 1989 Insulin-like growth factor-I action on growth hormone secretion and messenger ribonucleic acid levels : Interaction with somatostatin. Endocrinology 124:1794-1799

Narayanan N, Lussier B, French M, Moor B, Kraicer J 1989 Growth hormone-releasing factor-sensitive adenylate cyclase system of purified somatotrophs : Effects of guanine nucleotides, somatostatin, calcium, and magnesium.

Endocrinology 124:484-495

Negro-Vilar A, Lapetina EG 1985 1,2-Didecanoylglycerol and phorbol 12,13-dibutyrate enhance anterior pituitary hormone secretion *in vitro*. Endocrinology 117:1559-1564

Neumeyer JL, Baindur N, Bakthavachalam V, Yuan J, Madras BK, Kula NS, Campbell A, Baldessarini RJ 1992 Selective probes for characterization of dopamine D1 and D2 receptors. Neurochem Int [Suppl] 20:63S-68S

Newman CB, Wardlaw SL, Frantz AG 1985 Suppression of basal and stress-induced prolactin release and stimulation of luteinizing hormone secretion by α-melanocyte stimulating hormone. Life Sci 36:1661-1668

Niimi M, Takahara J, Sato M, Kawanishi K 1992 Identification of dopamine and growth hormone-releasing factor-containing neurons projecting to the median eminence of the rat by combined retrograde tracing and immunohistochemistry. Neuroendocrinology 55: 92-96

Nishioka RS, Kelley KM, Bern HA 1988 Control of prolactin and growth hormone secretion in teleost fishes. Zool Sci 5:267-280

Niznik HB 1987 Dopamine receptors : Molecular structure and function. Mol Cell Endocrinol 54:1-22

Nozaki M, Miyata K, Oota Y, Gorbman A, Plisetskaya EM 1988 Different cellular distributions of two somatostatins in brain and pancreas of salmonids, and their associations with insulin- and glucagon-secreting cells. Gen Comp Endocrinol 69:267-280

Ohlsson L, Lindstrom P 1990 The correlation between calcium outflow and growth hormone release in perifused rat somatotrophs. Endocrinology 126:488-497

Okada S, Chen WY, Wiehl P, Kelder B, Goodman HM, Guller S, Sonenberg M, Kopchick J 1992 A growth hormone (GH) analog can antagonize the ability of native GH to promote differentiation of 3T3-F442A preadipocytes and stimulate insulin-like and lipolytic activities in primary rat adipocytes. Endocrinology 130:2284-2290

Olivereau M, Ollevier F, Vandesande F, Verdonck M 1984 Immunocytochemical identification of CRF-like and SRIF-like peptides in the brain and pituitary of

cyprinid fish. Cell Tissue Res 237:379-382

Omeljaniuk RJ, Tonon MC, Peter RE 1989 Dopamine inhibition of gonadotropin and α-melanocyte-stimulating hormone release *in vitro* from the pituitary of the goldfish (*Carassius auratus*). Gen Comp Endocrinol 74:451-467

Onali P, Schwartz JP, Costa E 1981 Dopaminergic modulation of adenylate cyclase stimulation by vasoactive intestinal peptide in anterior pituitary. Proc Natn Acad Sci USA 78:6531-6534

Oyama H, Bradshaw RA, Gabbay KH, Permutt A 1980 Isolation and characterization of immunoreactive somatostatin I. J Biol Chem 255:2251-2254

Pan JX, Lechan RM, Lin HD, Sohn J, Reichlin S, Jackson IMD 1985 Multiple forms of human pancreatic growth hormone-releasing factor-like immunoreactivity in teleost brain and pituitary. Endocrinology 116:1663-1665

Pandiella A, Elahi FR, Vallar L, Spada A 1988 α1-adrenergic stimulation of *in vitro* growth hormone release and cytosolic free Ca^{2+} in rat somatotrophs. Endocrinology 122:1419-1425

Parker SL, Crowley WR 1992 Activation of central D-1 dopamine receptors stimulates oxytocin release in the lactating rat : Evidence for involvement of hypothalamic paraventricular and supraoptic nuclei. Neuroendocrinology 56:385-392

Parker DB, Sherwood NM 1990 Evidence of growth hormone-releasing hormone-like molecule in salmon brain, *Oncorhynchus keta* and *O. kisutch.* Gen Comp Endocrinol 79:95-102

Patel YC, Papachristou DN, Zingg HH, Farkas EM 1991 Regulation of islet somatostatin secretion and gene expression : Selective effects of adenosine 3',5'-monophosphate and phorbol esters in normal islets of Langerhans and in a somatostatin-producing rat islet clonal cell line 1027 B_2. Endocrinology 128:1754-1762

Peng C, Chang JP, Peter RE 1990 Growth hormone-releasing factor stimulates growth hormone secretion from pituitary cells of goldfish. Bull Can Soc Zool 21:79 (Abs)

Peng C, Chang JP, Yu KL, Wong AOL, Van Goor F, Peter RE, Rivier JE 1993 Neuropeptide Y stimulates growth hormone and gonadotropin-II secretion in the goldfish pituitary : Involvement of both presynaptic and pituitary cell actions.

Endocrinology (in press)

Peng C, Huang YP, Peter RE 1990 Neuropeptide Y stimulates growth hormone and gonadotropin release from the goldfish pituitary *in vitro.* Neuroendocrinology 52:28-34

Peter RE, Chang JP, Nahorniak CS, Omeljaniuk RJ, Sokolowska M, Shih SH, Billard R 1986 Interactions of catecholamines and GnRH in regulation of gonadotropin secretion in teleost fish. Recent Prog Horm Res 42:513-548

Peter RE, Nahorniak CS, Vale WW, Rivier JE 1984 Human pancreatic growth hormone-releasing factor (hpGRF) stimulates growth hormone release in goldfish. J Exp Zool 231:161-163

Peter RE, Yu KL, Marchant TA, Rosenblum PM 1990 Direct neural regulation of the teleost adenophypophysis. J Exp Zool 4 [Suppl]:84-98

Plisetskaya E, Pollock HG, Rouse JB, Hamilton JW, Kimmel JR, Andrews PC, Gorbman A 1986 Characterization of coho salmon (*Oncorhynchus kisutch*) islet somatostatins. Gen Comp Endocrinol 63:252-263

Plotsky PM, Vale W 1985 Patterns of growth hormone-releasing factor and somatostatin secretion into the hypophysial portal circulation of the rat. Science 230:461-463

Pickavance LC, Staines WA, Fryer JN 1992 Distribution and colocalization of neuropeptide Y and somatostatin in the goldfish brain. J Chem Neuroant 5:221-233

Pizzi M, D'Agostini F, Da Prada M, Spano PF, Haefely WE 1987 Dopamine D2 receptor stimulation decreases the inositol trisphosphate level of rat striatal slices. Eur J Pharmacol 136:263-264

Pontet A, Danger JM, Dubourg P, Pelletier G, Vaudry H, Calas A, Kah O 1989 Distribution and characterization of neuropeptide Y-like immunoreactivity in the brain and pituitary of the goldfish. Cell Tissue Res 255:529-538

Rainboth WJ 1991 Cyprinids of south east Asia. In : Winfield IJ, Nelson JS (eds) Cyprinid Fishes : Systematics, Biology and Exploitation. Chapman & Hall, Fish and Fisheries Series 3:156-211

Ramirez VD, Feder HH, Sawyer CH 1984 The role of brain catecholamines in the regulation of LH secretion : A critical inquiry. Front Neuroendocrinol 8:1-32

Rao DD, McKelvy J, Kebabian J, MacKenzie RG 1990 Two forms of the rat D2 dopamine receptor as revealed by the polymerase chain reaction. FEBS Lett 263:18-21

Raymond V, Leung PCK, Veilleux R, Lefevre G, Labrie F 1984 LHRH rapidly stimulates phosphatidylinositol metabolism in enriched gonadotrophs. Mol Cell Endocrinol 36:157-164

Rendon MC, Toro MJ, Mellado M, Montoya E 1988 Somatostatin stimulates phosphodiesterase in rat anterior pituitary and brain, and GH4C1 cells. Second Messengers and Phosphoproteins 12:75-81

Rettori V, Milenkovic L, Aquila MC, McCann SM 1990 Physiologically significant effect of neuropeptide Y to suppress growth hormone release by stimulating somatostatin discharge. Endocrinology 126:2296-2301

Reyl-Desmars F, Zeytin F 1985 Somatostatin inhibits growth hormone-releasing factor-stimulated adenylate cyclase activity in GH3 cells. Biochem Biophys Res commun 127:986-992

Richardson SB, Nguyen T, Hollander CS 1983 Dopamine stimulates somatostatin release from perifused hypothalamic cells. Am J Physiol 244:E560-E566

Richardson SB, Twente S 1991 Pre-exposure of rat anterior pituitary cells to somatostatin enhances subsequent growth hormone secretion. J Endocrinol 128:91-95

Riederer P, Sofic E, Konradi C, Kornhuber J, Beckmann H, Dietl M, Moll G, Hebenstreit G 1989 The role of dopamine in the control of neurobiological functions. In: Fluckiger E, Muller EE, Thorner MO (eds) The Role of Brain Dopamine. Basic and Clinical Aspects of Neuroscience (Springer-Verlag, New York) 3:1-15

Riederer P, Wuketich S 1976 Time course of nigrostriatal degeneration in Parkinson's disease. J Neural Transm 38:277-301

Rittmaster RS, Loriaux DL, Merriam GR 1987 Effect of continuous somatostatin and growth hormone-releasing hormone (GHRH) infusion on the subsequent growth

hormone (GH) response to GHRH. Neuroendocrinology 45:118-122

Rivas RJ, Nishioka RS, Bern HA 1986 *In vitro* effects of somatostatin and urotensin II on prolactin and growth hormone secretion in tilapia, *Oreochromis mossambicus.* Gen Comp Endocrinol 63:245-251

Robberecht P, Gillard M, Waelbroeck W, Camus JC, de Neef P, Christophe J 1986 Decreased stimulation of adenylate cyclase by growth hormone-releasing factor in the anterior pituitary of old rats. Neuroendocrinology 44:429-432

Root AW 1989 Neurophysiological regulation of the secretion of growth hormone. J Endocrinol Invest [Suppl] 12:3-7

Rosenthal SM, Silverman BL, Wehrenberg WB 1991 Exogenous growth hormone inhibits bovine but not murine pituitary growth hormone secretion *in vitro*: Evidence for a direct feedback of growth hormone on the pituitary. Neuroendocrinology 53:597-600

Samuels MH, Henry P, Ridgway EC 1992 Effects of dopamine and somatostatin on pulsatile pituitary glycoprotein secretion. J Clin Endocrinol Metab 74:217-222

Sawchenko PE, Swanson LW, Rivier J, Vale WW 1983 The distribution of growth hormone-releasing factor (GRF) immunoreactivity in the central nervous system of the rat: an immunohistochemical study using antisera directed against rat hypothalamic GRF. J Comp Neurol 237:100-115

Schwartz Y, Yamaguchi H, Goodman HM 1992 Growth hormone increases intracellular free calcium in rat adipocytes : Correlation with actions on carbohydrate metabolism. Endocrinology 131:772-778

Seeman P 1987a Dopamine receptors in human brain diseases. In: Creese I, Fraser CM (eds) Dopamine Receptors. Receptor Biochemistry and Methodology (Alan R. Liss, New York) 8:233-245

Seeman P 1987b Dopamine receptors and the dopamine hypothesis of schizophrenia. Synapse 1:133-141

Seeman P, Grigoriadis D, George SR, Watanabe M, Ulpian C 1986 Functional states of dopamine receptors. In : Woodruff GN, Poat JH, Roberts RJ (eds) Dopaminergic Systems and their Regulation. VCH, London, pp 97-109

Seeman P, Ulpian C, Grigoriadis D, Pri-Bar I, Buchman O 1985 Conversion of

dopamine D1 receptors from high to low affinity for dopamine. Biochem Pharmacol 34:151-154

Senogles SE, Benovic JL, Amlaiky N, Unson C, Milligan G, Vinitsky R, Spiegel AM, Caron M 1987 The D2-receptor of anterior pituitary is functionally associated with a pertussin toxin-sensitive guanine nucleotide binding protein. J Biol Chem 262:4860-4867

Serri O, Deslauriers N, Brazeau P 1987 Dual action of dopamine on growth hormone release *in vitro*. Neuroendocrinology 45:363-367

Sheppard MS, Eatock BA, Bala RM 1987 Characteristics of phorbol ester stimulated growth hormone release : Inhibition by insulin-like growth factor I, somatostatin, and low calcium medium and comparison with growth hormone releasing factor. Can J Physiol Pharmacol 65:2302-2310

Sheppard MS, Moor BC, Kraicer J 1985 Release of growth hormone (GH) from purified somatotrophs : Interaction of GH-releasing factor and somatostatin and role of adenosine 3',5'-monophosphate. Endocrinology 117:2364-2370

Shupnick MA, Greenspan SL, Ridgway EC 1986 Transcriptional regulation of thyrotropin subunit genes by thyrotropin-releasing hormone and dopamine in pituitary cells in culture. J Biol Chem 261:12675-12679

Sibley DR, Creese I 1983 Regulation of lipid binding to pituitary D-2 dopaminergic receptors. Effects of divalent cations and functional group modification. J Biol Chem 258:4957-4965

Sibley DR, DeLean A, Creese I 1982 Anterior pituitary dopamine receptors. Demonstration of interconvertible high and low affinity states of the D-2 dopamine receptor. J.Biol.Chem. 257: 6351-6361

Sibley DR, Monsma FJ Jr 1992 Molecular biology of dopamine receptors. Trends Pharmacol Sci 13:61-69

Sidhu A, Fishman PH 1990 Identification and characterization of functional D1 dopamine receptors in a human neuroblastoma cell line. Biochem Biophys Res Commun 166:574-579

Silverman BL, Kaplan SL, Grumbach MM, Miller WL 1988 Hormonal regulation of growth hormone secretion and messenger ribonucleic acid in cultured bovine

pituitary cells. Endocrinology 122:1236-1241

Simard M, Mirell CJ, Pekary AE, Drexler J, Kovacs K, Hershman M 1988 Hormonal control of thyrotropin and growth hormone secretion in a human thyrotrope pituitary adenoma studied *in vitro.* Acta Endocrinologica 119:283-290

Skottner A, Clark RG, Fryklund L, Robinson ILAF 1989 Growth response in a mutant dwarf rat to human growth hormone and recombinant human insulin-like growth factor I. Endocrinology 124:2519-2520

Slama A, Videau C, Kordon C, Epelbaum J 1992 Estradiol regulation of somatostatin receptors in the arcuate nucleus of the female rat. Neuroendocrinology 56:240-245

Smallridge RC, Kiang JG, Gist ID, Fein HG, Galloway RJ 1992 U-73122, an aminosteroid phospholipase C antagonist, noncompetitively inhibits thyrotropin-releasing hormone effects in GH3 rat pituitary cells. Endocrinology 131:1883-1888

Smith AI, Funder JW 1988 Proopiomelanocortin processing in the pituitary, central nervous system, and peripheral tissues. Endocr Rev 9:159-179

Sokoloff P, Giros B, Martres P, Bouthenet ML, Schwartz JC 1990 Molecular cloning and characterization of a novel dopamine receptor (D3) as a target for neuroleptics. Nature 347:146–151

Somoza GM, Peter RE 1991 Effects of serotonin on gonadotropin and growth hormone release from *in vitro* perifused goldfish pituitary fragments. Gen Comp Endocrinol 82:103-110

Sonntag WE, Forman LJ, Miki N, Trapp JM, Gottschall PE, Meites J 1982 L-Dopa restores amplitude of growth hormone pulses in old male rats to that observed in young male rats. Neuroendocrinology 34:163-168

Sopwith AM, Penny ES, Grossman A 1986 Normal circulating immunoreactive growth hormone releasing factor (hGRF) concentrations in patients with functional hypothalamic hGRF deficiency. Clin Endocrinol 24:395-400

Sowers JR, Catania A, Hershman M 1982 Evidence for dopaminergic control of circadian variations in thyrotropin (TSH) secretion in normal man. Clin Endocrinol (Oxf) 10:7-15

Soya H, Suzuki M 1988 Somatostatin rapidly restores rat growth hormone (GH) response attenuated by prior exposure to human GH-releasing factor *in vitro*. Endocrinology 122:2492-2498

Soya H, Suzuki M 1990 A possible role of hypothalamic somatostatin in the maintenance of rat pituitary responsiveness to growth hormone-releasing factor. Endocrinology 126:285-291

Spence JW, Sheppard MS, Kraicer J 1980 Release of growth hormone from purified somatotrophs : Interrelation between Ca^{++} and adenosine 3',5'-monophosphate. Endocrinology 106:764-469

Stachura ME, Tyler JM, Farmer PK 1988 Combined effects of human growth hormone (GH)-releasing factor-44 (GRF) and somatostatin (SRIF) on post-SRIF rebound release of GH and prolactin : A model for GRF-SRIF modulation of secretion. Endocrinology 123:1476-1482

Steiner RA, Illner P, Rolfs AD, Toivola PTK, Gale CC 1978 Noradrenergic and dopaminergic regulation of GH and prolactin in baboons. Neuroendocrinology 26:15-31

Steffey ME, Snyder GL, Barrett RW, Fink JS, Ackerman M, Adams P, Bhatt R, Gomez E, MacKenzie RG 1991 Dopamine D1 receptor stimulation of cyclic AMP accumulation in COS-1 cells. Eur J Pharmacol 207:311-317

Stojilkovic SS, Catt KJ 1992 Calcium oscillations in anterior pituitary cells. Endocr Rev 13:256-280

Stojilkovic SS, Izumi SI, Catt KJ 1988 Participation of voltage-sensitive calcium channels in pituitary hormone release. J Biol Chem 263:13054-13061

Stoof JC, Tilders FJH 1989 Dopamine receptors in the central nervous system. In: Fluckiger E, Muller EE, Thorner MO (eds) The Role of Brain Dopamine. Springer-Verlag, New York, Basic and Clinical Aspects of Neuroscience 3: 31-43

Strange PG 1987 Isolation and molecular characterization of dopamine receptors. In: Creese I, Fraser CM (eds) Dopamine Receptors. Alan. R. Liss. Inc., Receptor Biochemistry and Methodology 8:29-43

Sunahara RK, Guan HC, O'Dowd BF, Seeman P, Laurier LG, Ng G, George SR, Torchia J, Van Tol HHM, Niznik HB 1991 Cloning of the gene for a human

dopamine D5 receptor with higher affinity for dopamine than D1 receptor. Nature 350:614-619

Sunahara RK, Niznik HB, Weiner DM, Stormann TM, Brann MR, Kennedy JL, Gelernter JE, Rozmahel R, Yang Y, Israel Y, Seeman P, O'Dowd BF 1990 Human dopamine D1 receptor encoded by an intronless gene on chromosome 5. Nature 347:80-83

Suzuki R, Kaneko T, Hirano T 1991 Effects of osmotic pressure on prolactin and growth hormone secretion from organ-cultured eel pituitary. J Comp Physiol 161B:147-153

Sweeting RM, McKeown BA 1986 Somatostatin reduces plasma growth hormone levels during seawater adaptation in coho salmon (*Onchorhynchus kisutch*). Can J Zool 64:2062-2063

Tallo D, Malarkey WB 1981 Adrenergic and dopaminergic modulation of growth hormone and prolactin secretion in normal and tumor-bearing human pituitaries in monolayer culture. J Clin Endocrinol Metab 53:12781284

Tamai H, Komaki G, Matsubayashi S 1990 Effect of cholinergic muscarinic receptor blockade on human growth hormone (GH)-releasing hormone-(1-44)-induced GH secretion in anorexia nervosa. J Clin Endocrinol Metab 70:738-741

Tanoh T, Shimatsu A, Murakami Y, Ishikawa Y, Yanaihara N, Imura H 1991 Cholinergic modulation of growth hormone secretion induced by galanin in rats. Neuroendocrinology 54:83-88

Tannenbaum GS 1988 Somatostatin as a physiological regulator of pulsatile growth hormone secretion. Hormone Res 29:70-74

Tannenbaum GS 1990 Interrelationship of somatostatin and growth hormone-releasing hormone in the genesis of the rhythmic secretion of growth hormone. Acta Pœdiatr Scand 367 [Suppl]:76-80

Tannenbaum GS, Ling N 1984 The interrelationship of growth hormone (GH)-releasing factor and somatostatin in generation of the ultradian rhythm of GH secretion. Endocrinology 115:1952-1957

Tannenbaum GS, McCarthy GF, Zeitler P, Beaudet A 1990b Cysteamine-induced enhancement of growth hormone-releasing factor (GRF) immunoreactivity in

arcuate neurons: morphological evidence for putative somatostatin / GRF interactions within hypothalamus. Endocrinology 127:2551-2560

Tannenbaum GS, Painson JC, Lapointe M, Gurd W, McCarthy GF 1990a Interplay of somatostatin and growth hormone-releasing hormone in genesis of episodic growth hormone secretion. Metabolis[illegible]y [Suppl]:35-39

Tannenbaum GS, Painson JC, L[illegible]AMJ, Brazeau P 1989 Paradoxical enhancement of pituitary growth hormone (GH) responsiveness to GH-releasing factor in the face of high somatostatin tone. Endocrinology 124:1380-1388

Telford N, May PC, Sinha YN, Porter JC, Finch CE 1992 DOPA accumulates in the hypothalamic-hypophysial portal vessels and is taken into the anterior pituitary of NSD-1015-treated rodents. Neuroendocrinology 55:390-395

Terry LC, Rorstad OP, Martin JB 1980 The release of biologically and immunologically reactive somatostatin from perifused hypothalamic fragments. Endocrinology 107:794-800

Theill LE, Castrillo JL, Wu D, Karin M 1989 Dissection of functional domains of the pituitary-specific transcription factor GHF-1. Nature 342:945-948

Thorner MO, Vance ML 1989 Clinical aspects of dopamine in the regulation of human anterior pituitary function. In: Flukiger E, Muller EE, Thorner MO (eds) The Role of Brain Dopamine. Springer-Verlag, Berlin, Heidelberg, Basic and Clinical Aspects of Neuroscience 3:19-29

Tornquist K, Tashjian AH Jr 1991 Importance of transients in cytosolic free calcium concentrations on activation of Na^+/H^+ exchange in GH4C1 pituitary cells. Endocrinology 128:242-250

Toro MJ, Birnbaumer L, Redon MC, Montoya E 1988 Mechanism of action of somatostatin. Hormone Res 26:59-64

Torres I, Guaza C, Fernandez-Durango R, Borrell J, Charro AL 1982 Evidence for a modulatory role of catecholamines on hypothalamic somatostatin in the rat. Neuroendocrinology 35:159-162

Torsello A, Panzeri G, Cermenati P, Caroleo MC, Ghigo E, Camanni F, Muller EE, Locatelli V 1988 Involvement of the somatostatin and cholinergic systems in the mechanism of growth hormone autofeedback regulation in the rat. J Endocrinol

117:273-281

Towns R, Kostyo JL, Vogel T, Sakal E, Tchelet A, Maher R, Gertler A 1992 Evidence that the N-terminus of human growth hormone is involved in expression of its growth promoting, diabetogenic, and insulin-like activities. Endocrinology 130:1225-1230

Trudeau VL, Somoza GM, Nahorniak CS, Peter RE 1992 Interactions of estradiol with gonadotropin-releasing hormone and thyrotropin-releasing hormone in the control of growth hormone sectetion in the goldfish. Neuroendocrinology 56:483-490

Undie AS, Friedman E 1990 Stimulation of a dopamine D1 receptor enhances inositol phosphates formation in rat brain. J Pharmacol Exp Ther 253:987-992

Van Tol HHM, Bunzow JR, Guan HC, Sunahara RK, Seeman P, Niznik HB, Civelli O 1991 Cloning of the gene for a human dopamine D4 receptor with high affinity for the antipsychotic clozapine. Nature 350:610-614

Vasavan Nair NPV, Lal S, Eugenio H, Lizondo E, Thavundayil JX, Wood PL, Etienne P, Guyda H 1986 CCK-8 antagonizes apomorphine-induced growth hormone secretion in normal subjects. Horm Metabol Res 18:53-55

Vasilatos-Younken R, Cravener TL, Cogburn LA, Mast MG, Wellenreiter RH 1988 Effect of pattern of administration on the response to exogenous, pituitary-derived chicken growth hormone by Broiler-strain pullets. Gen Comp Endocrinol 71:268-283

Vaughan JM, Rivier J, Spiess J, Peng C, Chang JP, Peter RE, Vale W 1993 Isolation and characterization of hypothalamic growth hormone releasing factor from common carp, *Cyprinus carpio*. Neuroendocrinology 56:529-534

Verde G, Oppizzi G, Colussi G, Cremascoli G, Botalla L, Muller EE, Silvestrini F, Chiodini PG, Liuzzi A 1976 Effects of dopamine infusion on plasma levels of growth hormone in normal subjects and in agromegalic patients. Clin Endocrinol 5:419-423

Wakabayashi I, Miyazawa Y, Kanda M, Miki N, Demura R, Demura H, Shizume K 1977 Stimulation of immunoreactive somatostatin release from hypothalamic synaptosomes by high (K^+) and dopamine. Endocrinol Japan 24:601-604

Webster RA 1989 The catecholamines (noradrenaline and dopamine). In : Webster RA, Jordan CC (eds) Neurotransmitter, Drugs and Disease, Blackwell Scientific Publications, pp 95-125

Wehrenberg WB, Brazeau P, Luben R, Bohlen P, Guillemin R 1982 Inhibition of the pulsatile secretion of growth hormone by monoclonal antibodies to the hypothalamic growth hormone releasing factor (GRF). Endocrinology 111:2147-2153

Wehrenberg WB, Seifert H, Bilezikjian LM, Vale W 1986 Down-regulation of growth hormone releasing factor receptors following continuous infusion of growth hormone releasing factor. Neuroendocrinology 43:226-235

Wehrenberg WB, Wiviott SD, Voltz DM, Giustina A 1992 Pyridostigmine-mediated growth hormone release : Evidence for somatostatin involvement. Endocrinology 130:1445-1450

Weiner RI, Findell PR, Kordon C 1988 Role of classic and peptide neuromediators in the neuroendocrine regulation of LH and prolactin. In: Knobil E, Neill JD (eds) The Physiology of Reproduction. Raven Press, New York, pp 1235-1281

Weinshank RL, Adham N, Macchi M, Olsen MA, Branchek TA, Hartig PR 1991 Molecular cloning and characterization of a high affinity dopamine receptor ($D_{1\beta}$) and its pseudogene. J Biol Chem 266:22427-22435

Weiss J, Cronin MJ, Thorner MO 1987 Periodic interactions of GH-releasing factor and somatostatin can augment GH release *in vitro*. Am J Physiol 253:E508-E516

Willoughby JO, Day TA 1981 Central catecholamine depletion: Effects on physiological growth hormone and prolactin secretion. Neuroendocrinology 32:65-69

Willoughby JO, Menadue MF, Liebelt H 1987 Activation of serotonin receptors in the medial basal hypothalamus stimulates growth hormone secretion in the unanesthetized rat. Brain Res 404:319-322

Winiger BP, Wuarin F, Zahnd GR, Wollheim CB, Schlegel W 1987 Single cell monitoring of cytosolic calcium reveals subtypes of rat lactotrophs with distinct responses to dopamine and thyrotropin-releasing hormone. Endocrinology 121:2222-2228

Wood DF, Docherty K, Ramsden DB, Sheppard MC 1987 A comparison of the effects of bromocriptine and somatostatin on growth hormone gene expression in the rat anterior pituitary gland *in vitro.* Mol Cell Endocrinol 52:257-261

Yamauchi K, Nishioka RS, Young G, Ogasawara T, Hirano T, Bern HA 1991 Osmoregulation and circulating growth hormone and prolactin levels in hypophysectomized coho salmon (Oncorhynchus kisutch) after transfer to fresh water and seawater. Aquaculture 92:33-42

Yu KL, Nahorniak CS, Peter RE, Corrigan A, Rivier JE, Vale WW 1987 Brain distribution of radioimmunoassayable gonadotropin-releasing hormone in female goldfish : Seasonal variation and periovulatory changes. Gen Comp Endocrinol 67:234-246

Yu KL, Peng C, Peter RE 1991 Changes in brain levels of gonadotropin-releasing hormone and serum levels of gonadotropin and growth hormone in goldfish during spawning. Can J Zool 69:182-188

Yu KL, Peter RE 1992 Adrenergic and dopaminergic regulation of gonadotropin-releasing hormone release from goldfish preoptic-anterior hypothalamus and pituitary *in vitro.* Gen Comp Endocrinol 85:138-146

Yu KL, Rosenblum PM, Peter RE 1991 *In vitro* release of gonadotropin-releasing hormone from the brain preoptic-anterior hypothalamic region and pituitary of female goldfish. Gen Comp Endocrinol 81:256-267

Yu KL, Sherwood NM, Peter RE 1988 Differential distribution of two molecular forms of gonadotropin-releasing hormone in discrete brain areas of goldfish (*Carassius auratus*). Peptides 9:625-630

Zeitler P, Argente J, Chowen-Breed JA, Clifton DK, Steiner RA 1990b Growth hormone-releasing hormone messenger ribonucleic acid in the hypothalamus of the adult male rat is increased by testosterone. Endocrinology 127:1362-1368

Zeitler P, Tannenbaum GS, Cliton DK, Steiner RA 1991 Ultradian oscillations in somatostatin and growth hormone-releasing hormone mRNA in the brains of adult male rats. Proc Natl Acad Sci USA 88:8920-8924

Zeitler P, Vician L, Chowen-Breed JA, Argente J, Tannenbaum GS, Clifton DK, Steiner RA 1990a Regulation of somatostatin and growth-hormone-releasing hormone gene expression in the rat brain. Metabolism 39 [Suppl]:46-49

Chapter 2

Dopamine Stimulates Growth Hormone Release from the Pituitary of Goldfish through Dopamine D1 receptors [1]

2.1 Introduction

In mammals, the role of dopamine (DA) on growth hormone (GH) regulation is controversial; stimulatory (La Rossa *et al*, 1977), inhibitory (Cronin *et al*, 1984), or no effects (Kato *et al*, 1973) have been reported. In lower vertebrates, the effects of DA on GH release are still largely unknown. Our previous studies suggest that DA may be a GH-releasing factor in teleosts; serum GH levels in the goldfish can be elevated by intraperitoneal injection of the DA agonist apomorphine (Chang *et al*, 1985). Both DA and apomorphine are stimulatory to GH release from perifused goldfish pituitary fragments (Peter *et al*, 1990). Our recent experiments using goldfish pituitary cells in static culture also demonstrated that the DA D1 agonist SKF38393 mimics the GH-releasing effect of DA, but the D2 agonist LY171555 is not effective in this respect (Chang *et al*, 1990b). These results suggest that DA D1 receptors may be present in the anterior pituitary of vertebrates and mediating GH release. Our conclusions are substantiated by a recent report by Bluet-Pajot *et al* (1990), in which DA and the D1 agonist SKF38393 were found to be stimulatory to GH release in the rat, both *in vivo* and *in vitro*.

Besides acting as a GH-releasing factor, DA is also a gonadotropin (GTH)-release inhibitor in the goldfish, both *in vivo* (Chang and Peter, 1983) and *in vitro* (Chang *et al*, 1984). DA exerts its inhibitory effect by reducing GTH-releasing hormone (GnRH) receptor capacity in the pituitary (de Leeuw *et al*, 1989), inhibiting GnRH release from nerve terminals (Yu *et al*, 1991; Yu and Peter, 1991), and suppressing both spontaneous

[1] A version of this chapter has been published : Wong AOL, Chang JP, and Peter RE (1992), **Endocrinology** 130:1201-1210

as well as GnRH-ind d GTH-II release from gonadotrophs (Chang *et al*, 1984). In the goldfish, the negative effect of DA on GTH-II release is mediated via DA D2 receptors (Omeljaniuk *et al*, 1987; Omeljaniuk *et al*, 1989). It appears that DA inhibition on GTH-II release is a common phenomenon in teleosts (Peter *et al*, 1986; Lin *et al*, 1988).

In the present study, the receptor specificity for DA-stimulated GH release in the goldfish is further characterized using an *in vitro* perifusion system for pituitary fragments. Dose-dependency of the GH response to DA stimulation was examined. The GH-releasing actions of three D1 agonists, SKF38393, SKF77434 and SKF82958, and two D2 agonists, bromocriptine and LY171555, were also studied. To confirm that the D1 agonist-stimulated GH release is the result of ligand-receptor interaction, the action of increasing doses of (+)- and (-)-SKF38393 on GH release was tested. The receptor specificity was further investigated by monitoring the effects of two D1 antagonists, SCH23390 and SKF83566, and two D2 antagonists, domperidone and (-)-sulpiride, on the GH responses to DA and SKF38393. The release of GTH-II, which is known to be inhibited by activation of D2 receptors, was also assayed in some experiments to verify the receptor specificity for D1 and D2 drugs in the goldfish.

2.2 Materials and Methods

Animals Goldfish of the common or comet varieties were purchased from Ozark Fisheries (Stoutland, Mo., USA) or Grassyforks Fisheries (Martinsville, Ind., USA), and maintained in flow-through aquaria (1,800 liters) at 17 ^{o}C under a simulated natural photoperiod (Edmonton, Alberta) for at least 3 weeks prior to any experiments. The fish were fed to satiation daily with Ewos trout pellets. Fish of both sexes, with body weight ranging from 20 g to 30 g, were used in the present study. Since reproductive cycling is a characteristic of most teleosts, and these reproductive cycles can be reflected by pronounced variations in gonadal size, gonadal conditions of the goldfish, determined by the gonadosomatic index (GSI = weight of gonad / total body weight x 100 %) and morphological characteristics of the gonads, are reported separately for each individual experiment. The gonads, including the testes in the male and ovaries in the female, are categorized as being regressed when the GSI is ≤1.5% (May to November). Regressed

gonads are thin, translucent, and grayish in color. Ovaries undergoing gonadal recrudescence usually exhibit a grayish-green coloration with a GSI ranging from 1.5% to 8% (December to March). Small vitellogenic oocytes can be frequently identified in these recrudescent ovaries. Ovaries in the prespawning (or mature) phase (March to April) are larger, having a GSI between 8% to 16%. In this case, the vitellogenic oocytes are mature and clearly visible to the naked eye. The gonadal size in male fish is smaller, and rarely exceeds a GSI of 6%. Testes are considered to be recrudescent with a GSI between 1.5% to 3%, and prespawning (or mature) when the GSI is ≥3%. Testes in recrudescence and prespawning phase are white in color.

Reagents and test substances HEPES, dopamine, and bromocriptine were purchased from Sigma Chemical Company (St Louis, MO). SKF38393 (1-phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol hydrochloride), SKF77434 (7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine hydrochloride), SKF82958 (6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrobromide), SCH23390 (7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride), and SKF83566 (7-bromo-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride) were purchased from Research Biochemicals Inc. (Wayland, MA). LY171555 (quinpirole hydrochloride) and domperidone were gifts from Eli Lilly & Co. (Indianapolis, Ind) and Janssen Parmaceutica (Beerse, Belgium), respectively. Aliquots of salmon GnRH (sGnRH) stock solution (Peninsula Lab. Inc., Belmont, Calif.) in 0.1 M acetic acid were frozen at -25 °C, and diluted with the perifusion medium to 50 nM concentration immediately prior to use. All D1 or D2 drugs were first dissolved in dimethyl sulfoxide (DMSO), and the dilutions of the drugs were made up in Hank's Balanced Salt solution so that the final DMSO concentration was less than 0.1%. DMSO up to 0.1% concentration did not alter basal GH and GTH-II release from the pituitary cells of goldfish (Chang *et al*, 1990b).

Perifusion of pituitary fragments *In vitro* experiments were conducted using a perifusion system for goldfish pituitary fragments as described previously (MacKenzie *et al.*, 1984; Marchant *et al.*, 1989a). In brief, goldfish pituitary fragments (≈0.2 mm

in thickness) equivalent to 3 whole pituitaries were placed between 2 layers of 0.1 ml cytodex I beads (Sigma) in a 0.4 ml perifusion column constructed from disposable plastic syringes. The total dead volume of the perifusion system was about 0.75 ml. The fragments were perifused overnight (15-18 hr) at a flow rate of 5 ml/hr with M199 (Gibco, Grand Island, NY, USA) supplemented with Hank's salts, 25 mM HEPES and 56 U/ml nystatin (Sigma). Thereafter, the perifusion medium was switched to Hank's balanced salt solution containing 25 mM HEPES and 0.1 % BSA (Sigma), and the flow rate was increased to 15 ml/hr. After another 3 hr of perifusion, the basal GH and GTH-II release from the pituitary fragments remained relatively constant in the absence of any stimulation. Test substances were then added from a drug reservoir into the perifusion column through a 3-way stopcock. The perifusate from each column was collected in 5-min fractions, and stored frozen at -25 ^{o}C until the hormone contents were assayed. Each experiment was repeated several times using pituitary fragments in separate perifusion columns. Since sGnRH is a known stimulator for both GH and GTH-II release in the goldfish (Marchant *et al*, 1989a; Chang *et al*, 1990b), perifusion for each column was started and terminated with a 2-min pulse of 50 nM sGnRH to monitor the viability and responsiveness of pituitary fragments throughout the experiment. For dose-response studies, DA, D1 and D2 agonists were administered as 2-min pulses at 1 hour intervals, except where specified. For DA antagonist studies, 1-hour continuous perifusion of D1 or D2 antagonist was started one hour following the first pulse of DA or the D1 agonist SKF38393, and a second pulse of the stimulant was given half an hour later in the presence of the corresponding antagonist.

Radioimmunoassay GH levels were measured using a radioimmunoassay (RIA) for carp GH previously validated for the measurement of GH contents in the perifusates from *in vitro* experiments using goldfish pituitary fragments (Marchant *et al*,1989b). The RIA for GTH (Peter *et al*, 1984) is specific for the maturational GTH or GTH-II of the goldfish (Van Der Kraak *et al*, 1991). Samples from each experiment were measured in duplicate in the same RIA.

Data transformation Results from each individual column were expressed as a percentage of the average hormone content obtained in the first 6 fractions prior to any drug treatments (% pretreatment). This transformation was done to allow the pooling of data from separate columns of the same experiment without distorting the profile of hormone release during the course of perifusion. The hormone response was quantified by calculating the net change in hormone release after the drug treatment. The average hormone content (in % pretreatment) in the 3 fractions preceding each 2-min pulse of drug treatment was taken as the basal hormone level for that particular stimulation. Post-pulse fractions with hormone contents greater than the mean plus one SEM of the basal hormone level were included as a part of the hormone response. The net change in hormone release after drug treatment was defined as the sum of the differences between the hormone levels in the post-pulse fractions minus the respective prepulse basal hormone level.

Data analysis and statistics GH and GTH-II data (in % pretreatment) were analyzed by Student's t test or by analysis of variance (ANOVA) followed by Fisher's least significance difference (LSD) test. Differences were considered significant when $P < 0.05$. Dose-response curves for DA and SKF38393, and the corresponding values for the half-maximal effective dose (ED_{50}) and the maximum GH response were determined by the ALLFIT computer program developed by De Lean *et al* (1978).

2.3 Results

Effects of DA on GH release The GH response to 2-min pulses of DA was characterized using pituitary fragments prepared from sexually mature (=prespawning) fish. DA (0.1 nM to 5 μM) was administered either in increasing or decreasing doses in separate experiments (x4 columns each). No significant differences were found between the GH responses to the same dose of DA with the two protocols. Accordingly, only the GH release profile (mean ± SEM) for the experiments with increasing DA doses is presented (Fig. 2.1A) and the data obtained from the two protocols were pooled for the quantification of the GH response (Fig. 2.1B). The GH response to DA was dose-dependent, with an ED_{50} of 0.26 ± 0.06 μM. The minimal effective dose for DA to

induce GH release was 100 nM, and the maximal GH response to DA was estimated to be 185.6 ± 12.5 % pretreatment. The onset of the GH response was observed within 5 minutes after DA treatment, and the response had a duration of 15 - 20 minutes. In preliminary studies using the same experimental protocol (i.e., 2-min DA pulses at 1 hour intervals), prior exposure to 100 nM, 1 μM, or 5 μM of DA did not alter the GH response to subsequent DA challenges at the same dose, indicating that desensitization or potentiation did not occur in this perifusion protocol (data not shown). The GH response to sGnRH was not affected by previous exposure to DA, as no differences were found between the GH responses to the first and second sGnRH pulses (Fig. 2.1, A and B).

Effects of DA D1 agonists on GH and GTH-II release Effects of the DA D1 agonist (±)-SKF38393 on GH and GTH-II release were examined using pituitary fragments from sexually regressed fish. Doses of SKF38393 ranging from 0.05 nM to 10 μM were tested using a total of 16 perifusion columns. Preliminary studies demonstrated that pituitary fragments exposed to 2-min pulses of SKF38393 at (i) 60-min intervals when the dose of (±)-SKF38393 was less than 1 μM, or (ii) 90-min intervals when the dose was equal to or greater than 1 μM, did not cause desensitization in the GH response (data not shown). The D1 agonist (±)-SKF38393 stimulated GH release in a dose-dependent manner with an ED_{50} of 0.41 ± 0.12 μM (Fig. 2.2A, lower panel). The minimal dose of (±)-SKF38393 to stimulate GH release was 80 nM, and the GH response reached a maximum of 405.0 ± 36.1 % pretreatment at 1 μM and higher doses of (±)-SKF38393. In contrast, (±)-SKF38393 did not have any effects on basal GTH-II release (Fig. 2.2B), which indicates a selective action of this D1 agonist on GH release only. In all these experiments, no significant differences were observed in the GH or GTH-II responses between the first and second sGnRH pulses. The stereoselectivity of the GH-releasing effect of SKF38393 was also investigated using pituitary fragments from goldfish in mid-stages of gonadal recrudescence. (+)-SKF38393, at doses from 10 nM to 1 μM, stimulated GH release in a dose-dependent manner (Fig. 2.3A), but (-)-SKF38393 was not effective in this regard (Fig. 2.3B).

Using pituitary fragments from sexually regressed fish, another two DA D1 agonists SKF77434 (Fig. 2.4A) and SKF82958 (Fig. 2.4B) were found to have GH-

releasing activity similar to that of SKF38393. At doses ranging from 10 nM to 1 μM, both D1 agonists enhanced GH release in a dose-dependent manner, but did not alter the basal GTH-II release (data not shown). Estimation of ED_{50} values was not performed for these two D1 agonists, as no obvious maximal GH responses were observed within the dose range tested. Again, no differences were found in the GH responses between the first and second sGnRH pulses in these perifusions, indicating that prior treatment with either SKF77434 or SKF82958 did not alter the GH response to a subsequent sGnRH pulse.

Effects of DA D2 agonists on GH and GTH-II release The effects of two DA D2 agonists, bromocriptine and LY171555, on GH and GTH-II release were examined using pituitary fragments from sexually regressed fish. Repeated 2-min pulses of 0.1 % DMSO, the solvent used for both D1 and D2 drugs, did not alter the basal GH (Fig.2.5A) and GTH-II release (Fig. 2.6A, upper panel). Increasing doses of bromocriptine or LY171555 from 1 nM to 1 μM had no observable effects on basal GH release (Fig. 2.5, B and C); however, the basal GTH-II levels were attenuated in a dose-dependent manner (Fig. 2.6). The estimated ED_{50} values for the inhibitory effects of bromocriptine and LY171555 on GTH-II release were 1.0 ± 0.7 and 11.3 ± 8.1 nM, respectively. In these perifusion experiments, previous exposure to micromolar concentrations of the two D2 agonists significantly reduced the GTH-II but not the GH responses to subsequent sGnRH pulse.

Effects of D1 and D2 antagonists on DA-stimulated GH release The receptor specificity for DA-stimulated GH release was further characterized by using the D1 antagonist SCH23390 and the D2 antagonist domperidone. Pituitary fragments obtained from goldfish in late stages of gonadal recrudescence were used in these experiments. GH levels were consistently elevated by 2-min pulses of 0.5 μM DA. The D1 antagonist SCH23390 at 5 μM concentration had no noticeable effects on the basal GH secretion, but it blocked the GH-releasing action of 0.5 μM DA (Fig. 2.7A, upper panel). The GH response to DA was not affected by simultaneous perifusion with 5 μM of the D2

antagonist domperidone (Fig. 2.7A lower panel). However, domperidone alone caused a small but significant increase in basal GH levels; the mean GH contents in the six fractions collected before and after the beginning of domperidone perifusion were 90 ± 1 and 107 ± 3 % pretreatment, respectively ($P < 0.05$).

In contrast to the GH response, 2-min pulses of 0.5 μM DA caused a decrease in the basal GTH-II levels (Fig. 2.7B); the average GTH-II contents in the six fractions collected before and after the DA treatment were 98 ± 2 and 74 ± 3 % pretreatment, respectively ($P < 0.05$). In general, the GTH-II response to DA occurred after a lag time of 5 - 10 minutes, and the GTH-II levels were maximally suppressed at 15 - 20 minutes after DA treatment. The GTH-II-release inhibitory effect of DA was abolished by simultaneous perifusion with 5 μM of the D2 antagonist domperidone (Fig. 2.7B, lower panel), but not the D1 antagonist SCH23390 (Fig. 2.7B, upper panel). Neither of the two antagonists had any effects on the basal GTH-II levels or the GTH-II response to subsequent sGnRH stimulation.

Effects of D1 and D2 antagonists on SKF38393-stimulated GH release To further examine the specificity of D1 receptor activation on GH release, the effects of two D1 antagonists, SCH23390 and SKF83566, and two D2 antagonists, domperidone and (-)-sulpiride, on the GH response to the D1 agonist SKF38393 were studied using pituitary fragments prepared from goldfish in mid-stages of gonadal recrudescence. Administration of 2-min pulses of the D1 agonist SKF38393 at 0.5 μM consistently elevated the basal GH levels. This GH response was blocked in the presence of 5 μM of the D1 antagonists SCH23390 or SKF83566 (Fig. 2.8, upper panels); however, the D2 antagonists domperidone and (-)-sulpiride were not effective in this respect (Fig. 2.8, lower panels). The mean GH contents in the six fractions collected before and after the onset of perifusion with the D1 antagonist SKF83566 were 98 ± 2 and 147 ± 7 % pretreatment respectively ($P < 0.05$), indicating that this D1 antagonist at 5 μM concentration behaved as a partial agonist to increase basal GH release. No GH-releasing action was observed for the other D1 antagonist SCH23390 at the same concentration. Similar to our previous experiments (Fig. 2.7A, lower panel), treatment with 5 μM of the D2 antagonist domperidone, but not (-)-sulpiride, enhanced the basal GH release; the

average GH levels in the six fractions collected before and after the onset of domperidone perifusion were 89 ± 2 and 116 ± 7 % pretreatment, respectively ($P < 0.05$). Prior exposure to D1 or D2 antagonists did not affect the GH response to subsequent sGnRH challenge.

2.4 Discussion

In the present study, perifusion of goldfish pituitary fragments with DA resulted in differential actions on GH and GTH-II release; DA was stimulatory to GH, but inhibitory to GTH-II release. These results confirm our earlier findings in the goldfish that DA functions as a GTH-release inhibitor (for review, see Peter *et al*, 1986) as well as a GH-releasing factor (Chang *et al*, 1985; Chang *et al*, 1990b; Peter *et al*, 1990). Our data also demonstrate that the GH-releasing action of DA is dose-dependent, with an ED_{50} of 0.26 ± 0.06 μM. This stimulatory effect of DA can not be due to its subsequent conversion to norepinephrine or its cross-reactivity with receptors for norepinephrine or serotonin, as norepinephrine and serotonin are GH-release inhibitors in the goldfish (Chang *et al*,1985; Peter *et al*,1990; Somoza and Peter, 1991). Although a carp GH-releasing hormone (GHRH) has been purified (Vaughan *et al*, 1992) and found to increase GH release in the rainbow trout (Luo and McKeown, 1989) and the goldfish (Peng *et al*, 1990), the role of GHRH in goldfish GH regulation is still unclear.

In contrast to the nanomolar potency of the known peptidergic GH-releasing factors in the goldfish, such as sGnRH (ED_{50}, 2.5 ± 1.4 nM; Marchant *et al.*, 1989a), neuropeptide Y (ED_{50}, 0.5 ± 0.2 nM; Peng *et al.*, 1990), and thyrotropin-releasing hormone (ED_{50}, 0.5 ± 0.2 nM; Trudeau *et al.*, 1992), the ED_{50} for DA-stimulated GH release is in the micromolar concentration range. This may reflect the intrinsic properties of the DA receptor subtype mediating the GH response. In mammals, DA is known to act on two major receptor subtypes, the D1 and D2 receptors; the effective doses for biological responses mediated by DA D1 and D2 receptors are at the micromolar and nanomolar ranges, respectively (for review, see Kebabian and Calne, 1979; Stoof and Kebabian, 1984). The ED_{50} for DA-stimulated GH release in the present study is comparable to that of the known DA D1 systems in mammals, e.g., the DA- or D1 agonist SKF38393-stimulated cAMP release from the striatal tissue of the rat (ED_{50} for

SKF38393, 0.2 μM; see Stoof and Kebabian, 1981) or the cervical sympathetic ganglia of the rabbit (ED_{50} for DA, 6 - 10 μM; see Kebabian and Greengard, 1971). Evidence presented here suggests that DA stimulates GH release from the pituitary of goldfish via the D1 receptors. This hypothesis is further confirmed by our pharmacological studies; the dose-dependent GH-releasing effect of DA was mimicked by the D1 agonists SKF38393, SKF77434 and SKF82958, but not by the D2 agonists bromocriptine and LY171555. Furthermore, the GH response to DA or the D1 agonist SKF38393 was blocked specifically by the D1 antagonists SCH23390 and SKF83566; the D2 antagonists domperidone and (-)-sulpiride were not effective in this respect. With regard to the GH-releasing potency, SKF38393 had an ED_{50} of 0.41 ± 0.12 μM which was not different from that of DA. However, all the three D1 agonists had a higher efficacy in stimulating GH release than DA. Compared to the 1.8-fold increase of GH release induced by 1 μM of DA (i.e., the maximal GH response), the same dose of SKF38393, SKF77434 and SKF82958 elevated the GH release by 4-, 6.5- and 12-fold, respectively. The apparent discrepancy in GH-releasing efficacy may be due to differences in the corresponding affinity for D1 receptors in the goldfish pituitary; however, all these three D1 agonists and DA have similar binding affinities for D1 receptors in mammals (for review, see Seeman and Niznik, 1988). Perhaps, the synthetic D1 drugs have a higher resistance to degradation than DA, thereby contributing to their greater efficacy in GH release. A third DA receptor subtype, the D3 receptor, has been cloned recently, and its pharmacological properties are much closer to that of the D2 receptors than the D1 receptors (for review, see Sibley, 1991). Since specific agonists or antagonists for D3 receptors are not yet available, the possible actions of D3 receptors on GH release were not examined in the present study.

Although the site of action of DA D1 stimulation on GH release was not investigated in the present study, it is likely to be a direct action at the level of somatotrophs in the goldfish pituitary. This is supported by our previous findings that the D1 agonist SKF38393 induced GH release from pituitary cells in primary cell culture (Chang *et al*, 1990b). The possibility of an indirect action of DA on GH secretion through GnRH can also be eliminated, as DA has a negative effect on GnRH release from goldfish pituitary fragments via the D2 receptors (Yu and Peter, 1991).

A key assumption for the interpretation of our data is the receptor specificity of the D1 and D2 drugs in the goldfish, as all of the DA drugs have been validated using the mammalian animal models. It has been well established that activation of D2 receptors in the goldfish is inhibitory to GTH-II release, both *in vivo* (Omeljaniuk *et al*, 1987) and *in vitro* (Omeljaniuk *et al*, 1989). Our results demonstrated that all the three D1 agonists, SKF38393, SKF77434 and SKF82958, had no effects on basal GTH-II levels; however, the two D2 agonists, bromocriptine and LY171555, suppressed GTH-II release with estimated ED_{50} of 1.0 ± 0.7 nM and 11.3 ± 8.1 nM, respectively. Similarly, the GTH-release inhibitory effect of DA was blocked only by the D2 antagonist domperidone but not the D1 antagonist SCH23390. Taken together, the possibility of cross-reactivity of the D1 drugs on D2 receptors and D2 drugs on D1 receptors in the goldfish seems highly unlikely. This further substantiates our previous hypothesis that DA receptors resembling the mammalian D1 and D2 receptor subtypes are present in the pituitary of the goldfish (Chang *et al*, 1990b). Stereoselectivity consistent with the mammalian DA D1 systems has also been demonstrated in the present study; only the (+)- but not the (-)-enantiomer of the D1 agonist SKF38393 was stimulatory to GH release from the goldfish pituitary fragments, and again, the GH response was dose-dependent (for review of stereoselectivity of D1 receptors, see Kaiser, 1983). These observations strongly indicate that the DA D1 receptors are highly conserved during vertebrate evolution.

Unexpectedly, the D2 antagonist domperidone was found to have some degree of GH-releasing activity, but no effects on basal GTH-II release. The domperidone action on GH release could have been indirect through the GH-releasing effect of GnRH (Marchant *et al*, 1989a). The adenohypophysis of teleosts is unique in that it is directly innervated by neurosecretory fibers from the hypothalamus (Peter *et al.*, 1990). In the goldfish, GnRH-immunopositive fibers have been identified close to both GTH- and GH-secreting cells (Kah *et al.*, 1986). Since the D2 antagonist domperidone is known to release GnRH (Yu and Peter, 1991) and deplete DA contents (Sloley *et al*, 1991) in the goldfish pituitary, it is conceivable that domperidone exerts its effect by enhancing GnRH release from nerve terminals in the pituitary fragments. In our previous studies,

sGnRH stimulates GH and GTH-II release from dispersed goldfish pituitary cells with ED_{50} of 0.3 ± 0.1 and 1.9 ± 0.5 nM respectively (Chang *et al*, 1990a), indicating that the GH response to GnRH is more sensitive compared to the corresponding GTH-II response. The treatment with 5 μM domperidone might have induced a small release of GnRH sufficient to cause a GH but not a GTH-II response.

In summary, we have demonstrated that DA stimulates GH release from perifused goldfish pituitary fragments in a dose-dependent manner, and this effect can be mimicked by D1 but not D2 agonists. Similarly, the GH response to DA or the D1 agonist SKF38393 can be blocked only by D1 but not D2 antagonists. The GH response to SKF38393 has a stereoselectivity consistent with that of the mammalian DA D1 systems. These results confirm that the DA-stimulated GH release from the pituitary of goldfish is mediated through the D1 receptors. Our studies also provide a unique model for neuroendocrine regulation in vertebrates; the neurotransmitter DA differentially regulates the release of two distinct hormones, GH and GTH-II, in the same tissue (i.e., the anterior pituitary) through two different receptors, the D1 and D2 receptors, respectively. Although DA D1 receptors have never been identified in the pituitary of vertebrates, our results strongly indicate that this receptor subtype is present in the pituitary of goldfish and is involved in the regulation of GH release. The apparent similarities of the D1 receptor pharmacology between the goldfish and the mammals also suggests that the DA D1 receptors are highly conserved during vertebrate evolution.

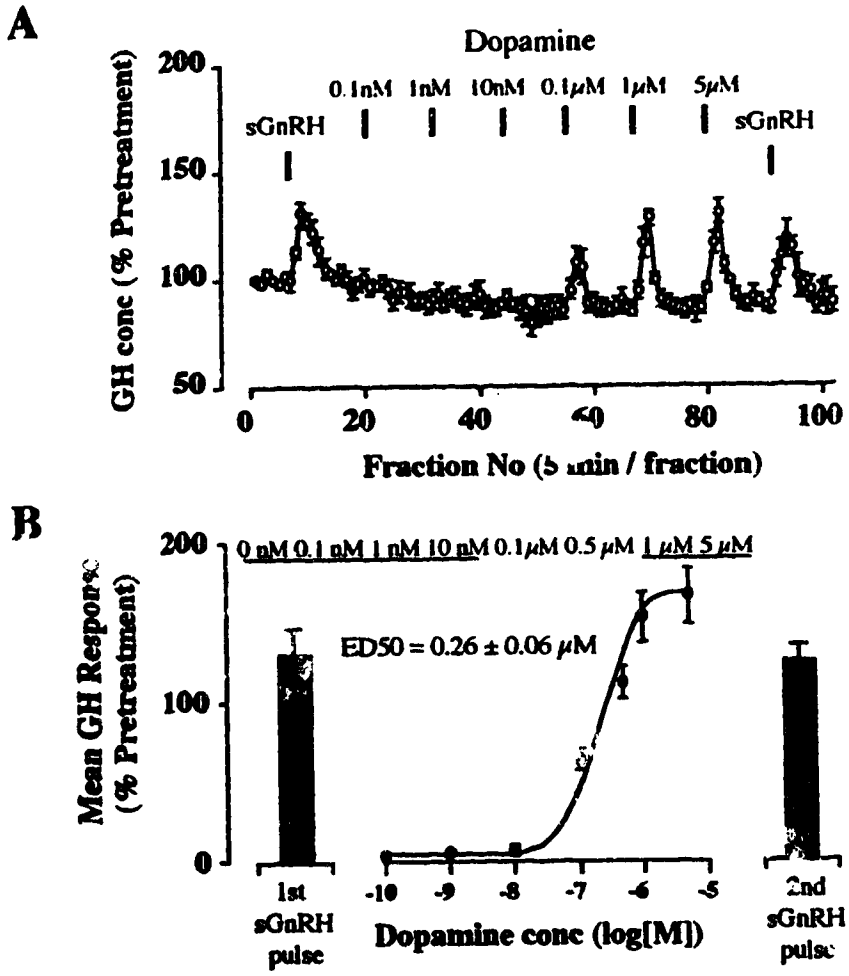


Fig. 2.1 Effects of dopamine (DA) on the growth hormone (GH) release from perifused goldfish pituitary fragments. Only the GH release profile of experiments with increasing doses of DA is presented (A). The average pretreatment GH level was 36.4 ± 2.2 ng GH / ml. Data from experiments with increasing and decreasing doses of DA administration (x4 columns each) were found to have no significant differences, and were pooled together to construct a dose-response curve (B). Doses of DA inducing similar GH responses are grouped within the same underscore ($P > 0.05$, ANOVA followed by Fisher's LSD test). All data are expressed as mean ± SEM.

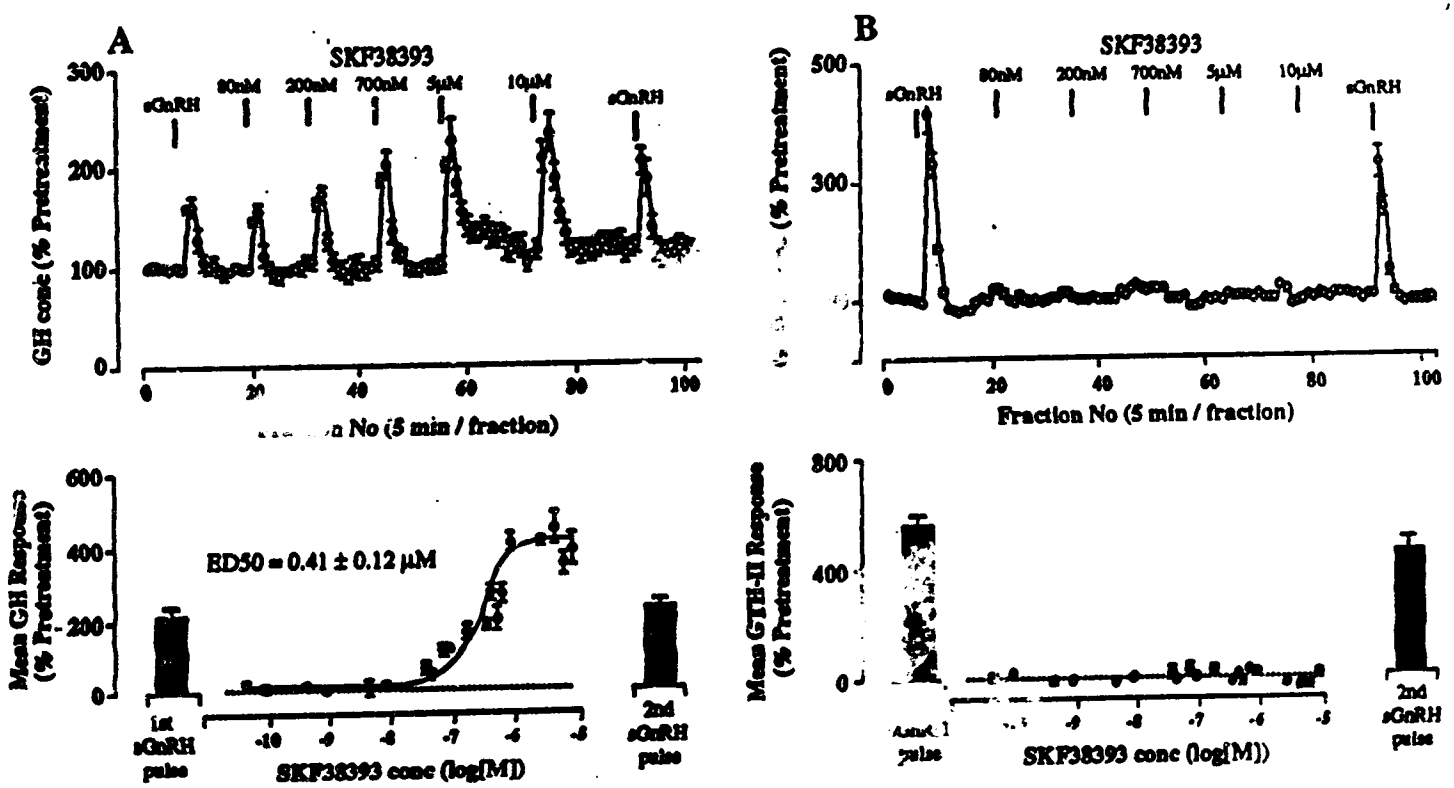


Fig. 2.2 Effects of the D1 agonist SKF38393 (0.05nM - 10 μM) on the GH and GTH-II release from perifused goldfish pituitary fragments. Four series of perifusion experiments with doses of SKF38393 ranging from 0.05 nM to 0.5 μM, 0.1 nM to 1 μM, 40 nM to 7 μM, and 80 nM to 10 μM were performed (x4 columns each). Only the GH (A, upper panel) and GTH-II release profiles (B, upper panel) with doses of SKF38393 from 80 nM to 10 μM are presented. The average pretreatment GH and GTH-II levels were 41.9 ± 1.1 ng GH / ml and 10.4 ± 0.6 ng GTH-II / ml, respectively. The GH responses to SKF38393 with doses ranging from 0.05 nM to 10 μM (a total of 16 columns) were pooled together to construct a dose-response curve (A, lower panel). The GTH-II responses for the same set of experiments are also presented (B, lower panel). All data are expressed as mean ± SEM (n = 4).

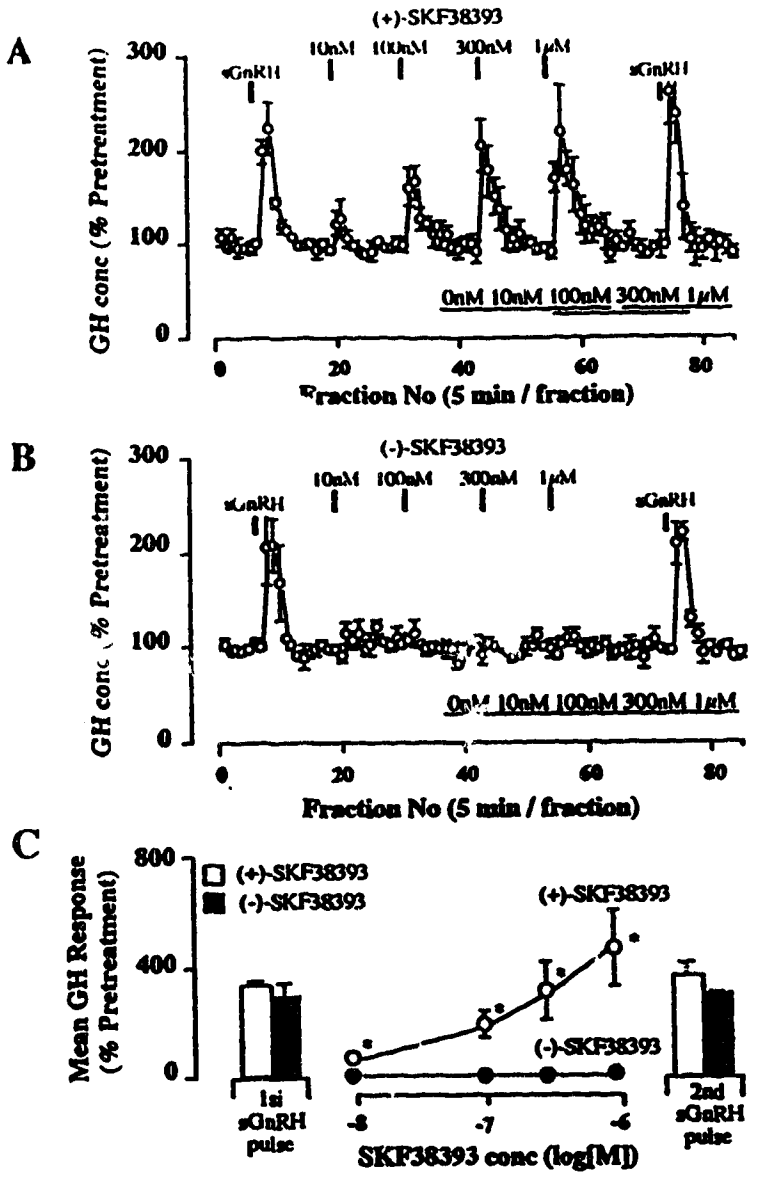


Fig. 2.3 Stereoselectivity of the GH-releasing action of the D1 agonist SKF38393. The GH release profiles for increasing doses (10 nM - 1 μM) of (+)-SKF38393 (A) and (-)-SKF38393 (B) are presented (x3 columns each). The mean pretreatment GH levels for experiments using (+)- and (-)-SKF38393 were 60.6 ± 1.8 and 41.8 ± 2.1 ng GH / ml, respectively. The GH responses were quantitated (C) and doses of SKF38393 giving similar GH responses were grouped within the same underscore ($P > 0.05$, ANOVA followed by Fisher's LSD test). The GH responses to the same dose of (+)- and (-)-SKF38393 are compared using the Student's t test (* ; $P < 0.05$). All data are expressed as mean ± SEM ($n = 3$).

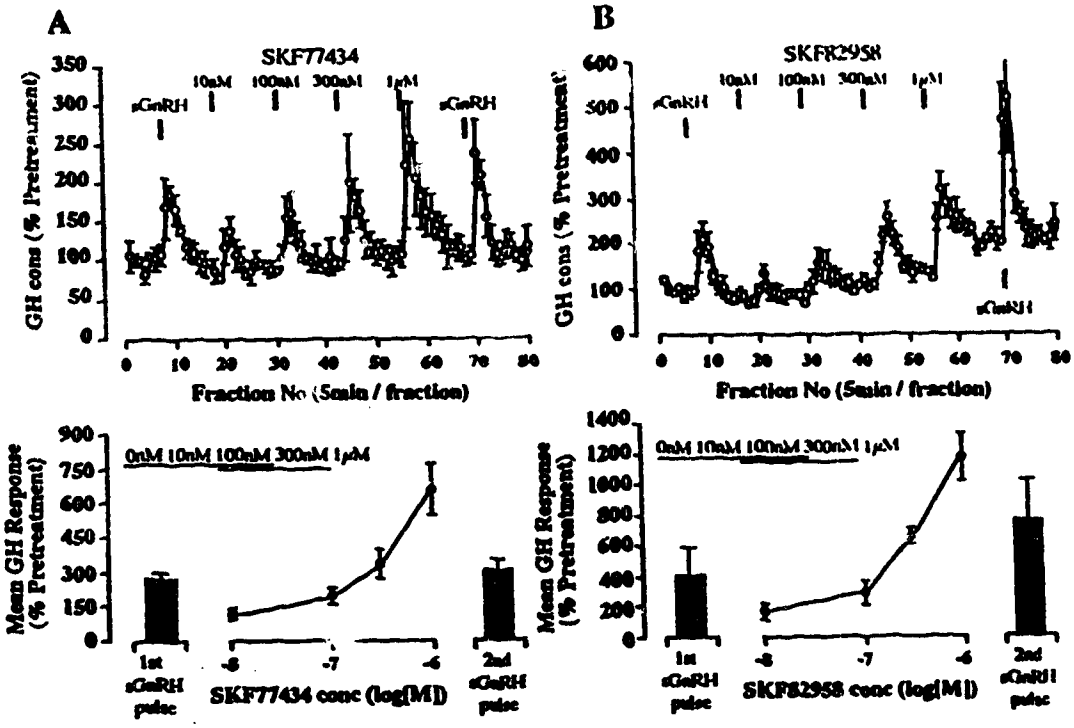


Fig. 2.4 Effects of the D1 agonists SKF77434 and SKF82958 on the GH release from perifused goldfish pituitary fragments. The GH release profiles to increasing doses (10 nM - 1 μM) of SKF77434 (A, upper panel) and SKF82958 (B, upper panel) are presented (x4 columns each). The mean pretreatment GH levels for experiments using SKF77434 and SKF82958 were 29.4 ± 2.8 and 34.7 ± 3.3 ng GH / ml, respectively. The GH responses to the two D1 agonists were quantitated (A and B, lower panels) and doses of the D1 agonist giving similar GH responses were grouped within the same underscore ($P > 0.05$, ANOVA followed by Fisher's LSD test). All data are expressed as mean ± SEM ($n = 4$).

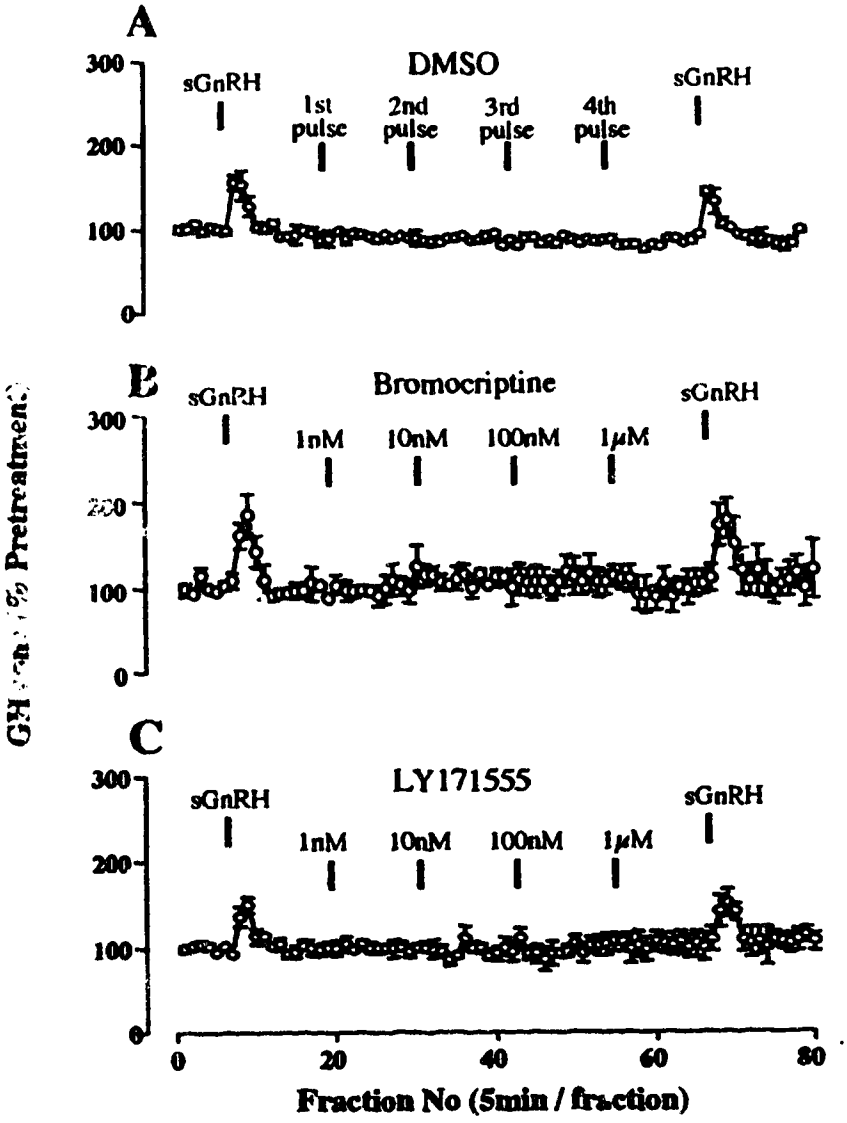


Fig. 2.5 Effects of repeated 2-min pulses of 0.1 % DMSO (A), increasing doses of bromocriptine (B) and LY171555 (C) on the GH release from perifused goldfish pituitary fragments. The mean pretreatment GH levels for the 3 experimental groups were 34.8 ± 0.6, 33.3 ± 2.5 and 39.8 ± 2.5 ng GH / ml, respectively. All data are expressed as mean ± SEM (n = 3).

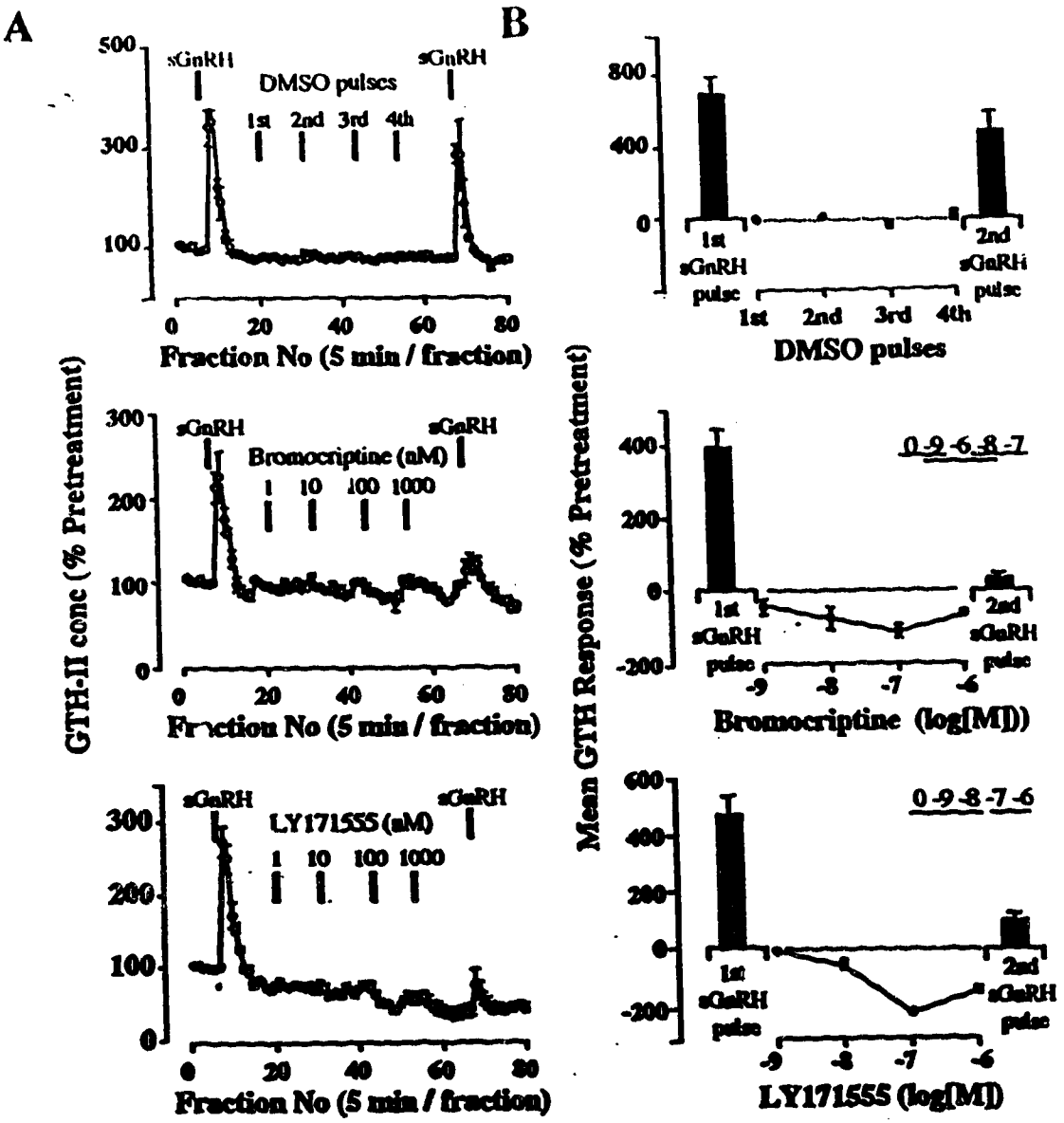


Fig. 2.6 Effects of the D2 agonists bromocriptine and LY171555 on the GTH-II release from perifused goldfish pituitary fragments. The GTH-II release profiles for repeated 2- min pulses of 0.1 % DMSO (A, upper panel), increasing doses of bromocriptine (A, middle panel) and LY171555 (A, lower panel) are presented (x3 columns each). The mean pretreatment GTH-II levels for the 3 experimental groups were 12.1 ± 0.4, 11.5 ± 0.8 and 14.4 ± 0.9 ng GTH-II / ml, respectively. The GTH-II responses to D2 agonists were quantitated as the net decrease in GTH-II contents in the 10 fractions after each 2-min pulse of drug treatment (B), and doses of the D2 agonist giving similar GTH-II responses were grouped within the same underscore ($P > 0.05$, ANOVA followed by Fisher's LSD test). The ED50s for the GTH-release inhibitory effect of bromocriptine and LY171555 were estimated to be 1.0 ± 0.7 nM and 11.3 ± 8.1 nM, respectively. All data are expressed as mean ± SEM (n = 3).

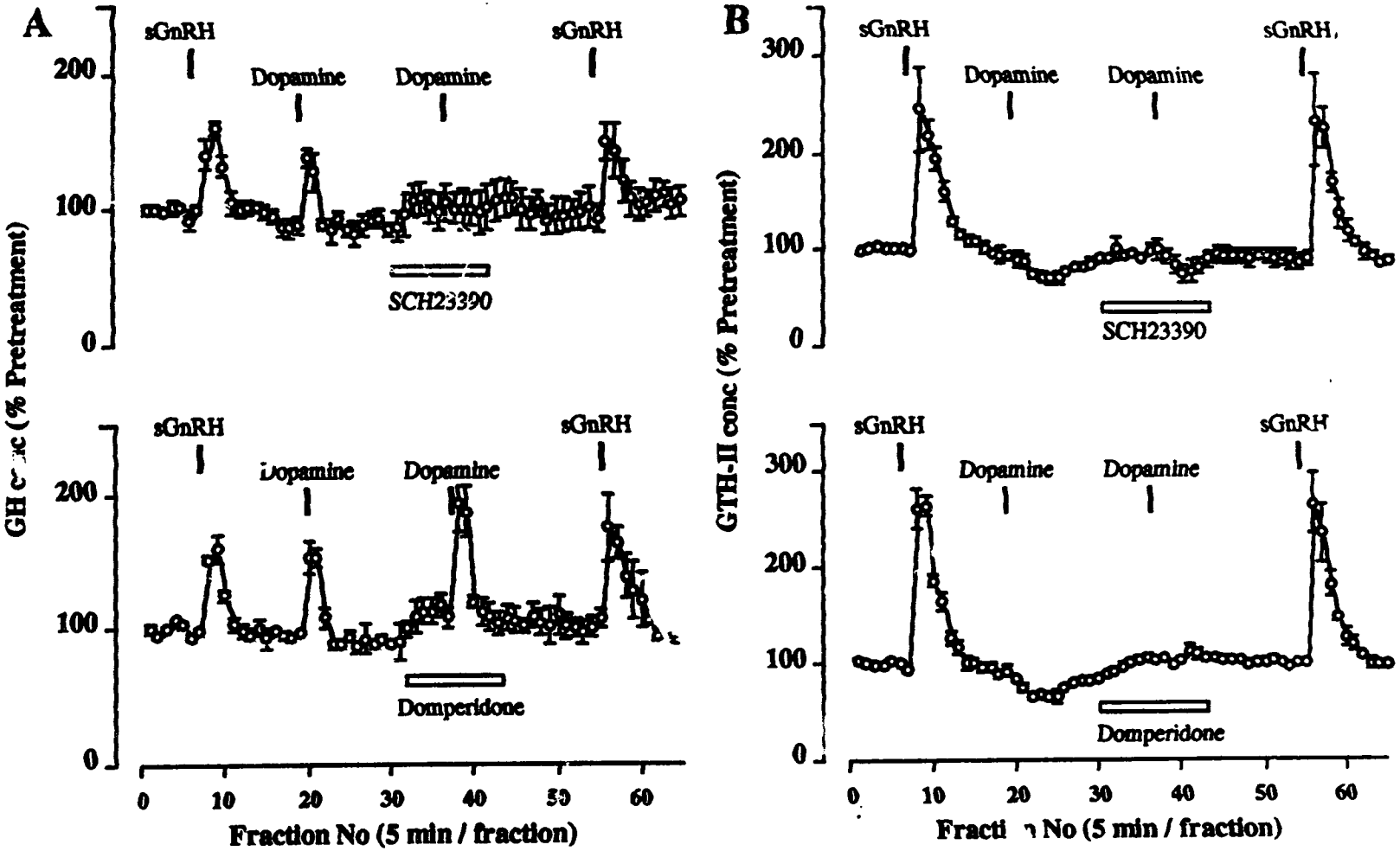


Fig. 2.7 Effects of the D1 antagonist SCH23390 (5μM, upper panels) and the D2 antagonist domperidone (5μM; lower panels) on the GH (A) and GTH-II responses (B) to 0.5 μM DA. The mean pretreatment GH and GTH-II levels were 46.4 ± 2.0 ng GH / ml and 22.1 ± 1.3 ng GTH-II / ml, respectively. Data for each antagonist perifusion were pooled from four separate columns and expressed as mean ± SEM (n = 4).

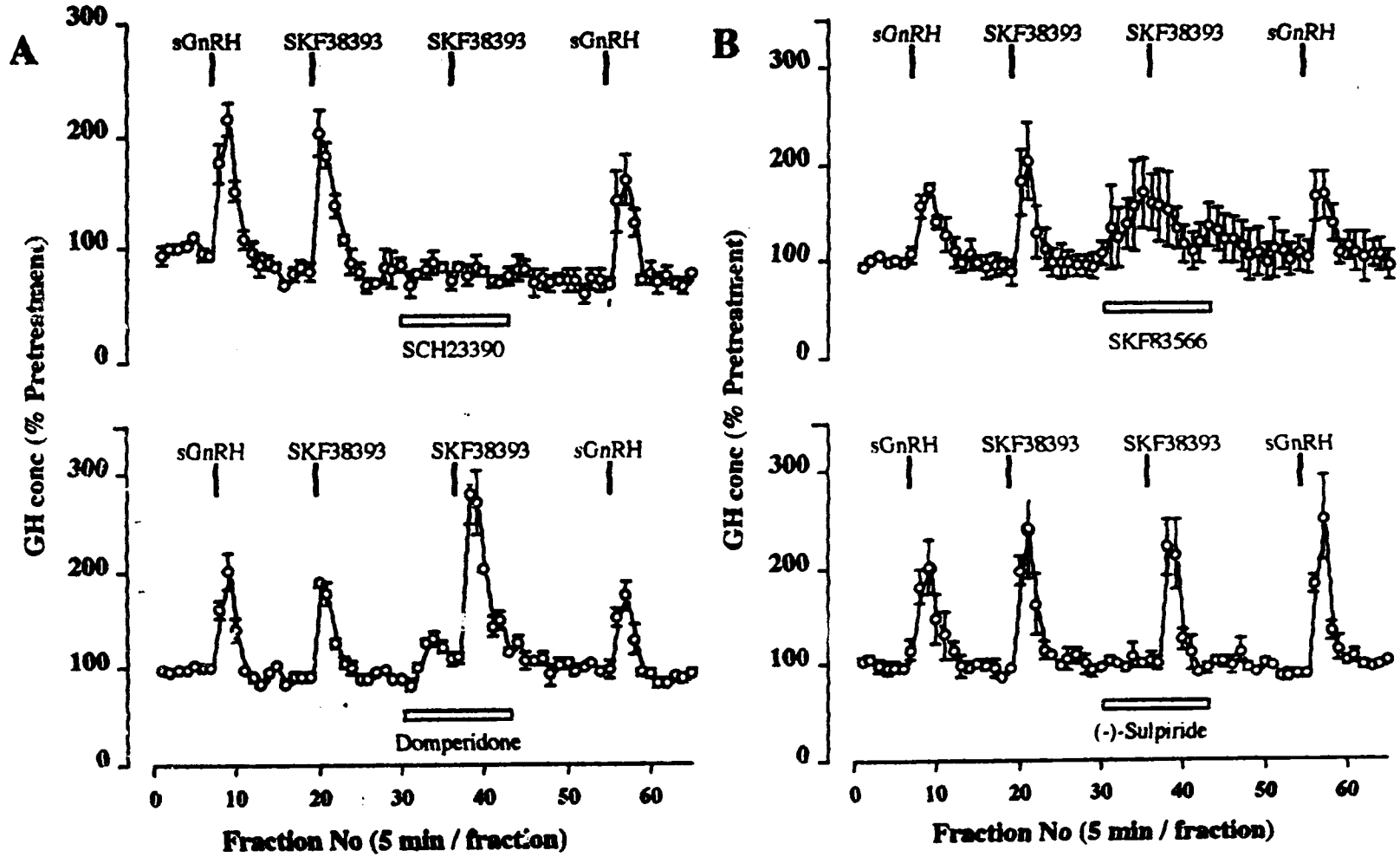


Fig. 2.8 Effects of D1 and D2 antagonists on SKF38393 (0.5 μM) stimulated GH release from perifused goldfish pituitary fragments. The GH release profiles for experiments using 5 μM of the D1 antagonists (upper panels), SCH23390 (A) and SKF83566 (B), and 5 μM of the D2 antagonists (lower panels), domperidone (A) and (-)-sulpiride (B) are presented (x4 replicates each). The mean pretreatment GH levels for the four series of perifusion experiments were 38.8 ± 2.1, 45.0 ± 1.4, 49.7 ± 1.4, and 39.5 ± 1.7 ng GH / ml, respectively. All data are expressed as mean ± SEM (n = 4).

2.5 References

Bluet-Pajot MT, Mounier F, Durand D, Kordon C 1990 Involvement of dopam ' D1 receptors in the control of growth hormone secretion in the rat. J E ' nol 127:191-196

Chang JP, Cook H, Freedman G, Wiggs AJ, Somoza GM, de Leeuw F r RE 1990a Use of pituitary cell dispersion method and primary culture system for the studies of gonadotropin-releasing hormone action in the goldfish *Carassius auratus*. I. Initial morphological, static, and cell column perifusion studies. Gen Comp Endocrinol 77:256-273

Chang JP, MacKenzie DS, Gould DR, Peter RE 1984 Effects of dopamine and norepinephrine on *in vitro* spontaneous and gonadotropin-releasing hormone induced gonadotropin release by dispersed cells or fragments of the goldfish pituitary. Life Sci 35:2027-2033

Chang JP, Marchant TA, Cook AF, Nahorniak CS, Peter RE 1985 Influences of catecholamines on growth hormone release in female goldfish, *Carassius auratus*. Neuroendocrinology 40:463-470

Chang JP, Peter RE 1983 Effects of dopamine on gonadotropin release in female goldfish, *Carassius auratus*. Neuroendocrinology 36:351-357

Chang JP, Yu KL, Wong ALC, Peter RE 1990b Differential actions of dopamine receptor subtypes on gonadotropin and growth hormone release *in vitro* in goldfish. Neuroendocrinology 51:664-674

Cronin MJ, Thorner MO, Hellman P, Rogol AD 1984 Bromocriptine inhibits growth hormone release from pituitary cells in primary culture. Proc Soc Exp Biol Med 175:191-195

de Leeuw R, Habibi HR, Nahorniak CS, Peter RE 1989 Dopaminergic regulation of pituitary gonadotropin-releasing hormone receptor activity in the goldfish (*Carassius auratus*). J Endocrinol 121:239-247

DeLean A, Munson PJ, Rodbard D 1978 Simultaneous analysis of families of sigmoidal curves : application of bioassay, radioligand assay, and physiological dose-response curves. Am J Physiol 235:E97-E102

Kah O, Breton B, Dulka JG, Nunez-Rodriguez J, Peter RE, Corrigan A, Rivier JE, Vale WW 1986 A re-investigation of the GnRH (gonadotrophin-releasing hormone) systems in the goldfish brain using antibodies to salmon GnRH. Cell Tissue Res 244:321-337

Kato Y, Dupre J, Beck JC 1973 Plasma growth hormone in the anesthetized rat : Effects of dibutyryl cyclic AMP, prostaglandin E1, adrenergic agents, vasopressin, chlorpromazine, amphetamine and L-dopa. Endocrinology 93:135-146

Kaiser C 1983 Stereoisomeric probes of dopamine receptor. In: Kaiser C, Kebabian JW (eds) Dopamine receptors. American Chemical Society, Washingston DC, ACS Symposium series 224:223-246

Kebabian JW, Calne DB 1979 Multiple receptors for dopamine. Nature 277:93-96

Kebabian JW, Greengard P 1971 Dopamine-sensitive adenyl cyclase : Possible role in synaptic transmission. Science 174:1346-1349

La Rossa J, Agrin R, Melby JC 1977 Apomorphine-stimulated growth hormone release. Am J Med 63:907-913

Lin HR, Van Der Kraak G, Zhou XJ, Liang JY, Peter RE, Rivier JE, Vale WW 1988 Effects of [D-Arg^6, Trp^7, Leu^8, Pro^9 NEt]-luteinizing hormone-releasing hormone (LHRH-A), in combination with pimozide or domperidone, on gonadotropin release and ovulation in the chinese loach and common carp. Gen Comp Endocrinol 69:31-40

Luo D, McKeown BA 1989 An antioxidant dependent in vitro response of rainbow trout (*Salmo gairdneri*) somatotrophs to carp growth hormone-releasing factor (GRF). Horm Metabol Res 21:690-692

MacKenzie DS, Gould JP, Peter RE, Rivier JE, Vale WW 1984 Response of superfused goldfish pituitary fragments to mammalian and salmon gonadotropin-releasing hormone. Life Sci 35:2019-2026

Marchant TA, Chang JP, Nahorniak CS, Peter RE 1989a Evidence that gonadotropin-releasing hormone also function as a growth hormone-releasing hormone in the goldfish. Endocrinology 124:2509-2518

Marchant TA, Dulka JG, Peter RE 1989b Relationship between serum growth hormone levels and the brain and pituitary content of immunoreactive somatostatin in the

goldfish, *Carassius auratus*. Gen Comp Endocrinol 73:458-468

Omeljaniuk RJ, Shih SH, Peter RE 1987 *In vivo* evaluation of dopamine receptor-mediated inhibition of gonadotropin secretion from the pituitary gland of the goldfish. J Endocrinol 114:449-458

Omeljaniuk RJ, Tonon MC, Peter RE 1989 Dopamine inhibition of gonadotropin and a melanocyte-stimulating hormone release *in vitro* from the pituitary of the goldfish (*Carassius auratus*). Gen Comp Endocrinol 74:451-467

Peng C, Chang JP, Peter RE 1990 Growth hormone-releasing factor (GRF) stimulates growth hormone (GH) secretion from pituitary cells of the goldfish. The 29th Annual meeting of the Canadian Society of Zoologists, Vancouver. Bulletin 21:79 (abstract)

Peng C, Huang YP, Peter RE 1990 Neuropeptide Y stimulates growth hormone and gonadotropin release from the goldfish pituitary *in vitro*. Neuroendocrinology 52:28-34

Peter RE, Chang JP, Nahorniak CS, Omeljaniuk RJ, Sokolowska M, Shih SH, Billard R 1986 Interactions of catecholamines and GnRH in regulation of gonadotropin secretion in teleost fish. Recent Prog Horm Res 42:513-548

Peter RE, Nahorniak CS, Chang JP, Crim LW 1984 Gonadotropin release from the pars distalis of the goldfish, *Carassius auratus*, transplanted beside the brain or into the brain ventricles : additional evidence for a gonadotropin release-inhibitory factor. Gen Comp Endocrinol 55:337-346

Peter RE, Yu KL, Marchant TA, Rosenblum PM 1990 Direct neural regulation of the teleost adenohypophysis. J Exp Zool (suppl) 4:84-89

Seeman P, Niznik MB 1988 Dopamine D1 receptor pharmacology. Pharmacology 2:161-170

Sibley DR 1991 Cloning of a 'D3' receptor subtype expands dopamine receptor family. Trend Pharmacol Sci 12:7-9

Sloley BD, Trudeau VL, Dulka JG, Peter RE 1991 Selective depletion of dopamine in the goldfish pituitary caused by domperidone. Can J Physiol Pharmacol 69:776-781

Somoza GM, Peter RE 1991 Effect of serotonin on gonadotropin and growth hormone

release from in vitro perifused goldfish pituitary fragments. Gen Comp Endocrinol 82:103-110

Stoof JC, Kebabian JW 1984 Two dopamine receptors : Biochemistry, physiology, and pharmacology. Life Sci 35:2281-2296

Stoof JC, Kebabian JW 1981 Opposing roles for D1 and D2 dopamine receptors in efflux of cAMP from rat neostriatum. Nature 294:266-268

Trudeau VL, Somoza GN, Nahorniak CS, Peter RE 1992 Interaction of estradiol with gonadotropin releasing hormone and thyrotropin releasing hormone in the control of growth hormone secretion in the goldfish. Neuroendocrinology 56:483-490

Van Der Kraak G, Suzuki K, Peter RE, Itoh H, Kawauchi H 1991 Properties of common carp gonadotropin I and gonadotropin II. Gen Comp Endocrinol 85:217-229

Vaughan J, Rivier J, Spiess J, Peng C, Chang JP, Peter RE, Vale W 1992 Isolation and characterization of hypothalamic growth hormone releasing factor from common carp, *Cyprinus carpio.* Neuroendocrinology 56:529-534

Yu KL, Rosenblum FM, Peter RE 1991 *In vitro* release of gonadotropin-releasing hormone from the brain preoptic-anterior hypothalamic region and pituitary of female goldfish. Gen Comp Endocrinol 81:256-267

Yu KL, Peter RE 1991 Adrenergic and dopaminergic regulation of gonadotropin-releasing hormone release from goldfish preoptic-anterior hypothalamus and pituitary *in vitro.* Gen Comp Endocrinol 85:138-146

Chapter 3

Actions of Dopamine as a Growth Hormone-Releasing Factor in the Goldfish [1]

3.1 Introduction

In mammals, dopamine (DA) functions as a neurotransmitter as well as a neurohormone. Secretion of DA into the hypothalamo-hypophyseal portal system has been reported in the rat (Gibbs and Neill, 1978). Its inhibitory effects on the release of pituitary hormones, such as prolactin, thyroid-stimulating hormone, and melanocyte-stimulating hormone are well documented (for review, see Tuomisto and Mannisto, 1985). Although internalization of [^{3}H]-DA has been demonstrated in somatotrophs (Rosenzweig and Kanwar, 1982), the role of DA on growth hormone (GH) release in mammals is still controversial, as stimulatory, inhibitory, and no effects have been reported (for review, see Muller, 1987).

In lower vertebrates, such as the teleost, even less is known about the actions of DA in GH regulation. In the goldfish, the anterior pituitary is under the direct innervation of a dopaminergic preoptico-hypophyseal pathway (Kah *et al.*, 1987), and the nerve terminals of DA neurons have been identified in close promixity to the somatotrophs (Kah *et al.*, 1984). The possible involvement of DA in GH regulation as suggested by these anatomical studies was confirmed by our previous findings that DA and its non-selective agonist apomorphine are stimulatory to GH release in the goldfish, both *in vivo* (Chang *et al.*, 1985) and *in vitro* (Peter *et al.*, 1990). More recently, we also provided *in vitro* evidence that the GH response to DA in the goldfish is mediated through the DA D1 receptors (Chang *et al.*, 1990b; Wong *et al.*, 1992). These results suggest that DA, besides its role as a neurotransmitter, may also function as a GH-releasing factor in teleosts.

---

1 A version of this chapter has been accepted for publication : Wong AOL, Chang JP, and Peter RE (1993) **Am J Physiol.**

In our previous studies, we used a pharmacological approach to demonstrate the GH-releasing actions of DA in the goldfish. However, evidence to substantiate the physiological role of DA as a GH-releasing factor is still lacking. In the present study, we put forward further evidence to support the hypothesis that DA, by acting through the DA D1 receptors in the pituitary, also functions as a GH-releasing factor in the goldfish. Seasonality of the GH responses to DA, and the actions of the GH-release inhibitor somatostatin (SRIF) on DA-stimulated GH release were examined. Receptor specificity for the GH-releasing effects of DA was confirmed *in vivo* using the non-selective DA agonist apomorphine, the D1 agonist SKF82958, and the D1 antagonist SCH23390. Effects of long-term feeding with apomorphine on the body growth of goldfish were also investigated.

3.2 Materials and Methods

Animals Goldfish of the common or comet varieties were purchased from Ozark Fisheries (Stoutland, MO, USA) or Grassyforks Fisheries (Martinsville, IN, USA), and maintained in flow-through aquaria (1,800 liters) at 17 °C under a simulated natural photoperiod (Edmonton, Alberta, Canada) for at least 3 weeks prior to any experiments. The fish were fed to satiation daily with Ewos trout pellets (pellet size 5P; Astra Chemicals Ltd., Mississauga, Ontario, Canada). Goldfish of both sexes, with body weight ranging from 15 g to 30 g, were used in the present study. Since reproductive cycling is a characteristic of most teleosts, and these reproductive cycles can be reflected by pronounced variations in gonadal size, gonadal conditions of the goldfish, determined by the gonadosomatic index (GSI = weight of gonad / total body weight x 100 %) as well as the morphological characteristics of the gonads, are reported separately for individual experiments. The gonad was categorized as sexually regressed if GSI was $\leq$ 1.5 %, as sexually recrudescent if GSI was between 1.5 % to 8 %, and as sexually mature (=prespawning) if GSI was $\geq$ 8 % (for details, refer to chapter 2).

Reagents and test substances Dopamine, apomorphine, and somatostatin were purchased from Sigma Chemical Company (St Louis, MO, USA). The D1 agonist SKF82958 and the D1 antagonist SCH23390 were purchased from Research

Biochemicals Inc. (Wayland, MA, USA). The D2 antagonist pimozide was a generous gift from Janssen Pharmaceutica (Beerse, Belgium). For *in vivo* experiments, apomorphine, pimozide, SKF82958 and SCH23390 were first dissolved in a minimal amount of dimethyl sulfoxide (DMSO), and subsequently diluted to appropriate concentrations with propylene glycol. Somatostatin was dissolved directly in 0.7 % saline. For *in vitro* experiments, dopamine and somatostatin were dissolved in perifusion medium (Hank's balanced salt solution supplemented with 25 mM HEPES and 0.1 % BSA). To avoid oxidation of the DA stock solution, DA was solublized 5 min before giving the 2-min pulses of drug treatment. Aliquots of salmon gonadotropin (GTH)-releasing hormone (sGnRH) (Peninsula Laboratories Inc., Belmont, CA, USA) in 0.1 M acetic acid stock solution were frozen at -25 °C, and diluted with perifusion medium to 50 nM concentration immediately prior to use. All other materials used in the experiments were obtained from commercial sources and were of the highest quality available.

In vitro experiments An *in vitro* perifusion system for goldfish pituitary fragments (Marchant *et al.*, 1989a) was used to investigate the seasonality of the GH-releasing actions of DA and the effects of SRIF on DA-stimulated GH release. In brief, goldfish pituitary fragments (≈0.2 mm in thickness) equivalent to 3 whole pituitaries were loaded between 2 layers of 0.1 ml cytodex I beads (Sigma) in a 0.4 ml perifusion column constructed from disposable plastic syringes. The total dead volume of the perifusion system was about 0.75 ml. The fragments were perifused overnight (15 - 18 hr) at a flow rate of 5 ml/hr with M199 (Gibco, Grand Island, NY, USA) supplemented with Hank's balanced salt, 25 mM HEPES and 56 U/ml nystatin (Sigma). Thereafter, the perifusion medium was switched to Hank's balanced salt solution containing 25 mM HEPES and 0.1 % BSA (Sigma), and the flow rate increased to 15 ml/hr. After another 3 hr of perifusion, the basal GH and GTH-II release from the pituitary fragments remained relatively constant in the absence of any stimulation. Test substances were then added from a drug reservoir into the perifusion column through a 3-way stopcock. Since sGnRH is a known stimulator for both GH and GTH-II release in the goldfish (Marchant *et al.*, 1989a), two-minute pulses of 50 nM sGnRH were given at the beginning and at

the end of each perifusion to monitor the responsiveness and viability of pituitary fragments throughout the course of perifusion experiment. DA from 0.1 nM to 5 μM concentrations was administered as two-minute pulses at one-hour intervals. The protocol used for DA perifusion has been previously validated to have no potentiating nor desensitizing effects on GH release within the doses tested (Wong *et al.*, 1992). For the studies of SRIF actions on DA-induced GH release, one-hour continuous perifusion of 100 nM SRIF was initiated one hour after the first two-minute pulse of 500 nM DA, and a second 500 nM DA pulse was given half an hour later in the presence of SRIF. Perifusates were collected in 5-minute fractions and stored at -25 °C until their GH contents were assayed. Since DA is known to inhibit GTH-II release in the goldfish via the D2 receptors (for review, see Peter *et al.*, 1986), GTH-II contents in the perifusates for SRIF perifusion experiments were also measured to serve as a negative control. Profiles of hormone release during the course of perifusion and the net hormone responses to drug treatment were expressed as "% pretreatment" as defined previously (Wong *et al.*, 1992). In brief, the GH and GTH-II data from each individual perifusion column were expressed as a percentage of the corresponding average hormone content obtained in the first six fractions of perifusates prior to any drug treatments. This transformation was done to allow pooling of the data from separate columns of the same experiment, without distorting the profile of hormone release during the course of perifusion. Hormone responses were quantified by calculating the net change in hormone release (i.e., area under the curve) after a particular drug treatment.

Pituitary fragments of the goldfish are known to contain nerve terminals from the hypothalamus (Peter *et al.*, 1990), and there is a possibility that the actions of SRIF on GH release may be exerted indirectly through these nerve terminals. To confirm that the influence of SRIF on DA-stimulated GH release is directly at the pituitary cell level, the effects of 0.1 nM to 10 nM SRIF on GH release were examined in the presence or absence of 1 μM DA using a static incubation system for goldfish pituitary cells (Chang *et al.*, 1990b). The preparation of pituitary cells has been previous validated to have no contamination of nerve terminals (Chang *et al.*, 1990a). Both the GH and GTH-II contents in culture medium were assayed, and the data were expressed as a percentage of the basal hormone release in the control wells without any drug treatments (as "%

control").

In vivo experiments Fish were anesthetized by submersion in 0.05 % tricaine methanesulfonate (Syndel Laboratories Ltd., Vancouver, B.C., Canada) prior to experimental handling. The effects of SRIF on DA-induced GH release *in vivo* were investigated by intraperitoneal (i.p.) injection of a non-selective DA agonist apomorphine (20 μg/g body weight) in the presence or absence of SRIF (1 μg/g body weight). The DA receptor subtype mediating the GH responses to apomorphine and the D1 agonist SKF82958 were also examined. Apomorphine (20 μg/g body weight) or SKF82958 (20 μg/g body weight) was injected (i.p.) in the presence or absence of 40 μg/g body weight of the D1 antagonist SCH23390 or the D2 antagonist pimozide. For each experiment, the fish were tagged with numbered metal clips on the operculum for individual identification. About 200 μl of blood was taken from the caudal vasculature of each fish using heparinized syringes just prior to injection of drugs and 5 hours after the drug treatment. Plasma was collected after centrifugation, and assayed for GH and GTH-II contents. For each individual fish, the plasma hormone concentration after drug treatment was expressed as a percentage of the hormone level before drug administration (as "% presample").

To examine the effects of orally administered apomorphine on body growth and plasma GH levels, apomorphine was dissolved in 1 % DMSO and absorbed into Ewos trout pellets (pellet size 5P) at a dose of 20 mg/g pellets. After overnight lyophilization, the apomorphine-treated pellets were gased with argon and stored at -25 °C. Trout pellets prepared in a similar manner with 1 % DMSO and stored under argon gas, but without apomorphine were used for the control treatment. To test whether apomorphine is orally active in the goldfish in terms of GH release, three groups of fish from the same stock were not fed for two days. In the morning of the third day, one group was sacrificed for blood sampling (control group), and the other two groups were fed at an excess ration of 5 % body weight with apomorphine- and vehicle-treated pellets, respectively. Five hours after feeding, blood samples were taken from these two groups of fish. Plasma samples were then collected, and assayed for both GH and GTH-II

contents. Owing to the stress of blood sampling on feeding behavior in the goldfish, presampling of blood prior to the feeding experiment was not performed, and the hormone data were simply expressed as ng hormone/ml plasma.

To investigate the effects of long-term feeding with apomorphine on the body growth of goldfish, two groups of fish from the same stock were accomodated in parallel tanks (~15 fish per each 96-liter flow-through aquarium) at 17 °C and under 16L:8D photoperiod, and were tagged for individual identification. The initial body weight and length for each fish were recorded as described previously (Marchant *et al.*, 1989a). The fish were fed in the morning at a ration of 5 % body weight per day, and the data of body weight and length were taken in the late evening. The fish were fed with normal trout pellets for the first 30 days (referred as the "initial phase"). After that, one group of fish was fed with apomorphine-treated pellets for another 55 days (referred as the "final phase"), while the other group served as the control and was fed with vehicle-treated pellets. Body weight and length for each fish were measured once every 10 days. By the end of the feeding experiment, data for body weight and length from individual fish were expressed as a percentage of the corresponding initial values.

Radioimmunoassay GH contents were measured using a radioimmunoassay (RIA) previously validated for the measurement of goldfish GH (Marchant *et al.*, 1989b). The RIA for GTH-II (Peter *et al.*, 1984) is specific for the maturational GTH or GTH-II of the goldfish (Van Der Kraak *et al.*, 1991). Samples from each experiment were measured in duplicate in the same RIA.

Data analysis and statistics Data for hormone contents and growth rates were analyzed by Student's *t* test or by analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test. Differences were considered significant when $P<0.05$. Dose-response curves for DA, and the corresponding values for the half-maximal effective dose (ED_{50}) and the maximal GH response were determined by ALLFIT program (DeLean *et al.*, 1978).

3.3 Results

Seasonality of GH response to DA stimulation GH responses to two-minute pulses of DA with doses ranging from 0.1 nM to 5 μM were characterized using pituitary fragments prepared from sexually mature (=prespawning) fish (x4 columns) and sexually regressed fish (x6 columns), respectively (Fig. 3.1A). In both cases, DA stimulated GH release in a dose-dependent manner. Characteristics of the two dose-response curves are compared in Table 3.1; ED_{50} values and slope factors showed no significant differences, whereas the maximal GH response was higher and the minimal dose of DA to trigger GH release was lower in the dose-response curve for sexually regressed fish. Data from a preliminary study using sexually recrudescent goldfish (x2 columns) are also presented; the dose-response curve was intermediate between the curves for sexually mature and regressed fish (Fig. 3.1B). Since only four data points were included in the curve for recrudescent fish, a detailed comparison with the dose-response curves from sexually mature and regressed fish was not performed. Previous exposure to DA did not alter the subsequent GH responses to sGnRH, as no significant differences were observed between the average GH responses to the first and second sGnRH pulses in the two perifusion experiments (118 ± 10 *vs* 124 ± 17 % pretreatment, respectively) (Fig. 3.1A).

SRIF on DA-stimulated GH release Pituitary fragments prepared from sexually regressed goldfish were used to study the actions of SRIF on DA-stimulated GH release (x4 columns). In the presence of 100 nM SRIF, basal GH release was significantly suppressed (mean GH levels for the six fractions before and after SRIF perifusion were 59 ± 1 and 22 ± 2 ng/ml, respectively; $P<0.05$) and the GH response to 500 nM DA was completely abolished (Fig. 3.2A). Previous exposure to SRIF also reduced the GH response to subsequent sGnRH stimulation, as the net GH response to the second sGnRH pulse (81 ± 17 % pretreatment) was significantly lower than that of the first one (177 ± 7 % pretreatment; $P<0.05$). This is in accordance with our previous findings that sGnRH-stimulated GH release in the goldfish can be blocked by SRIF (Marchant *et al.*, 1989a). However, the inhibitory effects of DA on basal GTH-II release and the GTH-II responses to sGnRH were not affected by SRIF treatment (Fig. 3.2B), indicating a

selective action of SRIF on GH release in the goldfish (for a review on DA inhibition on GTH-II release in teleosts, see Peter *et al.*, 1986).

To test the direct action of SRIF on DA-stimulated GH release at the pituitary cell level, the interactions between SRIF and DA were further examined using pituitary cells prepared from goldfish in the early stages of sexual recrudescence. SRIF at doses from 0.1 nM to 10 nM suppressed both basal and DA-stimulated GH release in a dose-dependent manner (Fig. 3.3A). In contrast, SRIF did not affect the inhibitory actions of DA on basal GTH-II secretion (Fig. 3.3B).

The inhibitory effect of SRIF on DA-stimulated GH release was also investigated *in vivo* using sexually regressed goldfish. Apomorphine, a non-selective DA agonist, administered at 20 µg/g body weight (i.p.) induced a significant increase in plasma GH levels (Fig. 3.4A), but at the same time reduced the plasma GTH-II levels (Fig. 3.4B). SRIF at a dose of 1 µg/g body weight (i.p.) significantly attenuated both the basal and apomorphine-stimulated GH release. Similar to the results of the preceding sections, SRIF did not affect the inhibitory actions of apomorphine on GTH-II release *in vivo*.

DA receptor subtype mediating the *in vivo* GH-releasing effects of apomorphine and SKF82958 The GH-releasing actions of the non-selective DA agonist apomorphine (20 µg/g body weight, i.p.) was abolished by simultaneous treatment with the D1 antagonist SCH23390 (40 µg/g body weight, i.p.) in sexually regressed goldfish; the D2 antagonist pimozide (40 µg/g body weight, i.p.) was not effective in this regard (Fig. 3.4A). The inhibitory action of apomorphine on GTH-II release, on the contrary, was not affected by the D1 antagonist SCH23390, but was abolished by the D2 antagonist pimozide (Fig. 3.4B). Pimozide alone significantly elevated the basal GTH-II levels, and the observation is consistent with our previous report that this D2 antagonist increases circulating GTH-II levels in the goldfish by antagonizing the inhibition of endogenous DA on GTH-II release (Peter *et al.*, 1986). However, both SCH23390 and pimozide had no observable effects on basal GH secretion.

The involvement of D1 receptors in mediating DA-stimulated GH release was further examined *in vivo* using a D1 specific agonist SKF82958. Administration (i.p.) of SKF82958 (20 µg/g body weight) into sexually recrudescent goldfish caused a

significant increase in plasma GH levels (Fig. 3.5). This GH response was abolished by simultaneous treatment with the D1 antagonist SCH23390 (40 μg/g body weight, i.p.), but not the D2 antagonist pimozide (40 μg/g body weight, i.p.). In sexually recrudescent goldfish, SCH23390 alone induced a significant drop in basal GH release ($P<0.05$); pimozide alone was not effective in this respect.

Oral administration of apomorphine on plasma GH levels and body growth Goldfish in late stages of gonadal recrudescence were used to study the effects of feeding with apomorphine on plasma GH and GTH-II levels. Feeding the goldfish with apomorphine-treated pellets significantly elevated the circulating levels of GH, but at the same time suppressed the basal GTH-II release (Fig. 3.6). These results indicate that orally administered apomorphine is active in affecting pituitary hormone release in the goldfish. No changes in plasma GH and GTH-II levels were observed in the group of fish fed with vehicle-treated pellets.

Actions of long-term feeding with apomorphine on the body growth of goldfish were also investigated using fish in late stages of gonadal recudescence. During the initial phase of the *in vivo* body growth experiment (i.e., day 0 - 30), no significant differences in terms of increases in body weight and length were found between the two groups of fish fed with normal trout pellets (Fig. 3.7). After the beginning of drug treatment (i.e., day 30 - 85), the group of fish fed with apomorphine-treated pellets demonstrated a greater increase in body weight (Fig. 3.7A) and length (Fig. 3.7B) than the control group fed with vehicle-treated pellets. Growth rates for the two treatment groups in terms of net increases in body weight and length per day are compared in Table 3.2. The growth rates for both body weight and length were significantly enhanced after feeding with apomorphine.

3.4 Discussion

In the present study, the non-selective DA agonist apomorphine was demonstrated to have differential actions on GH and GTH-II release in the goldfish; apomorphine treatment significantly elevated plasma GH levels, but at the same time suppressed GTH-

II release. The GH-releasing effect of apomorphine was mimicked by the D1 agonist SKF82958 and was abolished by simultaneous treatment with the D1 antagonist SCH23390. The D2 antagonist pimozide was not effective in blocking the GH responses to apomorphine or SKF82958, but was capable of blocking the inhibitory actions of apomorphine on GTH-II release. These results confirm our earlier findings that DA stimulates GH release in the goldfish via the D1 receptors (Chang *et al.*, 1990b; Wong *et al.*, 1992) and inhibits GTH-II release via the D2 receptors (for review, see Peter *et al.*, 1986). The absence of any GTH-II responses to SCH23390 and GH responses to pimozide indicates that the cross-reactivity of the D1 antagonist on D2 receptors or the D2 antagonist on D1 receptors is unlikely within the doses tested. Recently, the genes for three novel forms of DA receptor subtypes, designated as D3 (Sokoloff *et al.*, 1990), D4 (Van Tol *et al.*, 1991) and D5 receptors (Sunahara *et al.*, 1991), have been cloned. The pharmacological properties of the D3 and D4 receptors are comparable to the D2 receptors (Skoloff *et al.*, 1990; Van Tol *et al.*, 1991), whereas those of the D5 receptors are similar to the D1 receptors (Sunahara *et al.*, 1991). Since pharmacological probes specific for D3, D4, and D5 receptors are not yet available, the possible actions of these DA receptors on GH release in the goldfish were not examined in the present study.

In sexually recrudescent goldfish, administration (i.p.) of the D1 antagonist SCH23390 alone induced a significant decrease in plasma GH levels, indicating that DA D1 receptors are involved in the maintenance of basal GH release from the pituitary. However, this inhibitory effect was not observed when SCH23390 was given to fish in sexual regression. The absence of an inhibitory GH response in this case may be related to the presence of a high SRIF tone in sexually regressed goldfish. Hypothalamic and pituitary SRIF contents are known to be the highest when the goldfish is sexually regressed and the lowest during sexual recrudescence (Marchant *et al.*, 1989b). The absence of GH response to SCH23390 during sexual regression is likely due to a potent inhibitory effect of endogenous SRIF on DA-stimulated GH release. This hypothesis is supported by the results of the present studies on the interactions of SRIF and DA on GH release. Administration (i.p.) of the non-selective DA agonist apomorphine induced a significant rise in plasma GH levels, and this GH response was suppressed by simultaneous treatment with SRIF. Using perifused pituitary fragments and dispersed

pituitary cells under static incubation, the inhibitory actions of SRIF on DA-stimulated GH release were confirmed to be directly at the pituitary cell level. These results are also consistent with the previous immunohistochemistry studies in the goldfish, in which the nerve terminals of SRIF (Kah *et al.*, 1982) and DA neurons (Kah *et al.*, 1984; Kah *et al.*, 1987) have been identified in close proximity of the somatotrophs, although only rarely could direct synaptic contacts be observed. Together with the commonly accepted role of SRIF as a physiological GH-release inhibitor in vertebrates (for review, see Hall *et al.*, 1986), our data strongly indicate that DA is an integral part of the GH-regulating mechanism in the goldfish, and its GH-releasing effect can be negatively regulated by SRIF.

The role of DA as a GH-releasing factor is also supported by the demonstration of a distinct seasonality of DA-stimulated GH release in the goldfish. Using perifused goldfish pituitary fragments, both the magnitude and sensitivity of the GH response to DA were significantly enhanced in sexually regressed fish as compared to the mature (=presprawning) fish. The decreasing ability of DA to induce GH release with the gradual development of the gonad suggests that the responsiveness of the goldfish pituitary to DA stimulation may be under a negative modulation of gonadal factor(s). This is in accordance with the results of our recent *in vitro* perifusion studies using pituitary fragments from ovariectomized goldfish; the GH response to micromolar doses of DA in sexually mature female goldfish was greatly enhanced by surgical removal of the gonads (for details, see chapter 4). In mammals, gonadal steroids such as estrogen and progesterone are known to affect DA D1 receptor capacity (Tonnaer *et al.*, 1989), DA turnover (DiPaolo *et al.*, 1985; Yamaguchi *et al.*, 1991) and the excitability of DA neurons (Chiolo *et al.*, 1986). Gonadal steroids are also involved in the regulation of GH clearance (Badger *et al.*, 1991), as well as the synthesis of SRIF (Argente *et al.*, 1990) and GH-releasing hormone (Zeitler *et al.*, 1990). Whether similar actions of steroids also exist in the goldfish is not clear, and the identity of gonadal factor(s) responsible for the observed seasonality of DA-stimulated GH release still awaits further investigations.

The role of DA as a GH-releasing factor is further substantiated by our feeding experiment using sexually recrudescent goldfish. Similar to the results of mammalian

studies, the non-selective DA agonist apomorphine was found to be orally active in the goldfish (for a review on the pharmacology of apomorphine in mammals, see Colpaert *et al.*, 1976). Feeding the goldfish with apomorphine resulted in an elevation of the plasma GH levels, but at the same time the GTH-II levels were suppressed. Long-term feeding of the goldfish with apomorphine also induced a significant increase in the growth of body weight and length. It is conceivable that apomorphine enhanced the body growth of goldfish by stimulating GH release from the pituitary through DA D1 receptors. Our data, however, do not eliminate the possibility of other non-pituitary actions of apomorphine on body growth, as apomorphine is permeable to the blood-brain barrier (Colpaert *et al.*, 1976), and DA, by acting centrally, is known to regulate food intake in mammals (for review, see Morley, 1987). Nevertheless, the possibility of a growth enhancement as a result of an indirect action of apomorphine on feeding behavior seems unlikely in the present study because the effect of DA on feeding is mainly inhibitory (Bednar *et al.*, 1991).

In summary, we have demonstrated that the non-selective DA agonist apomorphine and the D1 agonist SKF82958 stimulate GH release in the goldfish *in vivo*, and this GH response can be blocked by the D1 antagonist SCH23390 but not the D2 antagonist pimozide. In accordance with its GH-releasing effect, apomorphine administered orally is capable of enhancing the body growth of goldfish. DA-stimulated GH release also exhibits a distinct pattern of seasonality, with the highest responsiveness and sensitivity to DA in sexually regressed fish, intermediate in recrudescent fish, and the lowest in sexually mature (=prespawning) fish. The GH responses to DA or apomorphine can be suppressed by simultaneous treatment with SRIF, and the inhibition of SRIF is confirmed to be directly at the pituitary cell level. These results strongly indicate that DA, by acting on D1 receptors in the pituitary, functions as a GH-releasing factor in the goldfish. The differential actions of apomorphine on GH and GTH-II release are also worth mentioning, as it may represent a potential application of non-selective DA analogs in fish farming. Supplementing the diet with long-lasting DA agonists, such as apomorphine, may slow down the reproductive activity of commercial fish by suppressing GTH-II secretion, and yet simultaneously enhance the body growth by increasing GH release.

Table 3.1
Comparison of the dose-response curves of dopamine-stimulated GH release in sexually mature and regressed goldfish.

	Sexually Regressed Fish :	Sexually Mature Fish :
Minimal Dose of DA for GH Response [a] **:**	10 nM	100 nM *
Half Maximal Effective Dose (ED50) :	274.1 ± 144.4 nM	128.2 ± 19.1 nM
Maximal GH Response :	983.8 ± 125.5 % Pretreatment	185.8 ± 6.9 * % Pretreatment
Slope Factor :	0.9 ± 0.3 % Pretreatment / nM DA	1.1 ± 0.2 % Pretreatment / nM DA

Statistical ananlysis by Student's t test or ANOVA followed by Fischer's LSD test. Asterisk (*) referred to significant difference at $P < 0.05$.

a The two values referred to the minimal dose of DA required to initiate a GH response which was significantly different from the basal GH release in the respective dose-response curves (as depicted in Fig. 3.1B).

Table 3.2
Comparison of growth rates in terms of net change in body weight and length per day between the groups of goldfish fed with apomorphine- and vehicle-treated pellets.

	Vehicle-treated group :	Apomorphine-treated group :
Growth rate for Body Weight : (% Initial Weight / Day)		
Initial Phase before treatment :	0.84 ± 0.16^{a}	0.91 ± 0.08^{a}
Final Phase after the beginning of treatment :	0.84 ± 0.09^{a}	1.45 ± 0.12^{b}
Growth rate for Body Length : (% Initial Length / Day)		
Initial Phase before treatment :	0.20 ± 0.03^{a}	$0.21 \pm .04^{a}$
Final Phase after the beginning of treatment :	0.21 ± 0.02^{a}	0.40 ± 0.07^{b}

Statistical analysis by t test for comparison of slope using regression coefficient.
(^{a} vs ^{b}, significant difference at $P < 0.05$)

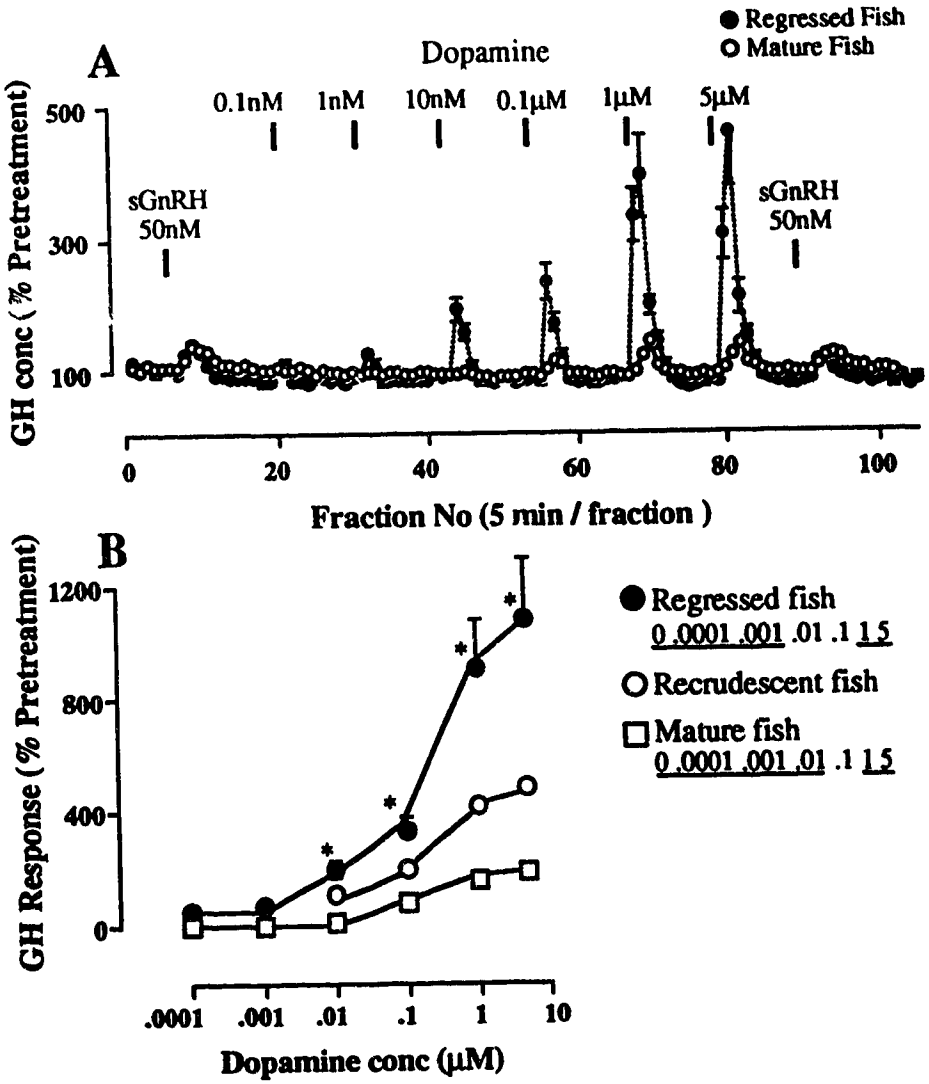


Fig. 3.1 Seasonality of growth hormone (GH) responses to dopamine (DA) stimulation in the goldfish. GH-release profiles to 2-min pulses of 0.1 nM to 5 μM DA using perifused pituitary fragments from goldfish in sexually regressed stages (x6 columns) and mature (prespawning) stages (x4 columns) are presented (A). GH responses, quantitated as area under the curve (in "% pretreatment"), were used to construct the dose-response curve by ALLFIT program (B). Data from a parallel experiment using sexually recrudescent goldfish (x2 columns, no SEM is presented) are also included for comparison. The average pretreatment GH levels for perifusions using sexually regressed and mature goldfish were 42 ± 3 and 36 ± 2 ng GH/ml, respectively. Doses of DA giving similar GH responses were grouped within the same underscore ($P>0.05$, ANOVA followed by Fischer's LSD test). GH responses to the same dose of DA are compared with respect to that of the mature goldfish using the Student's t test (*, $P<0.05$). All data, except that of the sexually recrudescent goldfish, are expressed as mean ± SEM ($n = 4 - 6$).

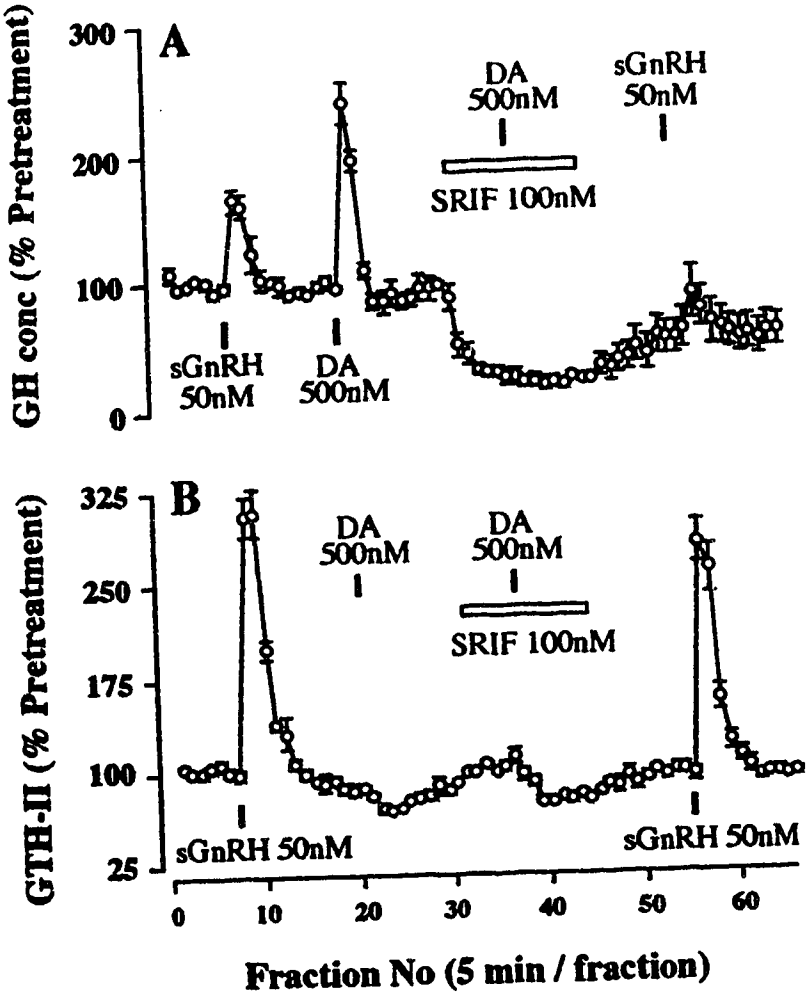


Fig. 3.2 Effects of somatostatin (SRIF) on dopamine (DA) stimulated growth hormone release from perifused goldfish pituitary fragments. Both growth hormone (GH) (A) and gonadotropin (GTH-II) data (B) are presented (x4 columns). The average pretreatment hormone levels for GH and GTH-II were 65 ± 3 ng GH/ml and 9 ± 1 ng GTH-II/ml, respectively. All data are expressed as mean ± SEM (n = 4).

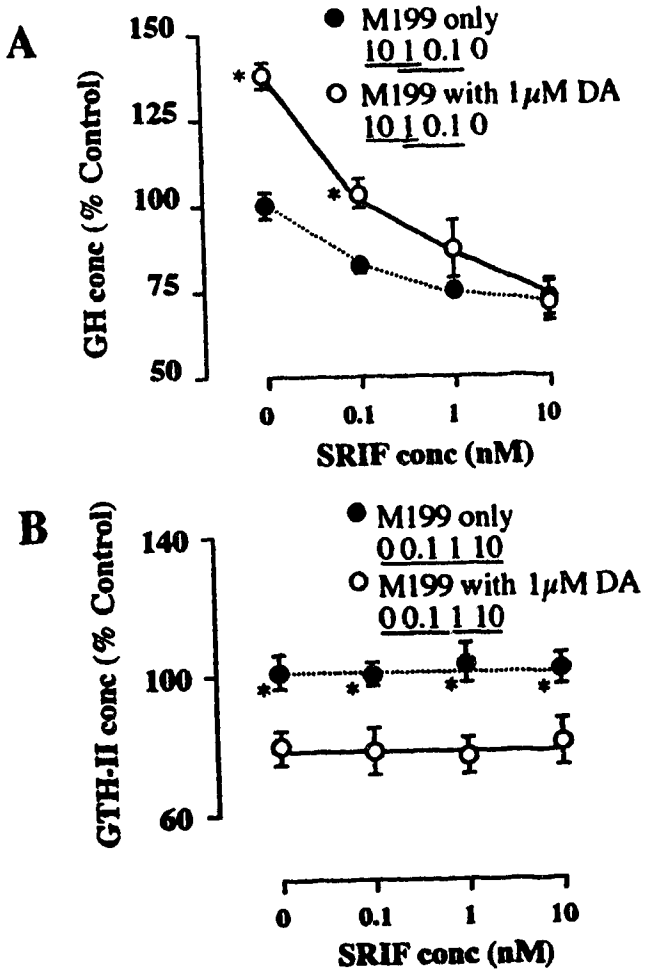


Fig. 3.3 Effects of somatostatin (SRIF) on basal and dopamine (DA)-stimulated growth hormone release from dispersed goldfish pituitary cells under static incubation. Both growth hormone (GH) (A) and gonadotropin (GTH-II) data (B) are presented (pooled data from 3 separated experiments, each with quadruplicate treatments). The average homone levels for GH and GTH-II in control wells were 429 ± 35 ng GH/ml and 69 ± 9 ng GTH-II/ml, respectively. Doses of SRIF giving similar hormone responses were grouped within the same underscore (P>0.05, ANOVA followed by Fischer's LSD test). Hormone responses to the same dose of SRIF with and without 1 μM DA were compared using Student's t test (*, P<0.05). All data are expressed as mean ± SEM (n = 12).

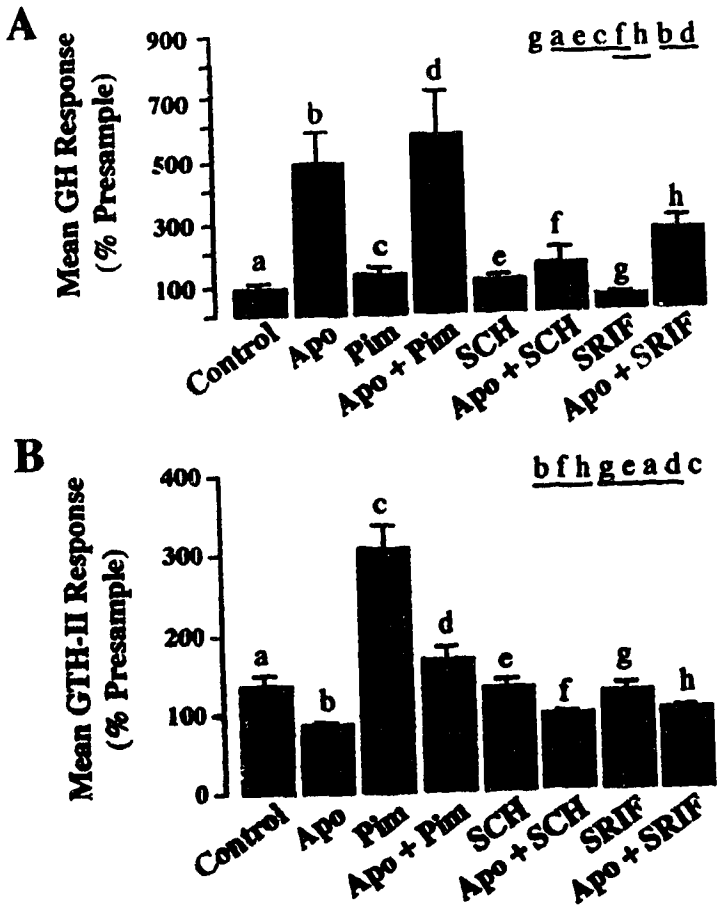


Fig. 3.4 *In vivo* actions of the D1 antagonist SCH23390 (SCH, 40 μg/g body weight), D2 antagonist pimozide (Pim, 40 μg/g body weight), and growth hormone (GH)-release inhibitor somatostatin (SRIF, 1 μg/g body weight) on the GH response to the non-selective dopamine agonist apomorphine (Apo, 20 μg/g body weight). Both GH (A) and gonadotropin (GTH-II) data (B) were transformed into "% presample" as described in the text, and expressed as mean ± SEM. The average presample GH and GTH-II levels in the plasma were 50 ± 14 ng GH/ml and 6.3 ± 0.3 ng GTH-II/ml, respectively. Owing to a limitation in the amount of plasma samples, the sample number for GTH-II data (n = 6 - 9) is less than that of the corresponding GH data (n = 8 - 10). Drug treatments giving similar hormone responses were grouped within the same underscore ($P>0.05$, ANOVA followed by Fischer's LSD test).

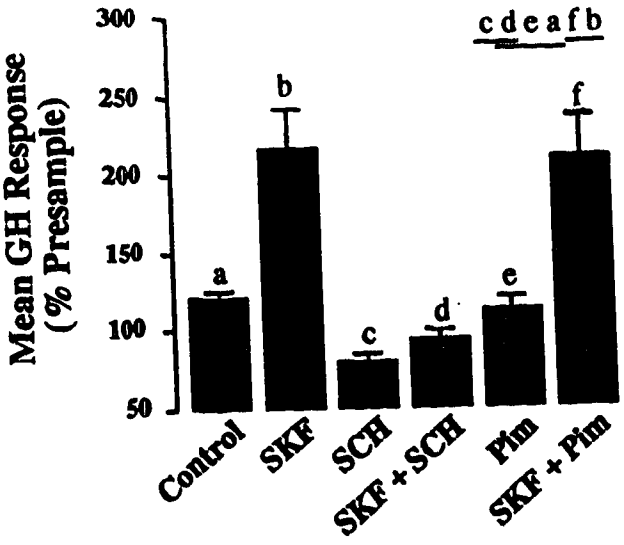


Fig. 3.5 *In vivo* actions of the D1 antagonist SCH23390 (SCH, 40 μg/g body weight) and the D2 antagonist pimozide (Pim, 40 μg/g body weight) on the growth hormone (GH) response to the D1 specific agonist SKF82958 (SKF, 20 μg/g body weight). GH data were transformed into "% presample" and expressed as mean ± SEM (n = 10). The average presample GH concentration was 9.2 ± 0.4 ng GH/ml. Drug treatments giving similar hormone responses were grouped within the same underscore (P>0.05, ANOVA followed by Fischer's LSD test).

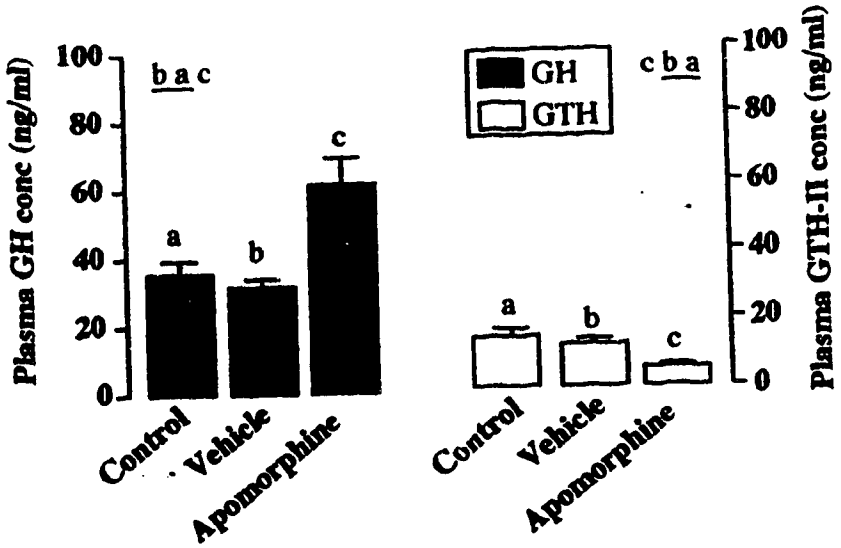


Fig. 3.6 Effects of feeding with apomorphine-treated pellets on plasma growth hormone (GH) and gonadotropin (GTH-II) levels in the goldfish. The control group was not fed for the first two days and blood-sampled on the morning of the third day. Another two groups of fish received similar treatment but were fed on the third day with vehicle- or apomorphine-treated pellets, and blood samples were taken 5 hours after feeding. All data are presented as mean ± SEM (n = 10). Treatments giving similar hormone responses were grouped within the same underscore (P>0.05, ANOVA followed by Fischer's LSD test).

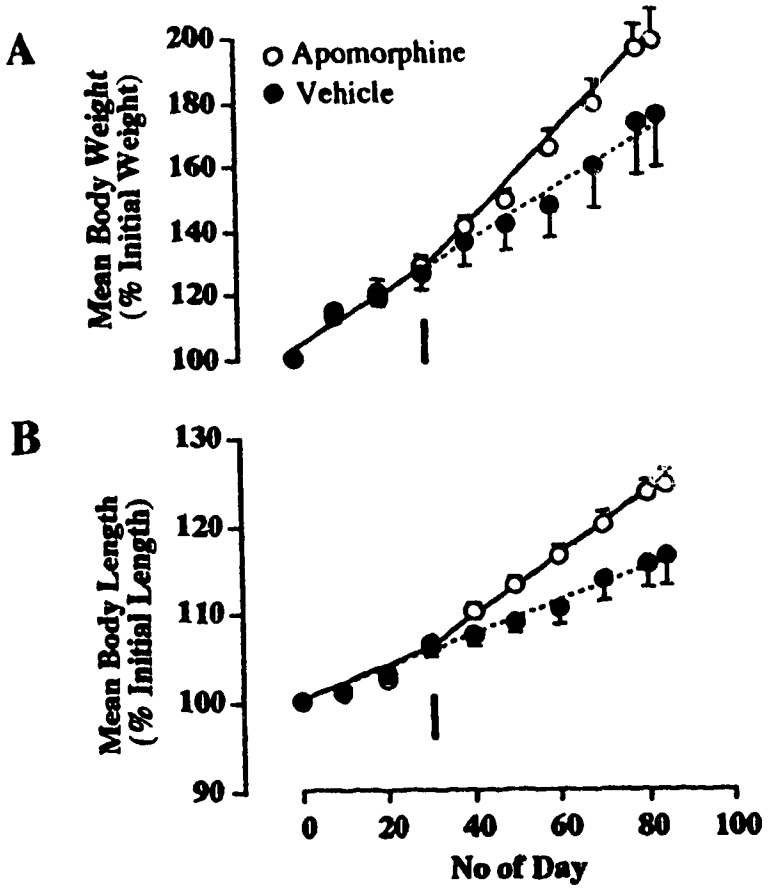


Fig. 3.7 Effects of long-term feeding with apomorphine-treated pellets on the body growth of goldfish. Body growth in terms of increase in body weight (A) and length (B) are presented. Average body weight and length of the fish at the beginning of experiment were 15.9 ± 0.8 g and 7.4 ± 0.1 cm, respectively. The bars in (A) and (B) represent the beginning of drug treatment. All data are presented as mean ± SEM (n = 13 - 15).

3.5 References

Argente J, Chowen-Breed JA, Steiner RA, Clifton DK 1990 Somatostatin messenger RNA in hypothalamic neurons is increased by testosterone through activation of androgen receptors and not by aromatization to estradiol. Neuroendocrinology 52:342-349

Badger TM, Millard WJ, Owens SM, LaRovere J, O'Sullivan D 1991 Effects of gonadal steroids on clearance of growth hormone at steady state in the rat. Endocrinology 128:1065-1072

Bednar I, Forsberg G, Linden A, Qureshi GA, Sodersten P 1991 Involvement of dopamine in inhibition of food intake by cholecystokinin octapeptide in male rats. J Neuroendocrinol 3:491-496

Tuomisto J, Mannisto P 1985 Neurotransmitter regulation of anterior pituitary hormones. Pharmacol Rev 37:249-33

Chang JP, Cook H, Freedman GL, Wiggs AJ, Somoza GM, de Leeuw R, Peter RE 1990a Use of a pituitary cell dispersion method and primary culture system for the studies of gonadotropin-releasing hormone action in the goldfish, *Carassius auratus*. I. Initial morphological, static, and cell column perifusion studies. Gen Comp Endocrinol 77: 256-273

Chang JP, Marchant TA, Cook AF, Nahorniak CS, Peter RE 1985 Influences of catecholamines on growth hormone release in female goldfish, *Carassius auratus*. Neuroendocrinology 40:463-470

Chang JP, Yu KL, Wong AOL, Peter RE 1990b Differential actions of dopamine receptor subtypes on gonadotropin and growth hormone release *in vitro* in goldfish. Neuroendocrinology 51:664-674

Chiolo LA, Glazer WM, Bunney BS 1986 Midbrain dopamine neurons : Electrophysiological studies on the acute effects of estrogen. In : Woodruff GN, Poat JA, Roberts PJ (eds) Dopaminergic Systems and their Regulation, VCH, London, pp 303-311

Colpaert FC, Van Bever WFM, Leysen JEMF 1976 Apomorphine : Chemistry, pharmacology, and biochemistry. Int Rev Neurobiol 19:225-268

DeLean A, Munson PJ, Rodbard D 1978 Simultaneous analysis of families of sigmoidal curves : Application of bioassay, radioligand assay, and physiological dose-response curves. Am J Physiol 235:E97-E102

DiPaolo T, Rouillard C, Bedard P 1985 17β-Estradiol at a physiological dose acutely increases dopamine turnover in rat brain. Eur J Pharmacol 117:197-203

Gibbs DM, Neill JD 1978 Dopamine levels in hypophyseal stalk blood in the rat are sufficient to inhibit prolactin secretion *in vivo*. Endocrinology 102:1895-1902

Hall TR, Harvey S, Scanes CG 1986 Control of growth hormone secretion in the vertebrates : A comparative survey. Comp Biochem Physiol 84A:231-253

Kah O, Dubourg P, Chamboll P, Calas A 1984 Ultrastructural identification of catecholaminergic fibers in the goldfish pituitary. (A high resolution radioautographic study after *in vitro* ^{3}H-dopamine administration). Cell Tissue Res 238:621-626

Kah O, Chambolle P, Dubourg P, Dubois MP 1982 Localisation immunocytochimique de la somatostatine dans le cerveau anterieur et l'hypophyse de deux Teleosteens, le Cyprin (*Carassius auratus*) et *Gambusia* sp. C R Acad Sci 294:519-524

Kah O, Dulka JG, Dubourg P, Thibault J, Peter RE 1987 Neuroanatomical substrate for the inhibition of gonadotrophin secretion in goldfish : Existence of a dopaminergic preoptico-hypophyseal pathway Neuroendocrinology 45:451-458

Marchant TA, Chang JP, Nahorniak CS, Peter RE 1989a Evidence that gonadotropin-releasing hormone also functions as a growth hormone-releasing hormone in the goldfish. Endocrinology 124:2509-2518

Marchant TA, Dulka JG, Peter RE 1989b Relationship between serum growth hormone levels and the brain and pituitary content of immunoreactive somatostatin in the goldfish, *Carassius auratus*. Gen Comp Endocrinol 73:458-468

Morley JE 1987 Neuropeptide regulation of appetite and weight. Endocr Rev 8:256-287

Muller EE 1987 Neural control of somatotropic function. Physiol Rev 67:962-1053

Peter RE, Chang JP, Nahorniak CS, Omeljaniuk RJ, Sokolowska M, Shih SH, Billard R 1986 Interactions of catecholamines and GnRH in regulation of gonadotropin secretion in teleost fish. Recent Prog Horm Res 42:13-548

Peter RE, Nahorniak CS, Chang JP, Crim LW 1984 Gonadotropin release from the pars distalis of the goldfish, *Carassius auratus*, transplanted beside the brain or into the brain ventricles : Additional evidence for a gonadotropin release-inhibitory factor. Gen Comp Endocrinol 55:337-346

Peter RE, Yu KL, Marchant TA, Rosenblum PM 1990 Direct neural regulation of the teleost adenohypophysis. J Exp Zool (suppl) 4:84-89

Rosenzweig L, Kanwar YS 1982 Dopamine internalization by and intracellular distribution within prolactin cells and somatotrophs of the rat anterior pituitary as determined by quantitative electron microscopic autoradiography. Endocrinology 111:1817-1829

Sokoloff P, Giros B, Martres MP, Bouthenet ML, Schwartz JC 1990 Molecular cloning and characterization of a novel dopamine receptor (D3) as a target for neuroleptics. Nature 347:146-151

Sunahara RK, Guan HC, O'Dowd BF, Seeman P, Laurier LG, Ng G, George SR, Torchia J, Van Tol HHM, Niznik HB 1991 Cloning of the gene for a human dopamine D5 receptor with higher affinity for dopamine than D1 receptor. Nature 350:614-619

Tonnaer JADM, Leinders L, Van Delft AML 1989 Ovariectomy and subchronic estradiol-17β administration decrease dopamine D1 and D2 receptors in rat striatum. Psychoneuroendocrinology 14:469-476

Van Der Kraak G, Suzuki K, Peter RE, Itoh H, Kawauchi H 1992 Properties of common carp gonadotropin I and gonadotropin II. Gen Comp Endocrinol 85:217-229

Van Tol HHM, Bunzow JR, Guan HC, Sunahara RK, Seeman P, Niznik HB, Civelli O 1991 Cloning of the gene for a human dopamine D4 receptor with high affinity for the antipsychotic clozapine. Nature 350:610-614

Wong AOL, Chang JP, Peter RE 1992 Dopamine stimulates growth hormone release from the pituitary of goldfish, *Carassius auratus*, through the dopamine D1 receptors. Endocrinology 130:1201-1210

Yamaguchi M, Koike K, Kadowaki K, Miyake A, Tanizawa O 1991 Short-term treatment with 17β-estradiol enhances spontaneous [^{3}H] dopamine release from

cultured rat tuberoinfundibular neurons. J Endocrinol Invest 14:187-191

Zeitler P, Argente J, Chowen-Breed JA, Clifton DK, Steiner RA 1990 Growth hormone-releasing hormone messenger ribonucleic acid in the hypothalamus of the adult male rat is increased by testosterone. Endocrinology 127:1362-1368

Chapter 4

Interactions of Somatostatin, Gonadotropin-Releasing Hormone, and the Gonads on Dopamine-Stimulated Growth Hormone Release in the Goldfish [1]

4.1 Introduction

In teleosts, the brain-pituitary axis is unique for the absence of a functional hypothalamo-hypophyseal portal blood system (for review, see Peter *et al.*, 1990). Unlike mammals, the anterior pituitary of bony fish is under direct innervation from the hypothalamus, in particular by aminergic and peptidergic nerve fibers (Ball, 1981). In the goldfish, nerve terminals immunoreactive to dopamine (DA) (Kah *et al.*, 1984), somatostatin (SRIF) (Kah *et al.*, 1982) and salmon gonadotropin-releasing hormone (sGnRH) (Kah *et al.*, 1986) have been identified in the close proximity of somatotrophs, suggesting that growth hormone (GH) release in the goldfish may be under the direct influence of these neuroendocrine factors. This hypothesis was confirmed by our subsequent findings that DA (Chang *et al.*, 1985; Peter *et al.*, 1990) and sGnRH (Marchant *et al.*, 1989a; Chang *et al.*, 1990b) are stimulatory to GH release in the goldfish, both *in vivo* and *in vitro*. The functional role of sGnRH as a GH-releasing factor is also substantiated by the recent demonstration that GnRH receptors are present in the goldfish somatotrophs (Cook *et al.*, 1991). SRIF, on the other hand, is inhibitory to basal GH release (Cook and Peter, 1984; Marchant *et al.*, 1987) and blocks the GH responses to sGnRH stimulation (Marchant *et al.*, 1989a). These observations are in accordance with the generally accepted physiological role of SRIF as a GH-release inhibitor in vertebrates (for review, see Nishioka *et al.*, 1988).

A distinct seasonality in circulating GH levels has been described in the goldfish; serum GH levels in the goldfish were found to be increasing during gonadal recrudescence, maintained at high levels throughout the spawning and post-spawning

[1] A version of this chapter has been submitted for publication : Wong AOL, Chang JP, and Peter RE (1993) **Gen Comp Endocrinol.**

season, and gradually returning to low levels when the fish become sexually regressed (Marchant and Peter, 1986). The mechanisms involved in the regulation of this seasonal GH cycle are still not fully understood. Recently, we have demonstrated that the GH-releasing effects of DA in the goldfish are mediated through DA D1 receptors (Chang *et al.*, 1990b; Wong *et al.*, 1992; Wong *et al.*, 1993a), and this DA D1 action also exhibits seasonal changes closely associated with the reproductive cycle (Wong *et al.*, 1993a,b). The responsiveness of GH release to DA stimulation was found to be the highest in sexually regressed fish, intermediate in recrudescent fish, and the lowest in sexually mature (=prespawning) fish (Wong *et al.*, 1992a,b), suggesting that the ability of DA to induce GH release may be influenced by the developing gonads. In the present study, we examined the *in vitro* interactions of DA with sGnRH and SRIF on GH release using perifused goldfish pituitary fragments as well as dispersed pituitary cells under static incubation. Possible influences of the gonads on DA-stimulated GH release were investigated by testing the responsiveness of pituitary fragments to DA stimulation after castration. Since DA is also known to inhibit basal and sGnRH-stimulated gonadotropin-II (GTH-II) release in the goldfish via DA D2 receptors (for review, see Peter *et al.*, 1986), GTH-II data were also collected in some of the experiments to serve as a parallel control.

4.2 Materials and Methods

Animals Goldfish of the common or comet varieties were purchased from Ozark Fisheries (Stoutland, MO, USA) or Grassyforks Fisheries (Martinsville, IN, USA), and maintained in flow-through aquaria (1,800 liters) at 17 °C under a simulated natural photoperiod (Edmonton, AB, Canada) for at least 3 weeks prior to any experiments. The fish were fed to satiation daily with Ewos trout pellets (Astra Chemicals Ltd., Mississauga, ONT, Canada). Goldfish of both sexes, with body weight ranging from 20 g to 30 g, were used in the present study. Since reproductive cycling is a characteristic of most teleosts, and these reproductive cycles can be reflected by pronounced variations in gonadal size, gonadal conditions of the goldfish, determined by the gonadosomatic index (GSI = weight of gonad / total body weight x 100 %) as well as the morphological characteristics of the gonads, are reported separately for individual

experiments. The gonad was categorized as sexually regressed if GSI was ≤ 1.5 %, as sexually recrudescent if GSI was between 1.5 % to 6 %, and as sexually mature (=prespawning) if GSI was ≥ 8 % (for details, see chapter 2).

Reagents and test substances HEPES, DA, and SRIF were obtained from Sigma Chemical Company (St Louis, MO, USA). The D1 agonist SKF38393 (1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine-7,8-diol hydrochloride) was purchased from Research Biochemicals Inc. (Wayland, MA, USA). SKF38393 was first dissolved into a minimal amount of dimethyl sulfoxide (DMSO), and subsequently diluted to 0.5 μM concentration with perifusion medium. The final concentration of DMSO was less than 0.1 %, and did not affect basal GH and GTH-II release from perifused goldfish pituitary fragments (Wong *et al.*, 1992). Aliquots of sGnRH (Peninsula Laboratories Inc., Belmont, CA, USA) in 0.1 M acetic acid stock solution were frozen at -25 °C, and diluted with perifusion medium or medium M199 to appropriate concentrations immediately prior to use.

Perifusion of pituitary fragments *In vitro* experiments were conducted using a perifusion system for goldfish pituitary fragments as described previously (MacKenzie *et al.*, 1984; Marchant *et al.*, 1989a). In brief, goldfish pituitary fragments (≈0.2 mm in thickness) equivalent to 3 whole pituitaries were placed between 2 layers of 0.1 ml cytodex I beads (Sigma) in a 0.4 ml perifusion column constructed from disposable plastic syringes. The total dead volume of the perifusion system was about 0.75 ml. The fragments were perifused overnight (15 - 18 hr) at a flow rate of 5 ml/hr with M199 (Gibco, Grand Island, NY, USA) supplemented with Hank's salts, 25 mM HEPES and 56 U/ml nystatin (Sigma). Thereafter, the perifusion medium was switched to Hank's balanced salt solution containing 25 mM HEPES and 0.1 % BSA (Sigma), and the flow rate was increased to 15 ml/hr. After another 3 hr of perifusion, the basal GH and GTH-II release from the pituitary fragments remained relatively constant in the absence of any stimulation. Test substances were then added from a drug reservoir into the perifusion column through a 3-way stopcock. To test for interactions, in most experiments the stimulants for GH release (i.e., DA at 0.5 μM, SKF38393 at 0.5 μM, or sGnRH at 100

nM) were administered as 2-min pulses before, during, and after the continuous perifusion of test substances (i.e., SRIF at 100 nM, DA at 1 μM, or sGnRH at 100 nM). To validate the kinetics of hormone release during long-term perifusion of DA and sGnRH, continuous 1-hour perifusion with 1 μM DA and 100 nM sGnRH was also tested. Perifusates from each column were collected in 5-min fractions, and stored at -25 °C until their hormone contents were assayed by radioimmunoassay (RIA). GH contents were assayed using a RIA for carp GH, which has been previously validated for the measurement of goldfish GH (Marchant *et al.*,1989b). The RIA for GTH (Peter *et al.*, 1984) is specific for the measurement of GTH-II in the goldfish (Van Der Kraak *et al.*, 1991). Hormone data were transformed into "% pretreatment" as defined previously (Wong *et al.*, 1992). In short, the hormone data from each individual column were expressed as a percentage of the mean hormone contents of the first 6 fractions at the beginning of the perifusion experiment prior to any drug treatment. This transformation was done to allow pooling of data from separate columns of the same experiment without distorting the profile of hormone release. Hormone responses were quantified by calculating the net change in hormone release (i.e., area under the curve) after a particular drug treatment (for details, see chapter 2).

Static incubation of dispersed pituitary cells Dispersed goldfish pituitary cells were prepared by trypsin / DNase treatment as described previously (Chang *et al.*, 1990a). Viability of pituitary cells was >90 % as indicated by trypan blue exclusion test. Pituitary cells were resuspended in plating medium (M199 with Earle's salts at pH 7.2, supplemented with 2.2 g/l sodium bicarbonate, 25 mM HEPES, 100,000 units penicillin/l, 100 mg streptomycin/l, and 1 % horse serum), and were cultured in 24-well Falcon Primaria plates (Becton Dickinson & Company, NJ, USA) at a density of 0.25 million cells/ml/well. After overnight incubation (15 - 18 hr) at 28 °C under 5% CO_2 and saturated humidity, the culture medium was decanted and replaced by testing medium (M199 with Hank's salts at pH 7.2, supplemented with 2.2 g/l sodium bicarbonate, 25 mM HEPES, 100,000 units penicillin/l, 100 mg streptomycin/l, and 0.1 % BSA). Drug treatment was initiated by adding testing medium with DA from 1 nM to 10 μM doses in

the presence or absence of 100 nM sGnRH. A reciprocal experiment with doses of sGnRH from 0.01 nM to 100 nM in the presence or absence of 1 μM DA was also conducted. Following a further incubation of 2 hours, 500 μl of testing medium was carefully removed from individual culture wells, and stored at -25 °C until their hormone contents were assayed. GH data were expressed as a percentage of the basal GH release in control wells without any drug treatment (as "% control").

Castration of goldfish Sexually mature goldfish were castrated as described previously (Kobayashi *et al.*, 1989) with minor modifications. After the fish was anesthetized with tricaine methanesulfonate (Syndel Laboratories Ltd., Vancouver, BC, Canada), an incision was made from a point near the anus to the level of the pectoral girdle along the medial line of the abdomen. The gonad, with the oviduct or the sperm duct attached, was carefully excised, and the wounds inside the abdomen as a result of castration were covered with non-antigenic gelfoam (Upjohn Company of Canada, Don Mills, ONT, Canada) presoaked with antibiotics (100,000 units penicillin/l, 100 mg streptomycin/l). The incision was sutured with sterilized silk, and treated with the antiseptic mercurochrome. The fish was then kept in water pretreated with 0.01 % malachite green (Sigma) and 0.05 g/l tetracycline (Sanofi Sante Animale, Victoriaville, PQ, Canada) for one week without feeding. Sham-operation was conducted in the control group in a similar manner without taking away the gonad. One month after castration, pituitary fragments were prepared for *in vitro* perifusion experiments. DA from 0.1 nM to 5 μM concentrations was administered as 2-min pulses at 1-hr intervals, the protocol for perifusion was previously validated to have no potentiating nor desensitizing effects within the doses tested (Wong *et al.*, 1992). Since sGnRH is known to stimulate both GH and GTH-II release in the goldfish (Marchant *et al.*, 1989a), sGnRH at 50 nM dose was given as 2-min pulses at the begining and at the end of the perifusion experiment to serve as an internal control for each individual column. Survival rate of the surgery was 93 % for the male fish and 78 % for the female fish. In the sham-operated groups, testosterone levels in the plasma were 3.76 ± 1.62 ng/ml for the male, and 1.88 ± 0.23 ng/ml for the female fish, respectively. However, testosterone levels in the castrated groups were not detectable (detection limit of the assay is ≈0.35 ng/ml), indicating that

the castration was complete.

Data analysis and statistics GH and GTH-II data were analyzed by Student's *t* test or by analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test. Differences were considered significant when $P<0.05$. Dose-response curves for DA-stimulated GH release were analyzed using the ALLFIT computer program (DeLean *et al.*, 1978).

4.3 Results

Continuous perifusion of DA and sGnRH on GH and GTH-II release Pituitary fragments from goldfish undergoing gonadal recrudescence were used to examine the actions of 1-hr continuous perifusion of 1 μM DA and 100 nM sGnRH on basal GH and GTH-II release (x4 columns each). DA induced a biphasic increase of GH release (Fig. 4.1A, upper panel), but at the same time suppressed basal GTH-II secretion (Fig. 4.1A, lower panel). The GH response to DA was composed of an initial peak followed by a plateau phase of a lower magnitude; the peak was reached 10 min after the beginning of DA perifusion, and the plateau phase was maintained at GH levels significantly higher than that of the basal secretion. Recovery of GH release to basal levels was observed 20 min after termination of DA treatment. sGnRH was stimulatory to both GH (Fig. 4.1B, upper panel) and GTH-II release (Fig. 4.1B, lower panel). The kinetics of GH and GTH-II release to sGnRH stimulation was also biphasic and similar to that of DA-induced GH release (Fig. 4.1A, upper panel).

Interactions of DA and sGnRH on GH and GTH-II release Goldfish in the early stages of gonadal recrudescence were used to study the interactions of DA and sGnRH on GH (Fig. 4.2, upper panels) and GTH-II release (Fig. 4.2, lower panels). 2-min pulses of sGnRH at 100 nM dose consistently stimulated GH release from perifused goldfish pituitary fragments (Fig. 4.2A, upper panel; x4 columns). Continuous perifusion of 1 μM DA elevated the basal GH levels before the second sGnRH pulse. However, the GH-releasing effects of the second sGnRH pulse were not affected by simultaneous treatment with DA, as no significant differences were observed among the net GH

responses to the first, second and third 100 nM sGnRH pulses (613 ± 69, 652 ± 27 and 559 ± 54 % pretreatment, respectively). Comparable results were also obtained with DA in the reciprocal experiment (Fig. 4.2B; x4 columns). Continuous perifusion of 100 nM sGnRH increased the basal GH levels before the second 2-min pulse of 1 μM DA (Fig. 4.2B, upper panel). Again, the net GH responses to the first, second and third DA pulses were not different from one another (983 ± 70, 886 ± 251 and 1028 ± 57 % pretreatment, respectively). These results indicate that the GH-releasing effects of DA and sGnRH are independent and additive at the pituitary level. In these experiments, GTH-II data were also collected as a parallel control; in both cases, DA consistently suppressed both basal and sGnRH-stimulated GTH-II release (Fig. 4.2, lower panels).

To demonstrate that the observed additivity between DA and sGnRH in terms of GH release was the result of a direct action at the pituitary cell level, studies using static incubation of pituitary cells prepared from goldfish undergoing gonadal recrudescence were also performed. DA (1 nM - 10 μM) stimulated GH release in a dose-dependent manner (Fig. 4.3A). The GH responses to various doses of DA were further enhanced by an average of 36 ± 1 % control in the presence of 100 nM sGnRH. This elevation in GH levels was not different from the net GH response to 100 nM sGnRH alone (37 ± 4 % control). Similar results were also observed in the reciprocal experiment (Fig. 4.3B). The dose-response curve for sGnRH-stimulated GH release (with sGnRH doses ranging from 0.01 nM to 0.1 μM) was shifted upward by an average of 64 ± 2 % control in the presence of 1 μM DA. The magnitude of this upward shift was similar to that of the GH response to 1 μM DA alone (59 ± 8 % control).

Effects of SRIF on DA D1-stimulated GH release Pituitary fragments prepared from sexually regressed goldfish were used to examine the actions of SRIF on DA-stimulated GH release (Fig. 4.4). SRIF at 100 nM dose suppressed basal GH levels and abolished the GH response to 500 nM DA (Fig. 4.4, upper panel). A full recovery of GH responsiveness to DA stimulation was observed 2 hours after termination of SRIF treatment, as no significant differences were found in the net GH responses to the first and third 500 nM DA pulses (177 ± 15 and 160 ± 18 % pretreatment, respectively).

Basal GTH-II levels as well as the inhibitory effect of DA on GTH-II secretion were not affected by SRIF (Fig. 4.4, lower panel), indicating that the actions of SRIF were selective to GH but not GTH-II release. Interactions of SRIF and DA on GH release were further investigated using the D1 agonist SKF38393 at 500 nM dose. In this study, the pituitary fragments for perifusion were prepared from goldfish in early gonadal recrudescence. Similar to the results of DA experiments, SRIF was inhibitory to the basal and SKF38393-induced GH release in the goldfish (Fig. 4.5).

Effects of castration on DA-stimulated GH release Sexually mature (=prespawning) male and female goldfish were used to study the influence of the gonads on DA-stimulated GH release. Results from male (Fig. 4.6) and female goldfish (Fig. 4.7) were similar. In both cases, DA consistently induced a dose-related GH release from the pituitary fragments of both castrated and sham-operated goldfish (Fig. 4.6A and 4.7A). Basal GH release (see legends of Fig. 4.6 and 4.7) and the net GH responses to higher doses of DA (i.e., 100 nM, 1 μM and 5 μM DA) were significantly enhanced after castration (Fig. 4.6B and 4.7B). ED_{50} values and the maximal GH responses, respectively, to DA stimulation were estimated to be : 134 ± 30 nM and 1024 ± 55 % pretreatment for sham-operated male fish; 132 ± 14 nM and 1792 ± 16 % pretreatment for castrated male fish; 90 ± 21 nM and 1784 ± 10 % pretreatment for sham-operated female fish; and 112 ± 15 nM and 3428 ± 40 % pretreatment for castrated female fish. The GH responses to the first and second 50 nM sGnRH pulses were found to be similar in magnitude in each individual perifusion experiment, indicating that the viability and responsiveness of pituitary fragments were maintained during the course of perifusion. In both male (Fig. 4.6C) and female goldfish (Fig. 4.7C), castration also reduced the GH responses to sGnRH stimulation.

4.4 Discussion

In the present study, DA was shown to have differential actions on GH and GTH-II secretion in the goldfish. Continuous perifusion of DA was stimulatory to GH but inhibitory to GTH-II release from the goldfish pituitary fragments. These results are consistent with our earlier findings that DA stimulates GH release via D1 receptors

(Chang *et al.*, 1990b; Wong *et al.*, 1992) and inhibits GTH-II release via D2 receptors (for review, see Peter *et al.*, 1986). sGnRH, on the contrary, was stimulatory to both GH and GTH-II release. The GH- (Marchant *et al.*, 1989a; Habibi *et al.*, 1992) and GTH-II-releasing effects of sGnRH (MacKenzie *et al.*, 1984; Peter *et al.*, 1985) have been previously reported in the goldfish, both *in vivo* and *in vitro*. The present study also demonstrated that the GH-releasing actions of DA and sGnRH were independent and additive at the pituitary cell level. Both the kinetics and magnitude of the GH responses to DA were not affected by simultaneous treatment with sGnRH, and *vice versa*. These observations are also in accordance with our previous findings that the GH responses of sGnRH and the D1 agonist SKF38393 are additive in perifused goldfish pituitary cells (Chang *et al.*, 1990b).

Continuous perifusion of goldfish pituitary fragments with sGnRH is known to cause receptor down-regulation and refractoriness to subsequent sGnRH stimulation (Habibi, 1991). The observation of a distinct GH response to DA stimulation during the prolonged exposure of sGnRH in the present study strongly suggests that the actions of DA and sGnRH to induce GH release in the goldfish are mediated through different mechanisms. In mammals, DA D1 receptors are coupled to the cAMP-producing enzyme adenylate cyclase via the stimulatory G-protein (for review, see Andersen *et al.*, 1990). It is commonly accepted that the D1 actions, such as DA D1-stimulated parathyroid hormone release (Brown and Hughes, 1983), are mediated through the cAMP-dependent pathway. In the goldfish, pharmacological agents that can elevate intracellular cAMP levels, e.g., forskolin and IBMX, are known to induce GH release from pituitary cells under static incubation (AOL Wong, JP Chang, and RE Peter, unpublished results; see chapter 6). Our recent studies also demonstrated that cAMP production in the goldfish pituitary cells was not affected by sGnRH treatment (Chang *et al.*, 1992), indicating that cAMP is not a second messenger mediating the GH-releasing actions of sGnRH. In the same animal model, the GH response to sGnRH can be suppressed by H7, a selective inhibitor for protein kinase C (PKC), suggesting that the stimulation of sGnRH on GH release is mediated by the PKC-dependent pathway (Chang *et al.*, 1991). Probably, the observed additivity of DA and sGnRH on GH release is due to the differential involvement of two separate signal transduction pathways.

Previously, we have demonstrated a distinct seasonality of DA-stimulated GH release from perifused goldfish pituitary fragments. The responsiveness of GH release to DA stimulation was found to be the highest in sexually regressed fish, intermediate in recrudescent fish, and the lowest in sexually mature (or prespawning) fish (Wong *et al.*, 1993a, b). These results lead to the speculation that the gradual development of the gonad may have a suppressive effect on DA-stimulated GH release. This hypothesis was confirmed in the present study, as the ability of DA to induce GH release was potentiated after castration of sexually mature goldfish. Similar observations were found in both male and female goldfish, and sexual dimorphism was not evident in this phenomenon (for a review on sexual dimorphism on GH regulation in mammals, see Jansson *et al.*, 1985). Based on the results of these *in vitro* studies, we conclude that the developing gonad exerts a negative modulating effect on DA D1-stimulated GH release in the goldfish.

In the present study, the GH responses to sGnRH pulses were significantly reduced after castration in both male and female goldfish, suggesting that the gradual maturation of the gonad also has a positive modulatory effect on the GH responses to sGnRH. In our preliminary studies, 2-min pulses of 50 nM sGnRH induced a net GH response of 94 ± 7, 197 ± 20, and 352 ± 36 % pretreatment from perifused pituitary fragments of sexually regressed, recrudescent, and mature (=prespawning) goldfish, respectively (AOL Wong, JP Chang, and RE Peter, unpublished results; see Appendix III). These results suggest that the responsiveness of the pituitary to sGnRH stimulation is enhanced when the gonad is fully mature (i.e., in prespawning stages). This is also in agreement with our previous findings that GnRH receptor capacity is the highest in the goldfish pituitary during the prespawning period (Habibi *et al.*, 1989) and the GH responses to sGnRH can be potentiated by estradiol implantation (Trudeau *et al.*, 1992). In mammals, especially in the rat, gonadal steroids such as estrogen are known to reduce DA D1 receptor capacity (Tonnaer *et al.*, 1989), stimulate GnRH mRNA expression (Park *et al.*, 1990), and modulate the pulsatility of GH release (for review, see Jansson *et al.*, 1985). In the goldfish, DA turnover in the pituitary, measured by the clearance rate of tissue DA contents after tyrosine hydroxylase inhibition, is low in sexually regressed compared to recrudescing fish, and is increased after estradiol and testosterone

implantation (Trudeau *et al.*, 1993). Therefore, it is conceivable that the modulatory actions of the gonads on DA- and sGnRH-stimulated GH release in the goldfish are mediated through sex steroids. More recently, immuno-reactivity to inhibin β_A and β_B subunits has been identified in the gonad of the goldfish (Ge *et al.*, 1993). Whether the gonadal peptides, such as inhibin and activin, also contribute to the modulatory effects of the gonad is not clear.

SRIF is generally accepted to be the major physiological GH-release inhibitor in vertebrates (for reviews, see Nishioka *et al.*, 1988). In the goldfish, SRIF is inhibitory to basal GH release (Cook and Peter, 1984; Marchant *et al.*, 1987) and blocks the GH responses to sGnRH (Marchant *et al.*, 1989a). In the current study, the GH responses to DA and the D1 agonist SKF-38393 were abolished by simultaneous treatment with SRIF, indicating that DA D1-stimulated GH release is also subject to the negative control of SRIF. Seasonal changes of SRIF contents in the goldfish hypothalamus are known to exhibit an inverse relationship with the serum GH levels, being the highest in sexually regressed fish and the lowest in sexually mature (=prespawning) fish (Marchant *et al.*, 1989b). The present demonstration of gonadal influences on DA- and sGnRH-stimulated GH release also indicates that the seasonality of DA D1 and sGnRH actions on GH secretion are tightly coupled with the reproductive cycle. These observations, as a whole, suggest that SRIF, DA, sGnRH, and gonadal factor(s) may interact at the pituitary level to control the seasonality of GH release in the goldfish.

In summary, we have demonstrated that the GH-releasing actions of DA and sGnRH in the goldfish are independent and additive at the pituitary cell level. The GH responses to DA D1-stimulation can be abolished by the physiological GH-release inhibitor SRIF. Castration in sexually mature (=prespawning) goldfish is stimulatory to DA-induced GH release, but inhibitory to the GH responses to sGnRH. These results indicate that SRIF, DA, sGnRH, and gonadal factor(s) interact at the pituitary level to regulate GH release in the goldfish. In the present study, the gonadal factors modulating the GH-releasing effects of DA and sGnRH have not been examined. The identification of these gonadal factors clearly warrants further investigation.

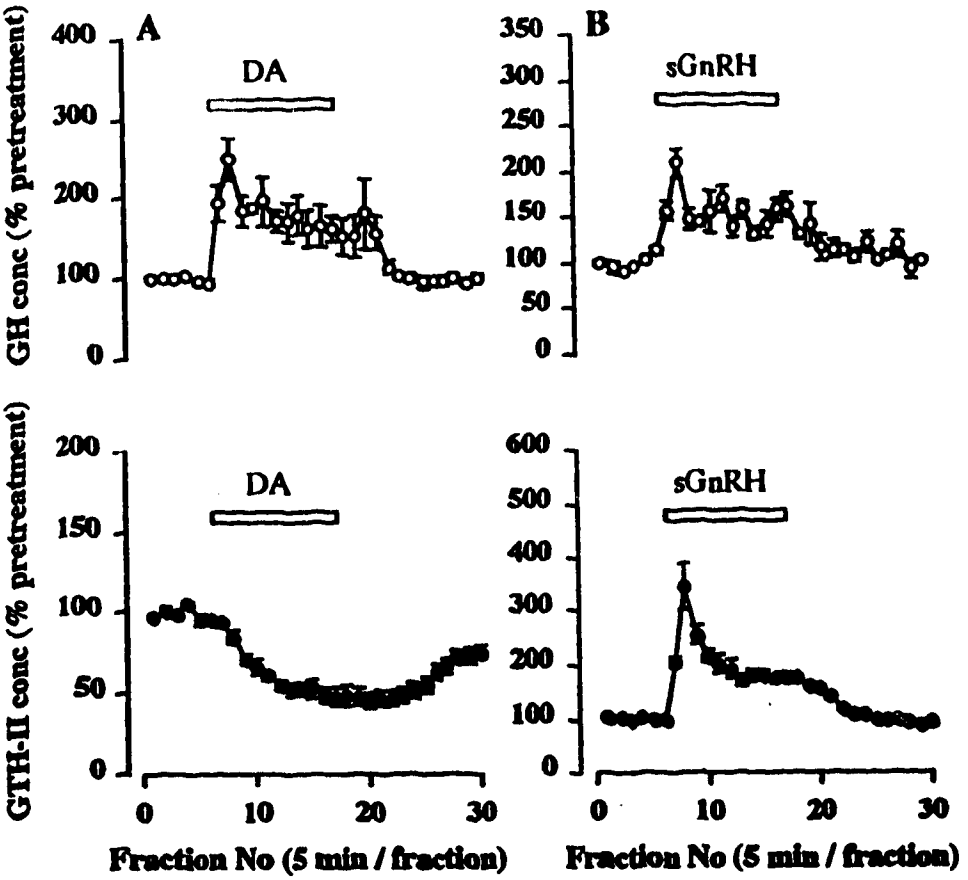


Fig. 4.1 Effects of of DA and sGnRH on GH and GTH-II release from perifused goldfish pituitary fragments. Pituitary fragments were perifused continuously for 1 hour with either 1 μM DA (A) or 100 nM sGnRH (B). Both GH (upper panels) and GTH-II release profiles (lower panels) are presented. Average pretreatment hormone levels were 46 ± 8 ng GH/ml and 12 ± 2 ng GTH/ml for perifusions with DA, and 58 ± 7 ng GH/ml and 10 ± 2 ng GTH-II/ml for perifusion with sGnRH, respectively. All data are expressed as mean ± SEM (n = 4).

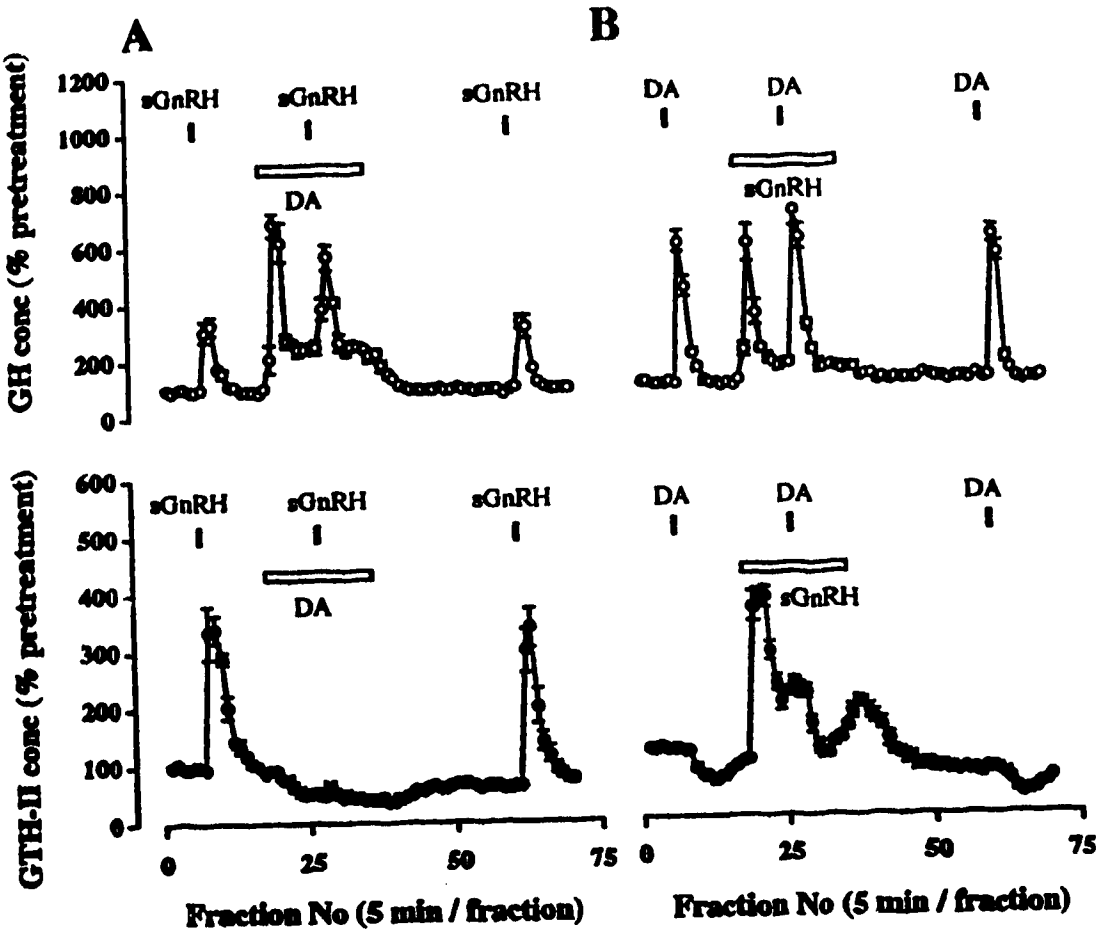


Fig. 4.2 Additivity of the GH-releasing actions of DA and sGnRH in perifused goldfish pituitary fragments. (A) sGnRH at 100 nM dose was administered as 2-min pulses before, during, and after 1.5 hours continuous perifusion with 1 μM DA. (B) A reciprocal experiment with DA at 1 μM dose administered as 2-min pulses before, during, and after 1.5 hours continuous perifusion with 100 nM sGnRH was also conducted. Both GH (upper panels) and GTH-II release profiles (lower panels) for the two experiments are presented. Average pretreatment GH and GTH-II levels were 64 ± 4 ng GH/ml and 15 ± 2 ng GTH-II/ml, respectively. All data are expressed as mean ± SEM (n = 4).

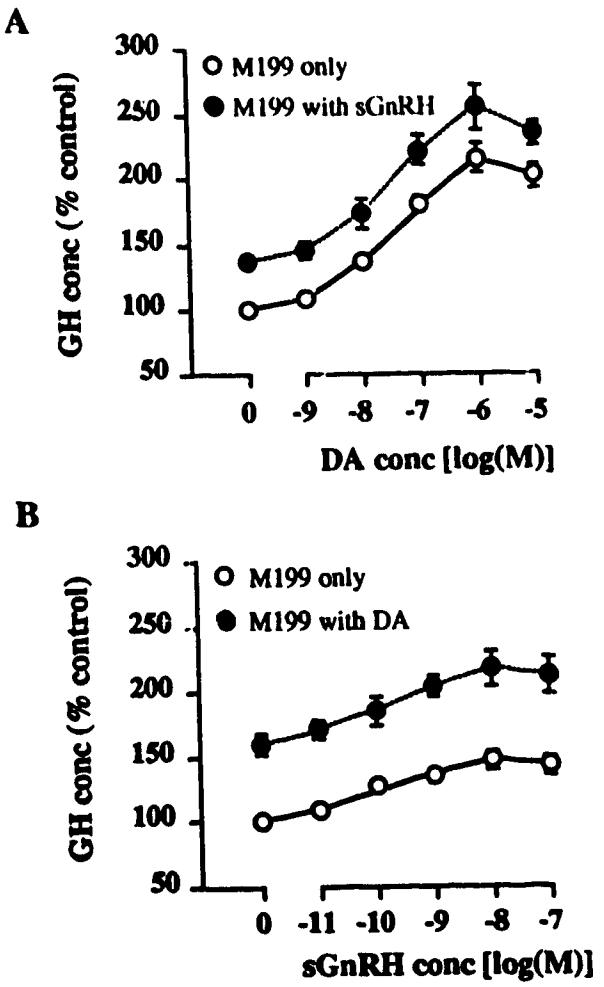


Fig. 4.3 Additivity of the GH-releasing effects of DA and sGnRH in dispersed goldfish pituitary cells under static incubation. GH responses to 2-hr incubation of DA (from 1 nM to 10 μM doses) in the presence or absence of 100 nM sGnRH (A), and sGnRH (from 0.01 nM to 100 nM doses) in the presence or absence of 1 μM DA (B) are presented. The average GH levels in the control wells for the two series of experiments were 1095 ± 88 and 1134 ± 138 ng GH/ml, respectively. GH data pooled from 3 separate experiments (each with quadruplicate treatments) are expressed as mean ± SEM (n = 12).

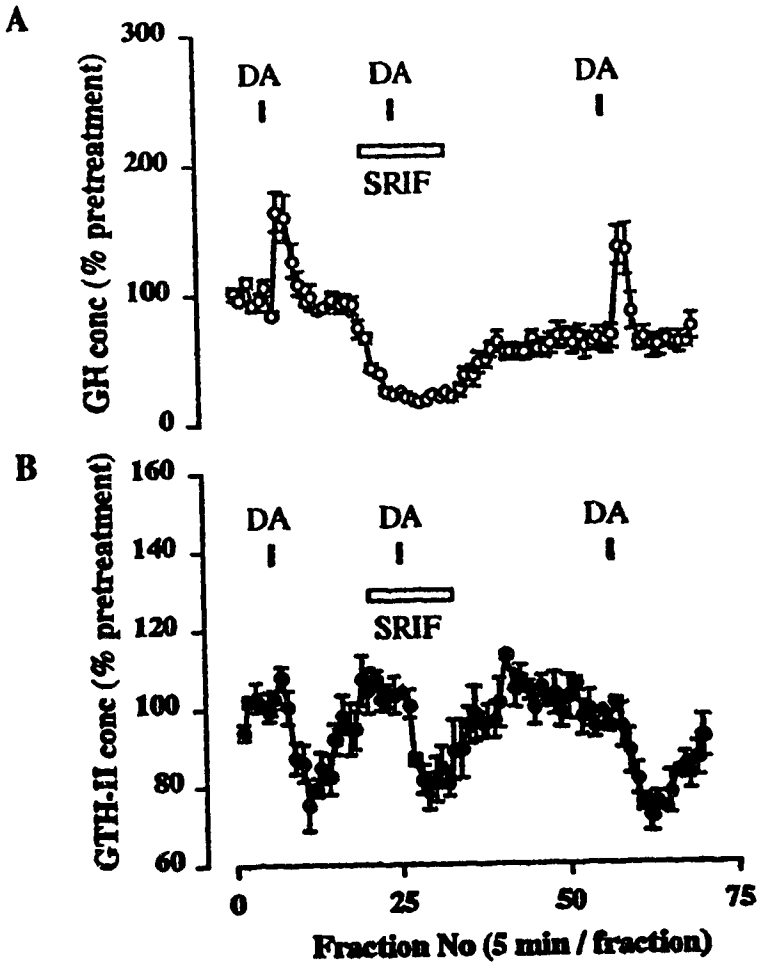


Fig. 4.4 Effects of SRIF on DA-stimulated GH release from perifused pituitary fragments of goldfish. DA at 500 nM dose was administered as 2-min pulses before, during, and after the 1-hr continuous perifusion of 100 nM SRIF. Both GH (A) and GTH-II (B) release profiles are presented. The average pretreatment GH and GTH-II levels were 24 ± 1 ng GH/ml and 9 ± 1 ng GTH-II/ml, respectively. All data are expressed as mean ± SEM (n = 3).

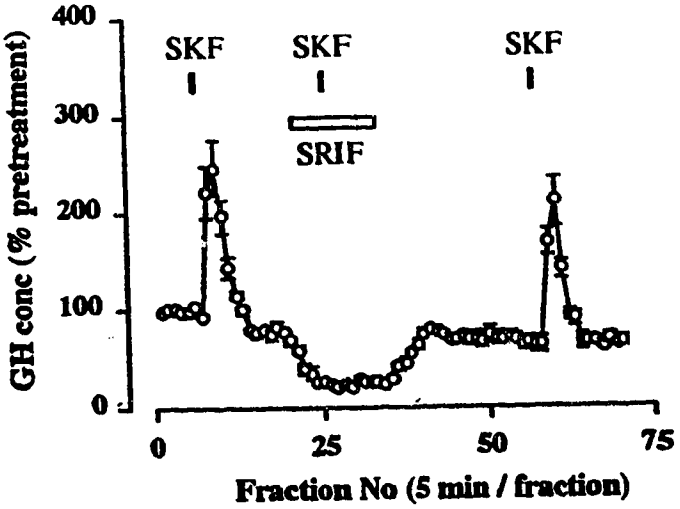


Fig. 4.5 Effects of SRIF on the D1 agonist SKF38393-stimulated GH release from perifused pituitary fragments of goldfish. SKF38393 (SKF) at 500 nM dose was administered as 2-min pulses before, during, and after the 1-hr continuous perifusion of 100 nM SRIF. The average pretreatment GH levels was 27 ± 2 ng/ml, and all GH data are presented as mean ± SEM (n = 4).

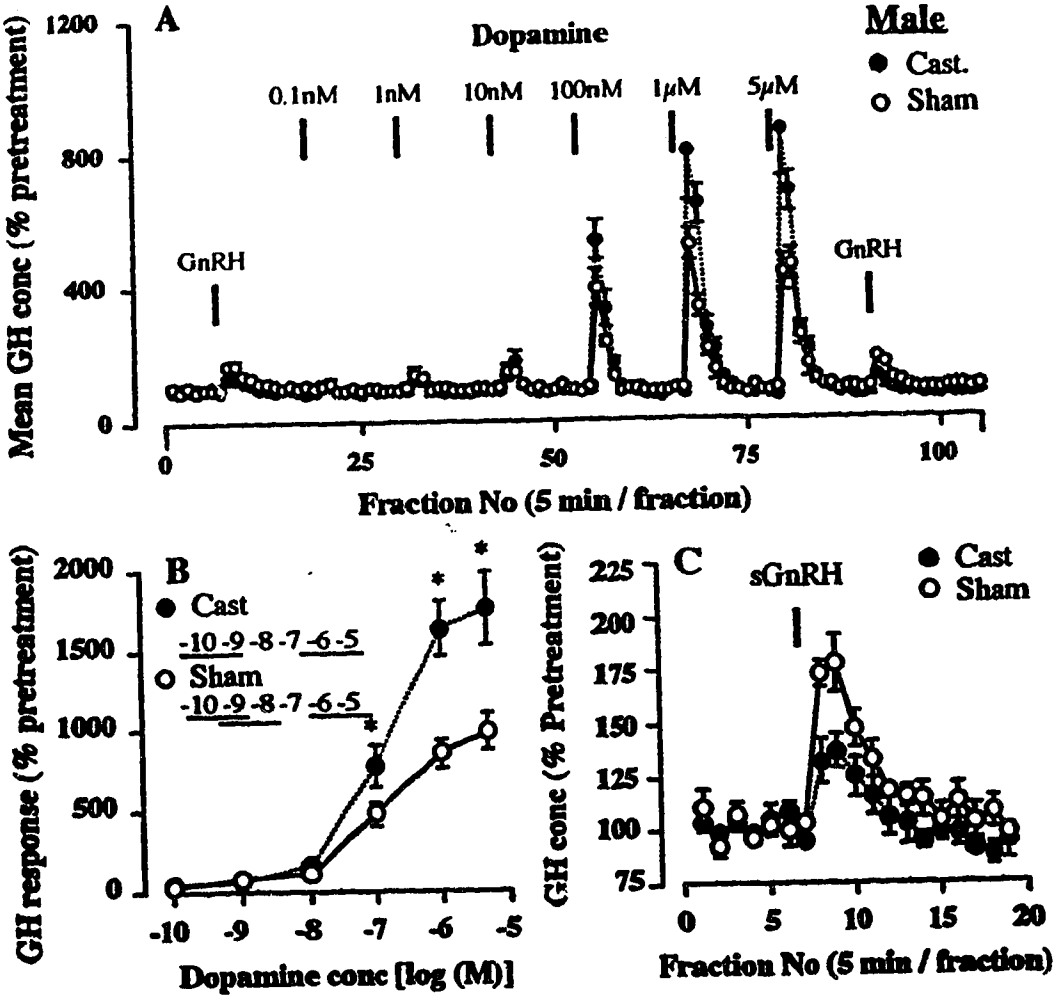


Fig. 4.6 Effects of castration on DA-stimulated GH release from perifused pituitary fragments of male goldfish. (A) DA from 0.1 nM to 5 μM concentrations was administered as 2-min pulses at 1 hour intervals. Average pretreatment GH levels for the castrated group (Cast) and sham-operated group (Sham) were 50 ± 7 and 29 ± 3 ng/ml, respectively. (B) The respective dose-response curves for the castrated and sham-operated groups were constructed using ALLFIT computer program. (C) Hormone release profile of the first 2-min 50 nM sGnRH pulse was magnified using a refined scale to highlight the effect of castration on sGnRH-stimulated GH release. All GH data are expressed as mean ± SEM (n = 4). Doses of DA giving similar magnitude of GH responses were grouped under the same underscore ($p>0.05$, ANOVA followed by Fisher's LSD test). GH responses to the same dose of DA were compared using Student's t test (*, $P<0.05$).

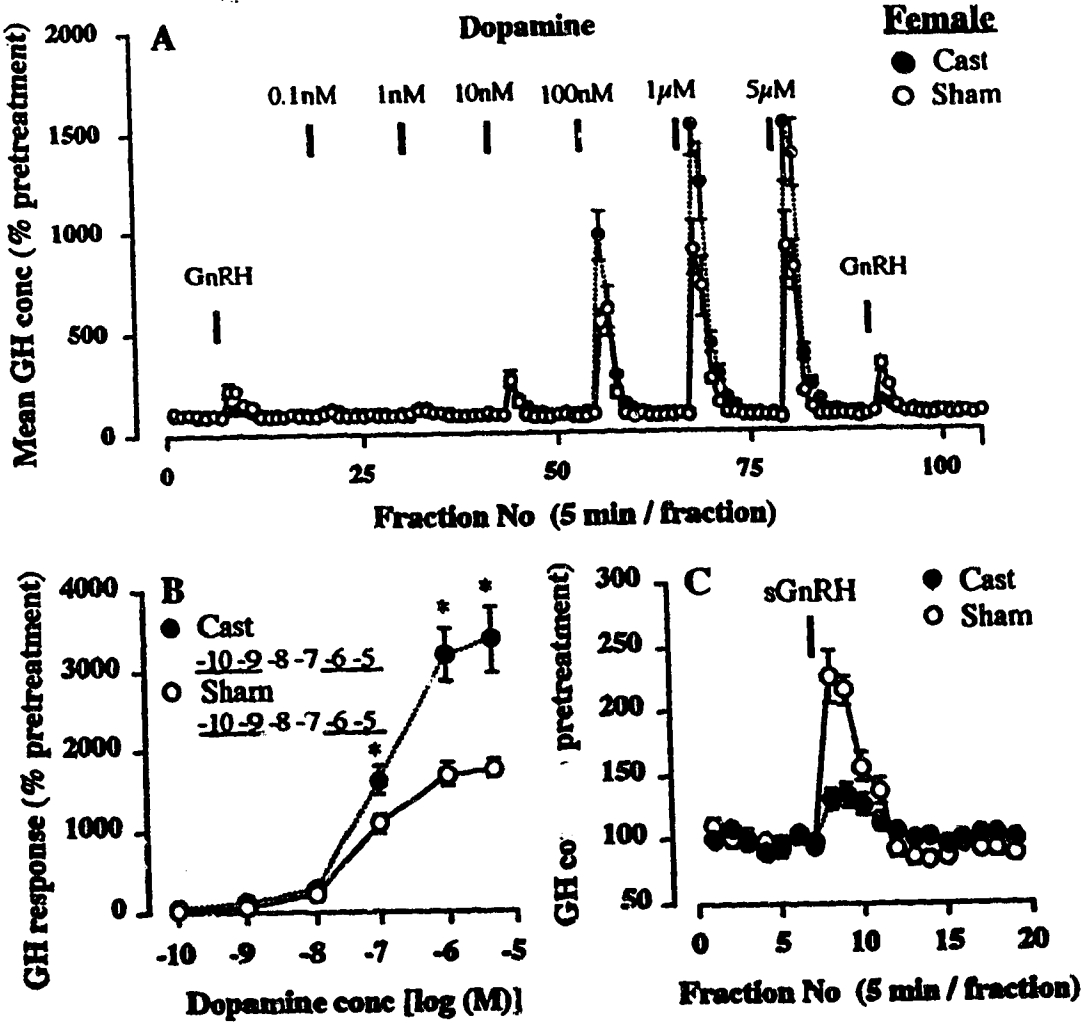


Fig. 4.7 Effects of castration on DA-stimulated GH release from perifused pituitary fragments of female goldfish. (A) DA from 0.1 nM to 5 μM concentrations was administered as 2-min pulses at 1 hour intervals. Average pretreatment GH levels for the castrated group (Cast) and sham-operated group (Sham) were 56 ± 4 and 42 ± 4 ng/ml, respectively. (B) The respective dose-response curves for the castrated and sham-operated groups were constructed using ALLFIT computer program. (C) GH release profile of the first 2-min 50 nM sGnRH pulse was magnified using a refined scale to highlight the effect of castration on sGnRH-stimulated GH release. All GH data are expressed as mean ± SEM (n = 4). Doses of DA giving similar magnitude of GH responses were grouped under the same underscore ($p>0.05$, ANOVA followed by Fisher's LSD test). GH responses to the same dose of DA were compared using Student's t test (*, $P<0.05$).

4.5 References

Andersen PH, Gingrich JA, Bates MD, Dearry A, Falardeau P, Senogles SE, Caron MG 1990 Dopamine receptor subtypes: beyond the D1/D2 classification. Trends Pharmacol Sci 11:231-236

Ball JN 1981 Hypothalamic control of the pars distalis in fishes. Gen Comp Endocrinol 44:135-170

Brown EM, Hughes DB 1983 D-1 dopamine receptor-mediated activation of adenylate cyclase, cAMP accumulation, and PTH release in dispersed bovine parathyroid cells. In: Kaiser C, Kebabian JW (eds) Dopamine Receptors. American Chemical Society, Washington DC, ACS Symposium Series 224:223-246

Chang JP, Cook H, Freedman GL, Wiggs AJ, Somoza GM, de Leeuw R, Peter RE 1990a Use of a pituitary cell dispersion method and primary culture system for the studies of gonadotropin-releasing hormone action in the goldfish, *Carassius auratus*. I. Initial morphological, static, and cell column perifusion studies. Gen Comp Endocrinol 77:256-273

Chang JP, Marchant TA, Cook AF, Nahorniak CS, Peter RE 1985 Influences of catecholamines on growth hormone release in female goldfish, *Carassius auratus*. Neuroendocrinology 40:463-470

Chang JP, Wong AOL, Van Der Kraak G, Van Goor F 1992 Relationship between cyclic AMP-stimulated and native gonadotropin-releasing hormone-stimulated gonadotropin release in the goldfish. Gen Comp Endocrinol 86:59-377

Chang JP, Jobin RM, de Leeuw R 1991 Possible involvement of protein kinase C in gonadotropin and growth hormone release from dispersed goldfish pituitary cells. Gen Comp Endocrinol 81:447-463

Chang JP, Yu KL, Wong ALO, Peter RE 1990b Differential actions of dopamine receptor subtypes on gonadotropin and growth hormone release *in vitro* in goldfish. Neuroendocrinology 51:664-674

Cook AF, Peter RE 1984 The effects of somatostatin on serum growth hormone levels in the goldfish, *Carassius auratus*. Gen Comp Endocrinol 54:109-113

Cook H, Berkenbosch JW, Fernhout MJ, Yu KL, Peter RE, Chang JP, Rivier JE 1991

Demonstration of gonadotropin releasing-hormone receptors on gonadotrophs and somatotrophs of the goldfish: an electron microscope study. Regul Pept 36:369-378

DeLean A, Munson PJ, Rodbard D 1978 Simultaneous analysis of families of sigmoidal curves: application of bioassay, radioligand assay, and physiological dose-response curves. Am J Physiol 235:E97-E102

Ge W, Cook H, Peter RE, Vaughan J, Vale W 1993 Immunocytochemical evidence for the presence of inhibin and activin-like proteins and their localization in goldfish gonads. Gen Comp Endocrinol (in press)

Habibi HR 1991 Homologous desensitization of gonadotropin-releasing hormone (GnRH) receptors in the goldfish pituitary: Effects of native GnRH peptides and a synthetic GnRH antagonist. Biol Reprod 44:275-283

Habibi HR, de Leeuw R, Nahorniak CS, Goos HJT, Peter RE 1989 Pituitary gonadotropin-releasing hormone (GnRH) receptor activity in goldfish and catfish: seasonal and gonadal effects. Fish Physiol Biochem 7:109-118

Habibi HR, Peter RE, Nahorniak CS, Raymond C, Milton DL, Millar RP 1992 Activity of vertebrate gonadotropin-releasing hormones and analogs with variant amino acid residues in positions 5,7 and 8 in the goldfish pituitary. Regul Pept 37:271-284

Jansson JO, Eden S, Isaksson O 1985 Sexual dimorphism in the control of growth hormone secretion. Endocrine Rev 6:128-150

Kah O, Breton B, Dulka JG, Nunez-Rodriguez J, Peter RE, Corrigan A, Rivier JE, Vale WW 1986 A re-investigation of the GnRH (gonadotrophin-releasing hormone) systems in the goldfish brain using antibodies to salmon GnRH. Cell Tissue Res 244:327-337

Kah O, Chambolle P, Dubourg P, Dubois MP 1982 Localisation immunocytochimique de la somatostatine dans le cerveau anterieur et l'hypophyse de deux Teleosteens, le Cyprin (*Carassius auratus*) et *Gambusia* sp. C.R. Acad Sci 294:519-524.

Kah O, Dubourg P, Chambolle P, Calas A 1984 Ultrastructural identification of catecholaminergic fibers in the goldfish pituitary. Cell Tissue Res 238:621-626

Kobayashi M, Aida K, Hanyu I 1989 Induction of gonadotropin surge by steroid hormone implantation in ovariectomized and sexually regressed female goldfish.

Gen Comp Endocrinol 73:469-476

MacKenzie DS, Gould DR, Peter RE, Rivier JE, Vale WW 1984 Response of superfused goldfish pituitary fragments to mammalian and salmon gonadotropin-releasing hormone. Life Sci 35:2019-2026

Marchant TA, Chang JP, Nahorniak CS, Peter RE 1989a Evidence that gonadotropin-releasing hormone also functions as a growth hormone-releasing factor in the goldfish. Endocrinology 124:2509-2518

Marchant TA, Dulka JG, Peter RE 1989b Relationship between serum growth hormone levels and the brain and pituitary content of immunoreactive somatostatin in the goldfish, *Carassius auratus*. Gen Comp Endocrinol 73:458-468

Marchant TA, Fraser RA, Andrews PC, Peter RE 1987 The influence of mammalian and teleost somatostatins on the secretion of growth hormone from goldfish (*Carassius auratus L*) pituitary fragments *in vitro*. Regul Pept 17:41-52

Marchant TA, Peter RE 1986 Seasonal variations in body growth rates and circulating levels of growth hormone in the goldfish, *Carassius auratus*. J Exp Zool 237:231-239

Nishioka RS, Kelly KM, Bern HA 1988 Control of prolactin and growth hormone secretion in teleost fish. Zool Sci 5:267-278

Park OK, Gugneja S, Mayo KE 1990 Gonadotropin-releasing hormone gene expression during the rat estrous cycle: Effects of pentobarbital and ovarian steroids. Endocrinology 127:365-372

Peter RE, Chang JP, Nahorniak CS, Omeljaniuk RJ, Sokolowska M, Shih SH, Billard R 1986 Interactions of catecholamines and GnRH in regulation of gonadotropin secretion in teleost fish. Recent Prog Horm Res 42:513-548

Peter RE, Nahorniak CS, Chang JP, Crim LW 1984 Gonadotropin release from the pars distalis of the goldfish, *Carassius auratus*, transplanted beside the brain or into the brain ventricles: additional evidence for a gonadotropin release-inhibitory factor. Gen Comp Endocrinol 55:337–346

Peter RE, Nahorniak CS, Sokolowska M, Chang JP, Rivier JE, Vale WW 1985 Structure-activity relationships of mammalian, chicken and salmon gonadotropin-releasing hormones *in vivo* in goldfish. Gen Comp Endocrinol 58:231-240

Peter RE, Yu KL, Marchant TA, Rosenblum PM 1990 Direct neural regulation of the teleost adenohypophysis. J Exp Zool [Suppl] 4:84-89

Tonnaer JADM, Leinders L, Van Delft AML 1989 Ovariectomy and subchronic estradiol-17β administration decrease dopamine D1 and D2 receptors in rat striatum. Psychoneuoendocrinology 14:469-476

Trudeau VL, Sloley BD, Wong AOL, Peter RE 1993 Interactions of gonadal steroids with brain dopamine and gonadotropin-releasing hormone in the control of gonadotropin-II secretion in the goldfish. Gen Comp Endocrinol 89:39-50

Trudeau VL, Somoza GM, Nahorniak CS, Peter RE 1992 Interactions of estradiol with gonadotropin-releasing hormone and thyrotropin-releasing hormone in the control of growth hormone secretion in the goldfish. Neuroendocrinology 56:483-490

Wong AOL, Chang JP, Peter RE 1992 Dopamine stimulates growth hormone release from the pituitary of goldfish, *Carassius auratus*, through the dopamine D1 receptors. Endocrinology 130:1201-1210

Wong AOL, Chang JP, Peter 1993a Dopamine functions as a growth hormone-releasing factor in the goldfish, *Carassius auratus* Fish Physiol Biochem (in press)

Wong AOL, Chang JP, Peter RE 1993b *In vitro* and *in vivo* evidence that dopamine exerts growth hormone releasing activity in the goldfish. Am J Physiol (in press)

Chapter 5

Characterization of D1 Receptors mediating Dopamine-Stimulated Growth Hormone Release from Goldfish Pituitary Cells [1]

5.1 Introduction

Physiological actions of dopamine (DA) are known to be mediated primarily by two DA receptor subtypes, the D1 and D2 receptors (Kebabian *et al.*, 1984). The two receptors are distinct membrane proteins (Strange, 1987), and can be differentiated based on their pharmacological properties. D1 receptors have a high binding affinity for phenyl benzazepines, such as SCH23390 and SKF83566, whereas D2 receptors are more selective to substituted benzamides and butyrophenones, such as (-)sulpiride and spiperone (Niznik, 1987; Stoof and Tilders, 1989). Other D2 drugs, especially LY171555 and domperidone, which have a high binding affinity and specificity for D2 receptors, are also frequently used in the research on DA receptors (for a recent review on the pharmacological probes for D1 and D2 receptors, see Neumeyer *et al.*, 1992).

In teleosts, the hypothalamo-pituitary axis is unique for the absence of a functional hypophyseal portal blood system, and the anterior pituitary is under the direct innervation from the hypothalamus (for review, see Peter *et al.*, 1990). In the goldfish, a dopaminergic preoptico-hypophyseal pathway innervating the adenohypophysis has been identified (Kah *et al.*, 1987), and DA nerve terminals are often located in close proximity to the somatotrophs (Kah *et al.*, 1986). These observations provide the anatomical basis for the possible involvement of DA in growth hormone (GH) regulation of the goldfish.

Previously, we have demonstrated that DA and the D1 agonists SKF38393 and SKF77434 stimulate GH release from perifused goldfish pituitary fragments. In contrast, the D2 agonist LY171555 is not effective in this regard. Furthermore, the GH

[1] A version of this chapter has been submitted for publication : Wong AOL, Chang JP, and Peter RE (1993) **Endocrinology**

responses to DA or the D1 agonist SKF38393 can be blocked by the D1 antagonists SCH23390 and SKF83566, but not the D2 antagonist domperidone (Wong *et al.*, 1992). Similar findings have been reported in *in vitro* studies using dispersed goldfish pituitary cells (Chang *et al.*, 1990b). Our recent *in vivo* studies also demonstrated that intraperitoneal (i.p.) injection of the non-selective DA agonist apomorphine and the D1 agonist SKF82958 increased plasma GH levels in the goldfish. These GH responses were suppressed by the D1 antagonist SCH23390 but not the D2 antagonist pimozide (Wong *et al.*, 1993). These results strongly suggest that DA stimulates GH release from the pituitary of goldfish through activation of D1 receptors.

DA D1 receptor binding sites have not been demonstrated in the pituitary of vertebrates. In the goldfish, our recent investigations have provided indirect evidence for the presence of D1 receptors in the pituitary of this particular animal model (Wong *et al.*, 1992; Wong *et al.*, 1993). In the present study, we examined the presence of D1-specific binding sites in the goldfish pituitary, and demonstrated that these binding sites have the expected properties of D1 receptors. To correlate the bioactivity in terms of DA D1-stimulated GH release with the binding characteristics of these putative "D1" receptors, dispersed pituitary cells from the goldfish and cell preparations enriched with somatotrophs were used for the receptor binding studies.

5.2 Materials and Methods

Animals Goldfish of the common or comet varieties were purchased from Ozark Fisheries (Stoutland, MO, USA) or Grassyforks Fisheries (Martinsville, IN, USA), and maintained in flow-through aquaria (1,800 liters) at 17 °C under a simulated natural photoperiod (Edmonton, AB, Canada) for at least 3 weeks prior to any experiments. The fish were fed to satiation daily with Ewos trout pellets (Astra Chemicals Ltd., Mississauga, ONT, Canada). Goldfish of both sexes, with body weight ranging from 20 to 30 g, were used in the present study. Since the seasonality of DA D1-stimulated GH release in the goldfish is known to be closely associated with the reproductive cycle (Wong *et al.*, 1993), gonadal conditions of the fish, characterized based on the gonadal morphology and gonadosomatic index (GSI = weight of gonad / total body weight x 100 %), are reported separately for individual experiments (for details, see chapter 2).

Reagents and test substances Radioactive ligand for the D1 receptor assay, [N-methyl-^{3}H]SCH23390 (N-methyl-^{3}H-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetra hydro-1H-3-benzazepine hydrochloride) ([^{3}H]SCH23390), was purchased from Amersham Canada Ltd. (Oakville, ONT, Canada). Dopamine, serotonin, and somatostatin (SRIF) were obtained from Sigma Chemical Company (St Louis, MO, USA). SKF38393 (1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine-7,8-diol hydro chloride), SKF83566 (7-bromo-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride), and the (+) and (-) enantiomers of SCH23390 (7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride) were purchased from Research Biochemicals Inc. (Wayland, MA, USA). LY171555 (trans-(-)-4aR-4,4a, 5,6,7,8,8a,9-octahydro-5-propyl-1H-pyrazolo[3,4-g]quinoline hydro choride) and domperidone were gifts from Eli Lilly & Co. (Indianapolis, IN, USA) and Janssen Pharmaceutica (Beerse, Belgium), respectively. The D1 and D2 drugs were first dissolved in a minimal amount of dimethyl sulfoxide (DMSO), and subsequently diluted to appropriate concentrations with Medium 199 (M199) for *in vitro* perifusion experiments, or with incubation medium for receptor binding studies. DMSO levels in the final solutions were always less than 0.1 %, and did not affect GH release from goldfish pituitary cells (Chang *et al.*, 1990b). Aliquots of salmon gonadotropin-releasing hormone (sGnRH) (Peninsula Laboratories, Belmont, CA, USA) in 0.1 M acetic acid stock solution were frozen at –25 °C, and made up to 50 nM solution immediately prior to use.

Preparation of dispersed pituitary cells Dispersed goldfish pituitary cells were prepared as described previously (Chang *et al.*, 1990a). In brief, goldfish pituitaries, diced into fragments about 0.5 mm in thickness, were exposed to controlled trypsin / DNAse treatment. Pituitary fragments were then mechanically dispersed in Ca^{2+}-deficient medium (M199 with Hank's salts prepared without the addition of $CaCl_2$, supplemented with 2.2 g/l sodium bicarbonate, 25 mM HEPES, 100,000 units penicillin/l, 100 mg streptomycin/l, and 0.3 % BSA; pH 7.2, GIBCO), and harvested by centrifugation at 200x g for 10 min at 4 °C. Cell yield of the dispersion procedures was

about 0.5 million cells per pituitary, and the viability of pituitary cells was >94 % as assayed by the trypan blue exclusion test.

Pituitary cells enriched with somatotrophs were prepared by a discontinuous density gradient as described by de Leeuw *et al.* (1984) with minor modifications (Chang and Jobin, 1993). Mixed populations of dispersed pituitary cells, which normally have about 20% of cells immunoreactive to GH antiserum, were gently layered on the top of a discontinuous Percoll gradient (with 40, 50, 60, 70, and 80 % Percoll) and centrifuged at 1400x g for 25 min at 17 °C. After centrifugation, pituitary cells collected at the interphase between 60 and 70 % Percoll were found to have the highest amount of cells immunoreactive to GH antiserum (≈70 %). Viability of these "enriched somatotrophs" after density gradient separation was always >92 % as indicated by the trypan blue exclusion test.

Perifusion of pituitary cells Dispersed pituitary cells were resuspended in plating medium (M199 with Earle's salt, supplemented with 2.2 g/l sodium bicarbonate, 25 mM HEPES, 100,000 units penicillin/l, 100 mg streptomycin/l, and 1 % horse serum; pH 7.2, GIBCO), and allowed to adhere onto preswollen cytodex beads (Cytodex I, Sigma) at 28 °C under 5 % CO_2 and saturated humidity. Cell attachment was >95 % after overnight incubation (15 - 18 hr). Cytodex beads with pituitary cells attached were loaded into 0.5 ml micro-columns (ACUSYST-S™, Endotronics Inc., Minneapolis, Minn., USA) as described previously (Chang *et al.*, 1990a). Two million cells per column was used for experiments using mixed populations of pituitary cells, and one million cells per column for experiments using enriched somatotrophs. The total dead volume of the perifusion system was about 1.7 ml. Pituitary cells were perifused with testing medium (M199 with Hank's salts, supplemented with 2.2 g/l sodium bicarbonate, 25 mM HEPES, 100,000 units penicillin/l, 100 mg streptomycin/l, and 0.1 % BSA; pH 7.2, GIBCO) at a flow rate of 15 ml/hr. After 3 hours of perifusion, basal GH release from pituitary cells remained relatively stable in the absence of any stimulation. Test substances were then added from a drug reservoir into the perifusion column through a 3-way stopcock. Perifusates from each column were collected in 5-min fractions, and stored at -25 °C until their hormone contents were assayed by a GH radioimmunoassay

(Marchant *et al.*, 1989a) . GH data were expressed as "% pretreatment" as defined previously (Wong *et al.*, 1992). In brief, GH data from each individual column were expressed as a percentage of the mean GH contents of the first 6 fractions of perifusates at the beginning of perifusion experiment (i.e., prior to any drug treatment). This transformation was done to allow pooling of data from separate columns of the same experiment without distorting the profile of hormone release. GH responses were quantified by calculating the net change in GH release (i.e., area under the curve) after a particular drug treatment (for details, see chapter 2).

Radioreceptor assay for goldfish D1 receptors Radioreceptor assay for D1 receptors in the goldfish pituitary was modified from the method described by Billard *et al.* (1984). Pituitary cells were detached from culture plates by replacing the culture medium with M199 containing Hank's salt (GIBCO) and 2 mM EGTA. About 0.5 - 0.8 million cells were aliquoted into each polypropylene assay tube (12 x 75 mm), and centrifuged at x200 g for 10 min at 4 °C. After centrifugation, the supernatant was removed, and the pellet resuspended in 0.8 ml ice-cold incubation medium (50 mM Tris buffer at pH 7.4, supplemented with 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM EDTA). Methylcellulose, which was included in the original buffer system described by Billard *et al.* (1984), was eliminated, as it significantly reduced both the non-specific and specific binding in our assay system. Incubation was initiated by adding 0.1 ml [^{3}H]SCH23390 (80 Ci/mmol, 1 nM in the final solution) and 0.1 ml incubation medium with 100 µM (+)SCH23390 (10 µM in the final solution to define non-specific binding) or with the appropriate concentrations of D1 or D2 drugs. In the present study, [^{3}H]SCH23390 at the final concentrations from 0.6 to 1.4 nM produced a high specific binding of about 68 % of the total radioligand bound (data not shown). Therefore, [^{3}H]SCH23390 at 1 nM dose was routinely used for the rest of the binding studies. After 2 hours of incubation at 4 °C, bound and free radioactive ligand were separated by rapid filtration, using a No.1225 Sampling Manifold (Millipore), through GF/B filters (Whatman) presoaked with incubation medium. Separation time was always less than 1.5 sec for the filtration system. The filters were then rinsed 2 times with 5 ml ice-cold

incubation medium. Our preliminary studies have demonstrated that 2 times rinsing reduced the non-specific binding without affecting the specific binding (Fig. 5.1). After drying the filters in a fumehood for 30 min, 5 ml of scintillation cocktail (0.2 g POPOP and 4 g PPO in a liter solution of toluene with 30 % Triton X-100) was added, and ^3H radioactivity in the GF/B filters measured the next day in a liquid scintillation counter. The recoverable radioactivity from the polypropylene tubes used in these experiments was found to be 96.3 ± 1.2 %. The stability of radioligand during the 2-hr incubation period was also tested. [^{3}H]SCH23390 (1 nM) was incubated with or without goldfish pituitary cells (1 million cells/ml/tube) for 2 hours at 4 °C. Supernatants of the two groups were collected after centrifugation, and loaded onto silica gel G plates (Fisher) for thin layer chromatography (TLC) as described previously (Omeljaniuk and Peter, 1989). TLC chromatograms were visualized by exposing to XAR films (Kodak) at -25 °C for 7 days. The results of TLC analysis did not indicate the occurrence of [^{3}H]SCH23390 degradation by incubation with goldfish pituitary cells (Fig. 5.2).

Autoradiography of goldfish D1 receptors Frozen sections of the goldfish pituitary (20 µm in thickness) were prepared at -20 °C using a Histostat™ cryostat (AO Scientific Instruments, Buffalo, N.Y., USA), and thaw-mounted onto precleaned and subbed microscope slides. Slide-mounted consecutive pituitary sections were placed in an incubation chamber, and incubated for 60 min at 4 °C with 1 nM of [^{3}H]SCH23390 in the presence or absence of the D1 antagonist SCH23390 (10 µM) or the D2 antagonist sulpiride (10 µM). These sections were then washed 2 times (1 min) with ice-cold incubation buffer (50 mM Tris buffer at pH 7.4, supplemented with 120 mM NaCl, 5mM KCl, 2 mM $CaCl_2$, 1mM $MgCl_2$, and 1 mM EDTA), and rapidly dipped in ice-cold distilled water to remove excess inorganic salts. Based on our preliminary studies, the above rinsing procedures consistently produced a specific binding of about 61 % of the total radioligand bound (data not shown). After drying for 30 min under a cool air stream, the pituitary sections were divided into two groups. In one group, the pituitary sections were wiped from the glass slides with GF/B filters. Thereafter, the filters with tissue fragments attached were incubated with 5 ml scintillation cocktail to measure the radioactivity of the bound [^{3}H]SCH23390. The other group of pituitary sections

previously exposed to radioligand with or without 10 μM SCH23390 were apposed with cover-slips precoated with NTB-2 emulsion (Kodak). After 45 days of exposure at -25 °C, the cover-slips were detached to develop the autoradiogram.

Data analysis and statistics GH data were analyzed by analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test. Differences were considered significant when $P<0.05$. Receptor binding affinity (K_d) and capacity (B_{max}) were determined using a weighted, non-linear least-squares curve-fitting program (LIGAND) for one- or two-site binding model as described by Munson and Rodbard (1980). IC_{50} values for D1 and D2 drugs were estimated by the four-parameter logistic equation using the ALLFIT program developed by DeLean *et al.* (1978).

5.3 Results

Effects of DA and the D1 agonist SKF38393 on GH release from goldfish pituitary cells To demonstrate that the pituitary cell preparation used for receptor binding studies was viable, we examined the actions of DA and the D1 agonist SKF38393 on GH release from goldfish pituitary cells under column perifusion (Fig. 5.3). Dispersed pituitary cells for these experiments were prepared from goldfish in late stages of gonadal regression. Similar to the results of our previous studies using perifused goldfish pituitary fragments (Wong *et al.*, 1992), 5-min pulses of DA (1 μM) and SKF38393 (1 μM) consistently stimulated GH release from goldfish pituitary cells. The D2 agonist LY171555 (1 μM), which served as a negative control for the experiment, was not effective in this regard.

Time course of [^{3}H]SCH23390 binding to dispersed goldfish pituitary cells Dispersed pituitary cells were prepared from post-spawning goldfish in early stages of gonadal regression. Specific [^{3}H]SCH23390 binding to pituitary cells increased rapidly with time, and reached an equilibrium within 20 min of incubation (Fig. 5.4). Thereafter, the specific binding remained stable for the following 1.5 hours. Maximal specific binding was about 57 % of the the total radioligand bound. The binding data (from 0 - 30 min) were fitted to a straight line ($R^2 = 0.97$) with an observed association rate constant (K_{obs}) of 0.073 /min based on a pseudo first-order kinetics (Fig. 5.4, inset). Since the

variability of data appeared to be reduced by using a longer incubation time, a 2-hr incubation period at 4 °C was routinely used for the subsequent binding studies.

[^{3}H]SCH23390 binding to increasing amount of goldfish pituitary cells Dispersed pituitary cells were prepared from goldfish undergoing gonadal recrudescence. Total and specific [^{3}H]SCH23390 binding increased gradually with the number of pituitary cells used in the binding study (Fig. 5.5). A linear correlation (R^2 = 0.98) between the amount of pituitary cells and specific binding of [^{3}H]SCH23390 was observed up to the level of 1 million cells/ml/tube.

Stereoselectivity of [^{3}H]SCH23390 binding and displacement with domperidone and serotonin Dispersed pituitary cells were prepared form goldfish undergoing gonadal regression. [^{3}H]SCH23390 binding was displaced in a dose-dependent manner by both (+) and (-)SCH23390 (Fig. 5.6A). IC_{50} values for (+) and (-)SCH23390, as deduced by the ALLFIT program, were 14 ± 4 and 553 ± 60 nM, respectively. These results indicate that (+)SCH23390 is more potent than (-)SCH23390 in displacing [^{3}H]SCH23390. The displacement curve for (+)SCH23390 was analyzed by Scatchard plot using the LIGAND program (Fig. 5.6B). The binding data were best fitted with a one-site model with K_d and B_{max} values of 33 ± 8 nM and 250 ± 88 pmole/million cells, respectively. The corresponding Hill plot (Fig. 5.6B, inset) was linear with a slope factor of 1.04, suggesting that (+)SCH23390 interacts with its binding sites in a non-cooperative manner.

The D2 antagonist domperidone displaced [^{3}H]SCH23390 binding from goldfish pituitary cells in a dose-dependent manner with an IC_{50} of 1.0 ± 0.2 μM (Fig. 5.6A). Serotonin, however, was even less effective in this respect; the first significant displacement of [^{3}H]SCH23390 binding was observed only at 10 μM dose of serotonin. When compared to the displacement curve of (+)SCH23390, the rank order of potency in displacing the radioligand was (+)SCH23390 > domperidone >> serotonin.

Effects of DA and SRIF on GH release from enriched somatotrophs Specific binding of [^{3}H]SCH23390 was also studied using pituitary cells enriched with somatotrophs. To

examine the responsiveness of this pituitary cell preparation, the actions of DA and SRIF on GH release were tested using enriched somatotrophs under column perifusion (Fig. 5.7). Increasing concentrations of DA (0.01 - 1 μM) administered as 2-min pulses at 1 hour intervals stimulated GH release from enriched somatotrophs in a dose-dependent manner (Fig. 5.7A). The protocol for perifusion has been previously validated to have no potentiating nor desensitizing effects within the dose range tested (Wong *et al.*, 1992). Goldfish in the late stages of gonadal recrudescence were used to prepare the enriched somatotrophs for this experiment.

Since sGnRH was previously shown to induces GH release in the goldfish (Marchant *et al.*, 1989b), 2-min pulses of 50 nM sGnRH were given at the beginning and at the end of the DA dose-response study to serve as an internal control. No significant differences were found between the GH responses to the first and second sGnRH pulses (206 ± 18 and 224 ± 15 % pretreatment, respectively), indicating that the responsiveness and viability of enriched somatotrophs were maintained during the perifusion experiment.

In a separate experiment (Fig. 5.7B), enriched somatotrophs were prepared from goldfish in mid-stages of gonadal recrudescence. DA at 1 μM dose was given as 2-min p[illegible] during the 1 hour continuous perifusion of SRIF (100 nM). SRIF treatment suppressed the basal GH levels and abolished the GH responses to DA.

Saturation of D1 binding sites in enriched somatotrophs with [^{3}H]SCH23390 Enriched somatotrophs were prepared from goldfish in gonadal regression. Specific binding of [^{3}H]SCH23390 increased gradually with increasing doses of the radioligand, and subsequently reached a plateau at 10.8 nM [^{3}H]SCH23390 (Fig. 5.8A). The binding data, as analyzed by Scatchard plot using the LIGAND program, were best fitted with a one-site model with K_d and B_{max} values of 11 ± 3 nM and 789 ± 138 pmole/million cells, respectively (Fig. 5.8B). The corresponding Hill plot (Fig. 5.8B, inset) also yielded a linear fit with a slope factor of 0.99.

Displacement of [^{3}H]SCH23390 binding from enriched somatotrophs with SKF83566 and LY171555 Enriched somatotrophs were prepared from goldfish undergoing gonadal regression. Both the D1 antagonist SKF83566 and the D2 agonist LY171555 displaced [^{3}H]SCH23390 binding in a dose-dependent manner (Fig. 5.9). The corresponding IC_{50} values for SKF83566 and LY171555, as deduced by the ALLFIT program, were found to be 71 ± 2 nM and 9.1 ± 1.0 μM, respectively. These data demonstrate that SKF83566 is at least 100 fold more potent than LY171555 in displacing [^{3}H]SCH23390 binding from goldfish somatotrophs.

Autoradiographic mapping of [^{3}H]SCH23390 binding sites in the goldfish pituitary Sexually regressed goldfish were used to prepare frozen sections of the pituitary for autoradiographic studies. The D1 antagonist SCH23390 (10 μM) significantly reduced the binding of [^{3}H]SCH23390 (1 nM) in goldfish pituitary sections (Fig. 5.10). The D2 antagonist sulpiride (10 μM), however, was not effective in this regard. In the present study, the specific binding of [^{3}H]SCH23390 accounted for about 60 % of the total radioligand bound. Representative autoradiograms of pituitary sections previously exposed to [^{3}H]SCH23390 (1 nM) with 10 μM SCH23390 (Fig. 5.11A), and [^{3}H]SCH23390 (1 nM) alone are also presented (Fig. 5.11B). Specific binding of [^{3}H]SCH23390 was localized predominantly in the proximal pars distalis (PD), and was not evident in the neurointermediate lobe (NIL) of the goldfish pituitary. Since the radioactivity signal in the proximal pars distalis was so intense, it was not possible to correlate the source of radioactivity with a specific cell type.

5.4 Discussion

In the goldfish, DA has been demonstrated to stimulate GH release, both *in vivo* (Wong *et al.*, 1993) and *in vitro* (Peter *et al.*, 1990; Wong *et al.*, 1992). Our previous pharmacological studies indicate that the GH-releasing effects of DA are mediated through DA D1 receptors (Chang *et al.*, 1990b; Wong *et al.*, 1992). In the current investigation, we provide the first direct evidence for the presence of D1-specific binding sites in the goldfish pituitary cells, and demonstrate that these binding sites have properties similar to the mammalian D1 receptors. These binding sites associated with

the D1 radioligand [^{3}H]SCH23390 in a rapid manner with K_{obs} of 0.073 /min. Such a rapid association of [^{3}H]SCH23390 has been previously reported for D1 receptors in the striatum of rats (Billard *et al.*, 1984) and the cerebral cortex of rabbits (Reader *et al.*, 1989). In the goldfish model, specific binding of [^{3}H]SCH23390 increased gradually with increasing amount of pituitary cells used in the incubation. The demonstration of "tissue linearity" in the present study indicates that the assay system for goldfish D1 receptors (i.e., at least up to 1 million cells/ml/tube level) is relatively clear of artifacts caused by receptor or ligand degradation. This is also supported by the results of TLC analysis, in which the chromatographical properties of [^{3}H]SCH23390 were not affected by previous incubation with pituitary cells. (For a detailed discussion of tissue linearity in radioreceptor assay, see Burt, 1985)

The D1 binding sites in the goldfish pituitary were saturable, and had a high binding affinity for [^{3}H]SCH23390. Based on Scatchard analysis using the LIGAND program, K_d values of the D1 binding sites in dispersed pituitary cells and enriched somatotrophs of the goldfish were estimated to be 33 ± 8 nM and 11 ± 3 nM, respectively. These results are comparable to the nanomolar K_d values of the mammalian D1 receptors as reported in the rat striatum (Billard *et al.*, 1984), rabbit cerebral cortex (Reader *et al.*, 1989), and human neuroblastoma cells (Sidu and Fishman, 1990). In mammals, the D1 receptors always exhibit a higher selectivity for the (+) but not (-) enantiomer of phenylbenzazepine derivatives (for reviews, see Kaiser, 1983; Iorio *et al.*, 1986). In the goldfish pituitary cells, the binding affinity of the putative "D1" receptors for (+)SCH23390 was 38 fold higher than that of (-)SCH23390. This is also in accordance with our previous findings that (+) but not (-)SKF38393 is effective in stimulating GH release from perifused goldfish pituitary fragments (Wong *et al.*, 1992). The demonstration of a similar binding affinity and stereoselectivity between the goldfish and mammalian D1 systems strongly suggests that DA D1 receptors are highly conserved during vertebrate evolution.

The specific binding of [^{3}H]SCH23390 in goldfish pituitary cells was reversible, and could be displaced with high affinity by the D1-selective ligands, (+)SCH23390 and SKF83566. The D2-specific ligands, LY171555 and domperidone, were much less effective in this regard. Since a cross-reactivity of [^{3}H]SCH23390 with serotonin 5HT$_2$

receptors has been reported in the frontal cortex of rats (Barnett *et al.*, 1988) and the thoracic aorta of rabbits (Ohlstein and Berkowitz, 1985), the effects of increasing doses of serotonin on specific binding of [^{3}H]SCH23390 were also examined in the present study. The IC_{50} of serotonin, as deduced by the ALLFIT program (by assuming a complete displacement occurred at 1 mM serotonin), was estimated to be >100 μM, indicating that the specific binding of [^{3}H]SCH23390 as a result of "non-selective" association with 5HT2 receptors was unlikely in the goldfish "D1" system. Based on the comparison of IC_{50} values, the relative binding affinity for the "D1" receptors in the goldfish pituitary is (+)SCH23390 > SKF83566 >> (-)SCH23390 > domperidone > LY171555 >> serotonin. A similar rank order of potency in the pharmacological profile for D1 receptors has been reported in the caudate nucleus (Seeman and Ulpian, 1986) and striatum of the rat (Iorio *et al.*, 1986), and the cerebral cortex of rabbits (Reader *et al.*, 1989). Although the gene sequences of some novel forms of mammalian DA receptors, such as D3 (Sokoloff *et al.*, 1990), D4 (Van Tol *et al.*, 1991), and D5 receptors (Sunahara *et al.*, 1991), have been cloned recently, selective pharmacological probes for these receptors are not yet readily available, and the possible existence of these receptors in the goldfish pituitary was not examined in the present study (for a recent review on the molecular biology of DA receptors, see Sibley and Monsma, 1992).

In the present study, the binding data of [^{3}H]SCH23390 were best fitted with a one-site receptor model, and the results of Hill plot analysis indicated that the radioligand associated with "D1" receptors in a non-cooperative manner. In mammals, the presence of high and low affinity binding sites for D1 receptors has been reported, and these binding sites were later confirmed to be the high and low affinity binding states of D1 receptors (for review, see Seeman *et al.*, 1986). By incubating a membrane preparation of calf caudate nucleus with non-hydrolyzable GTP analogs, the high affinity state of D1 receptors can be completely converted into the low affinity state (Seeman *et al.*, 1985). Similar conversion from the high to low affinity binding state was also observed for D2 receptors in a membrane preparation of the bovine pituitary (Sibley *et al.*, 1982), but not in intact pituitary cells (Sibley *et al.*, 1983). It is generally accepted that the two binding states represent the differential coupling of DA receptors with the G-proteins (Senogles *et al.*, 1987), and can only be demonstrated by using a membrane preparation for

receptor binding studies (Sibley *et al.*, 1983). In the goldfish, our preliminary studies using a membrane preparation of the whole pituitary produced a very low level of specific binding (~1.2 % of the total radioligand bound), which precludes any accurate measurements to examine the high and low affinity binding states for the goldfish "D1" receptors.

The "D1" receptors in the goldfish model were confirmed to mediate DA-stimulated GH release at the pituitary cell level. DA stimulated GH release from goldfish pituitary cells under column perifusion, and this GH response could be mimicked by the D1 agonist SKF38393 but not the D2 agonist LY171555. Using an enriched somatotroph preparation, the GH-releasing actions of DA were found to be dose-dependent, and under the direct inhibition of the GH-release inhibitor SRIF. Similar inhibitory effects of SRIF on DA-stimulated GH release have been previously reported in the goldfish, both *in vivo* and *in vitro* (Wong *et al.*, 1993). Furthermore, the ED_{50} of SKF38393-stimulated GH release was estimated to be 26 ± 7 nM in mixed populations of goldfish pituitary cells (A.O.L. Wong, J.P. Chang, and R.E.Peter, unpublished results; see chapter 6), which is in the same dose range of the binding affinity of [^{3}H]SCH23390 illustrated in the current study (K_d = 33 ± 8 nM for mixed populations of pituitary cells, and 11 ± 3 nM for enriched somatotrophs).

In the present study, we also examined the differential distribution of D1 binding sites in the goldfish pituitary. Specific binding of [^{3}H]SCH23390 in the frozen pituitary sections could be displaced by the D1 antagonist SCH23390 but not the D2 antagonist sulpiride. Specific binding of [^{3}H]SCH23390 was localized in the proximal pars distalis of the goldfish pituitary. On the contrary, the neurointermediate lobe did not demonstrate any significant levels of specific binding. In the goldfish, the distribution of pituitary cells immunoreactive to GH antiserum is confined to the proximal pars distalis of the pituitary (Cook *et al.*, 1983). The overlap in distribution between the somatotrophs and "D1" receptors suggests that the "D1" receptors are indeed present in the somatotrophs and functionally correlated with the GH-releasing actions of DA in the goldfish. This hypothesis is also supported by our findings that the binding capacity of [^{3}H]SCH23390 in enriched somatotrophs (B_{max} =789 ± 138 pmole/million cells) was always higher than

that of mixed populations of pituitary cells (B_{max} = 250 ± 88 pmole/million cells).

In summary, the present study has provided the first direct evidence for the existence of D1-specific receptor binding sites in goldfish pituitary cells. These "D1" receptors are saturable, stereo-specific, and selective for the D1 ligands. The association of these "D1" receptors with [^{3}H]SCH23390 is rapid and reversible, and exhibits a binding affinity with K_d values in the nanomolar range. These "D1" receptors are localized in the pars distalis, overlapping with the distribution of somatotrophs in the goldfish pituitary. These newly characterized "D1" receptors can be functionally correlated with DA-stimulated GH release in the goldfish. Since DA D1 receptors have not been previously demonstrated in the pituitary of vertebrates, the "D1" system in the goldfish pituitary represents a unique model to study the role of D1 receptors in the regulation of pituitary hormones. The apparent similarities of the D1 receptor pharmacology between the goldfish and mammals also suggests that the DA D1 receptors are highly conserved during vertebrate evolution.

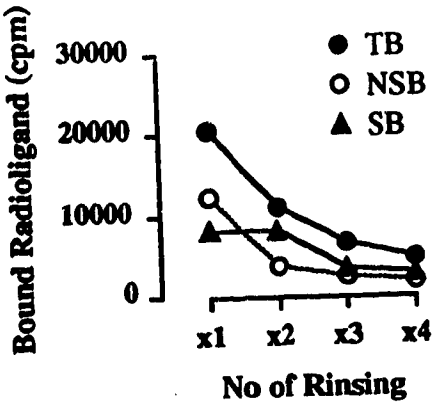


Fig. 5.1 Effects of repeated rinsing on specific binding of [^{3}H]SCH23390. Dispersed goldfish pituitary cells at 0.5 million cells per tube were incubated with 1 nM [^{3}H]SCH23390 for 2 hou[illegible]4 °C. After rapid filtration to remove the unbound [^{3}H]SCH2339[illegible], GF/B filters were rinsed repeatedly with 5 ml ice-cold incubation buffer as the number indicated. Data for total bound (TB), non-specific binding (NSB), and specific binding (SB) are the means of two replicate tubes (n = 2).

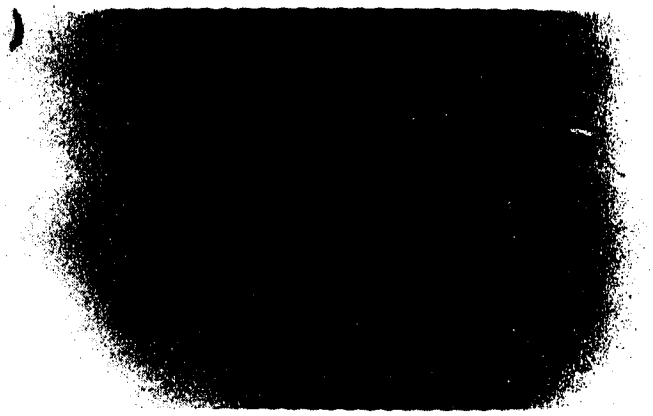


Fig. 5.2 TLC analysis of [^{3}H]SCH23390 previously incubated with goldfish pituitary cells. [^{3}H]SCH23390 (1 nM) was incubated (a) without and (b) with goldfish pituitary cells (1 million cells/ml/tube) for 2 hours at 4 °C. Supernatants of the two groups were loaded in silica gel G plate for TLC analysis, and the results of TLC chromatogram were visualized by exposing to XAR film. The experiment was repeated two times, and only the representative results are presented.

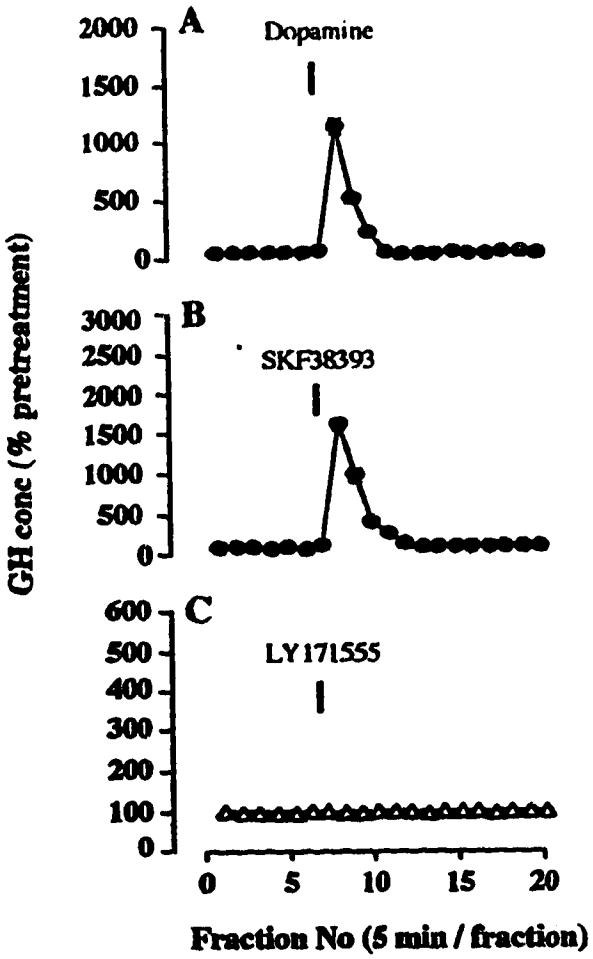


Fig. 5.3 Effects of (A) dopamine, (B) the D1 agonist SKF38393, and (C) the D2 agonist LY171555 on GH release from perifused goldfish pituitary cells. Drug treatments were administered as 5-min pulses at 1 μM dose as indicated by the black bars (x4 columns each). The mean pretreatment GH level for the experiment was 14 ± 2 ng/ml. All GH data are expressed as mean ± SEM (n =4).

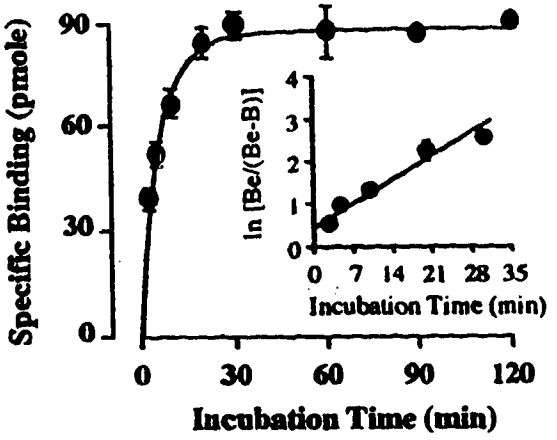


Fig. 5.4 Time course of [^{3}H]SCH23390 binding to goldfish pituitary cells. Pituitary cells at 0.7 million cells per tube were incubated at 4 °C with 1 nM [^{3}H]SCH23390 for 0 to 120 min. The inset is the pseu first-order association plot of the binding data with K_{obs} of 0.073 ... an. All data are expressed as mean ± SEM (n = 3).

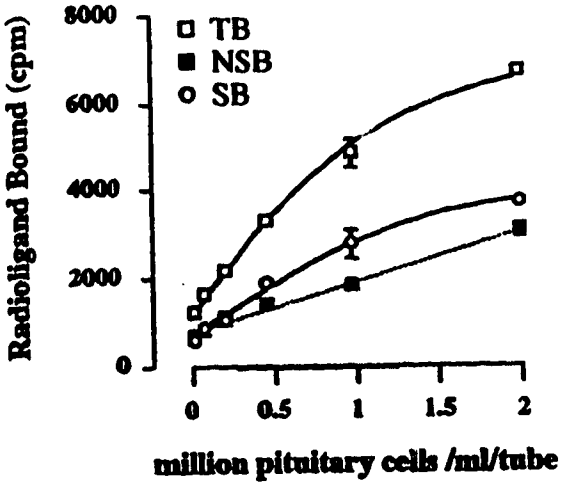


Fig. 5.5 [^{3}H]SCH23390 binding to increasing amount of goldfish pituitary cells. Pituitiary cells were incubated with 1 nM [^{3}H]SCH23390 for 2 hours at 4 °C. Data for total bound (TB), non-specific binding (NSB), and specific binding (SB) are expressed as mean ± SEM (n = 3).

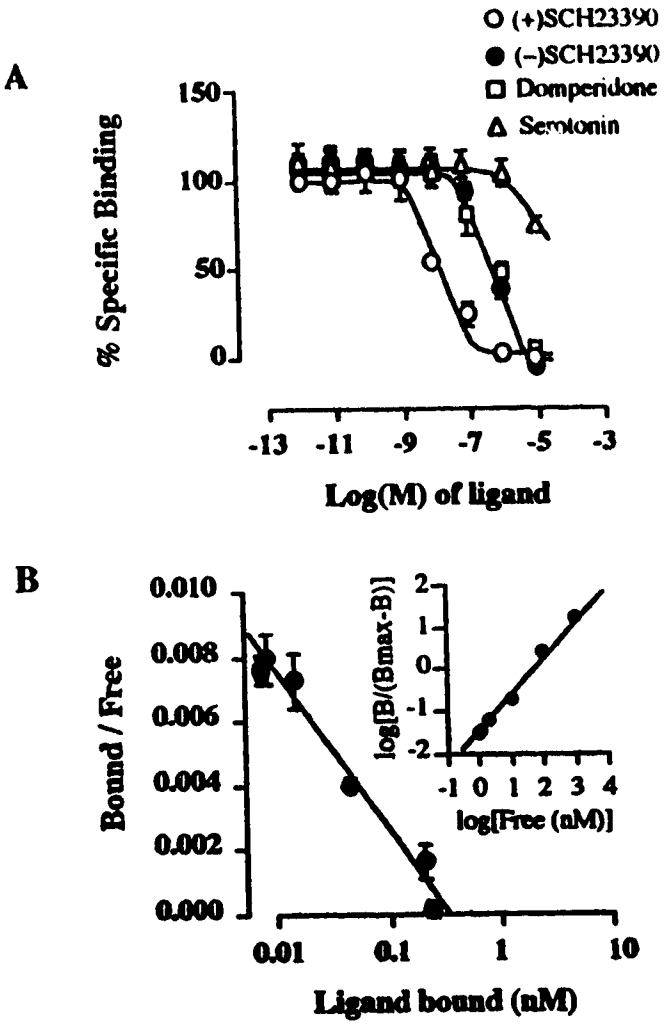


Fig. 5.6 Displacement of bound [^{3}H]SCH23390 with increasing doses of (+) and (−)SCH23390, domperidone and serotonin. Goldfish pituitary cells at 0.85 million cells per tube were incubated with 1nM [^{3}H]SCH23390 for 2 hours at 4 °C in the presence of SCH23390, domperidone, and serotonin (A). Displacement curves were expressed as a percentage of the maximal specific binding for [^{3}H]SCH23390. The IC_{50s} for (+)SCH23390, (-)SCH23390, domperidone, and serotonin were estimated to be 14 ± 4 nM, 553 ± 60 nM, 1.0 ± 0.2 μM, and >100 μM, respectively. Scatchard plot for (+)SCH23390 was constructed using the LIGAND program (B), the corresponding values for Kd and Bmax were 33± 8 nM and 250 ± 88 pmole/million cells, respectively. The inset represents the Hill plot analysis of the binding data fitted to a straight line ($R^2 = 0.98$) with a Hill coefficient of 1.04. All data are expressed as mean ± SEM (n = 3).

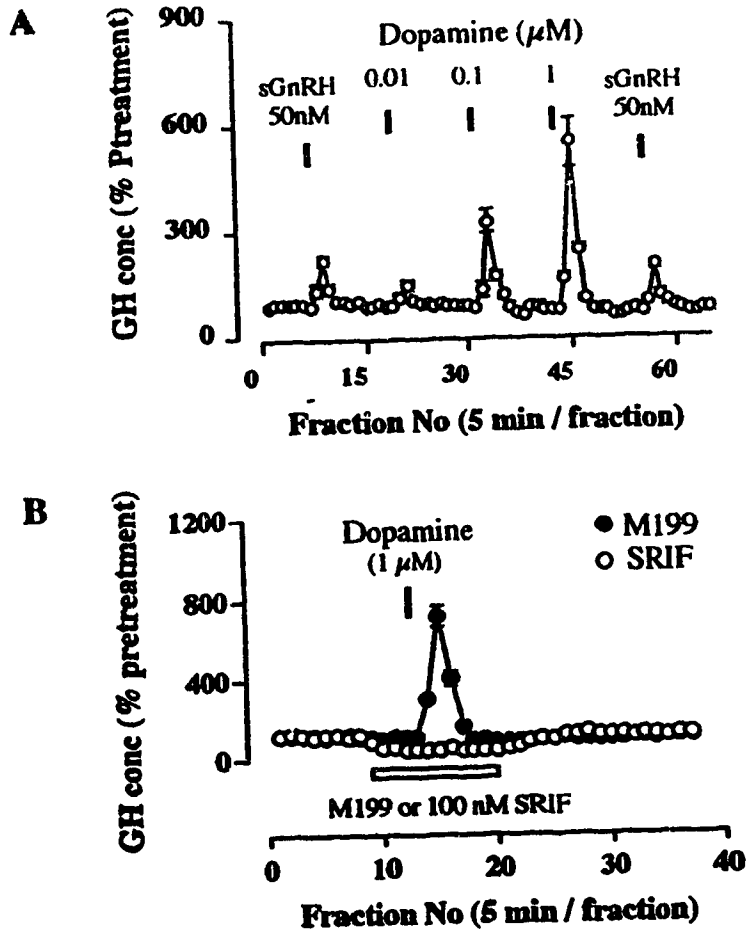


Fig. 5.7 Effects of dopamine (DA) and somatostatin (SRIF) on GH release from perifused goldfish pituitary cells enriched with somatotrophs. (A) DA was administered as 2-min pulses from 0.01 to 1 μM at 1 hour intervals (x4 columns). Salmon gonadotropin-releasing hormone (sGnRH) at 50 nM was given as 2-min pulses at the beginning and at the end of the perifusion experiment to serve as an internal control. (B) 2-min pulses of DA (1 μM) were administered in the presence or absence of the 1-hr continuous perifusion of SRIF (100 nM) (x4 columns each). The average pretreatment GH level for the perifusion experiments was 14 ± 2 ng/ml. All GH data are expressed as mean ± SEM (n = 4).

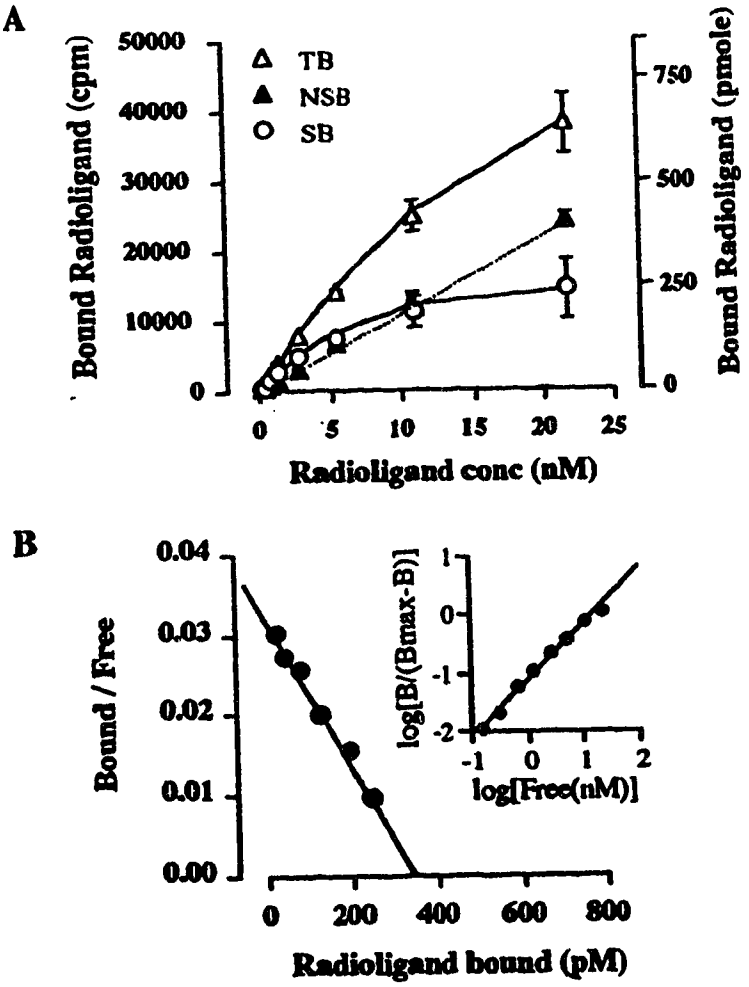


Fig. 5.8 Saturation of D1 binding sites with [^{3}H]SCH23390 in goldfish pituitary cells enriched with somatotrophs. Enriched somatotrophs at 0.5 million cells per tube were incubated with increasing amount of [^{3}H]SCH23390 for 2 hours at 4 °C (A). Scatchard plot was constructed using the LIGAND program (B), the corresponding values for Kd and Bmax were 11 ± 3 nM and 789 ± 138 pmole/million cells, respectively. The inset represents the Hill plot analysis of the binding data fitted to a straight line (R^2 = 0.99) with a Hill coefficient of 0.97. All data are expressed as mean ± SEM (n = 3).

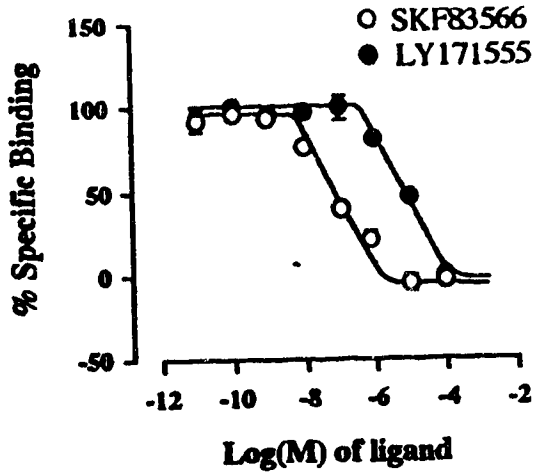


Fig. 5.9 Displacement of bound [^{3}H]SCH23390 with increasing doses of the D1 antagonist SKF83566 and the D2 agonist LY171555. Enriched somatotrophs from the goldfish at 0.5 million cells per tube were incubated with 1 nM [^{3}H]SCH23390 for 2 hours at 4 °C in the presence of either SKF83566 or LY171555. The IC_{50}s of SKF83566 and LY171555 were 71.3 ± 2.3 nM and 9.1 ± 1.0 μM, respectively. All data are expressed as mean ± SEM (n = 3).

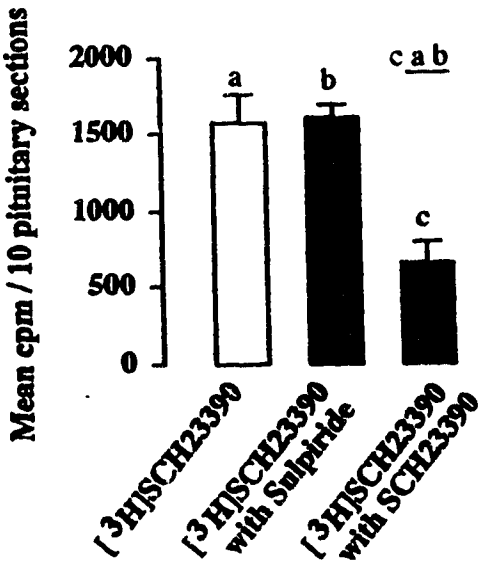


Fig. 5.10 Displacement of bound [^{3}H]SCH23390 from goldfish pituitary sections with the D1 antagonist SCH23390 and the D2 antagonist sulpiride. Consecutive pituitary sections were exposed to [^{3}H]SCH23390 (1 nM) alone, [^{3}H]SCH23390 (1 nM) with sulpiride (10 μM), and [^{3}H]SCH23390 (1 nM) with SCH23390 (10 μM), respectively. After incubation for 1 hr at 4 °C, radioactivity of these pituitary sections were measured using a liquid scintillation counter. 10 pituitary sections for each individual drug treatment were run at the same time, and the experiment was repeated six times. The data were pooled and expressed as mean ± SEM (n = 6).

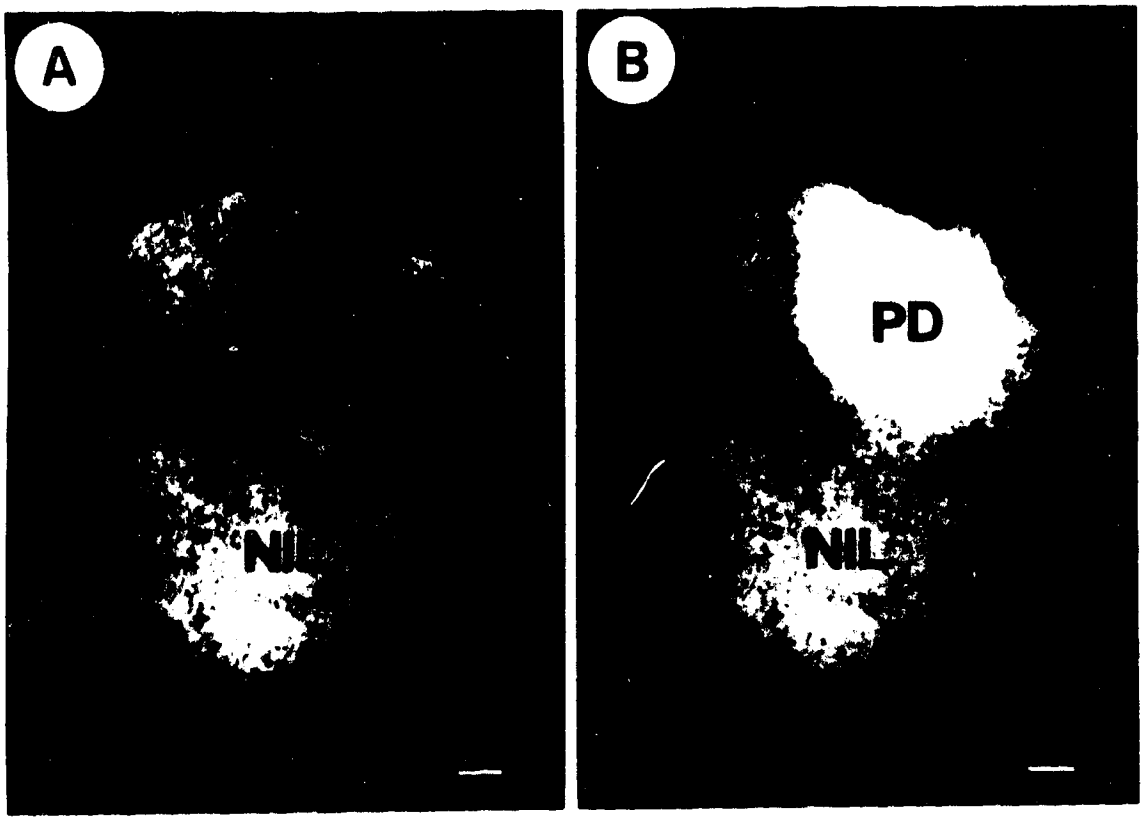


Fig. 5.11 Differential distribution of [^{3}H]SCH23390 specific binding in the goldfish pituitary. Frozen sections of the goldfish pituitary were previously incubated (A) with [3H]SCH23390 (1 nM) in the presence of 10 μM SCH23390, and (B) [^{3}H]SCH23390 (1 nM) alone. Binding of [^{3}H]SCH23390 was visualized by autoradiography. Results presented are the autoradiograms under dark field microscopy. Specific binding of [^{3}H]SCH23390 was localized in the proximal pars distalis (PD), but not in the neurointermediate lobe (NIL).
(Calibration bar = 0.1 mm)

5.5 References

Barnett A, McQuade R, Chipkin R, Vemulapalli S, Chiu P 1988 The effects of SCH23390 and related benzazepines on other CNS and peripheral receptor systems. In : Spano PF, Biggio G, Toffano G, Gessa GL (eds) Central and Peripheral Dopamine Receptors: Biochemistry and Pharmacology. Liviana Press, Padova, pp 69-79

Billard W, Ruperto V, Crosby G, Iorio LC, Barnett A 1984 Characterization of the binding of ^{3}H-SCH23390, a selective D-1 receptor antagonist ligand, in rat striatum. Life Sci 35:1885-1893

Burt DR 1985 Criteria for receptor identification. In : Yamamura HI, Kuhar MJ (eds) Neurotransmitter Receptor Binding (2nd ed), Raven Press, New York, pp 41-60

Chang JP, Cook H, Freedman GL, Wiggs AJ, Somoza GM, de Leeuw R, Peter RE 1990a Use of a pituitary cell dispersion method and primary culture system for the studies of gonadotropin-releasing hormone action in the goldfish, *Carassius auratus*. I. Initial morphological, static, and cell column perifusion studies. Gen Comp Endocrinol 77:256-273

Chang JP, Jobin RM 1993 Teleost pituitary cells: Isolation, culture, and use. In : Hochachka PW, Mommsen TP (eds) Biochemistry and Molecular Biology of Fishes (vol.3): Analytical Techniques. Elsevier, Amsterdam (in press)

Chang JP, Yu KL, Wong AOL, Peter RE 1990b Differential actions of dopamine receptor subtypes on gonadotropin and growth hormone release *in vitro* in goldfish. Neuroendocrinology 51:664-674

Cook H, Cook AF, Peter RE, Wilson SW 1983 Ultrastuctural immunocytochemistry of growth hormone cells in the goldfish pituitary gland. Gen Comp Endocrinol 50:348-353

De Lean A, Munson PJ, Rodbard D 1978 Simultaneous analysis of families of sigmoidal curves : Application to bioassay, and physiological dose-response curves. Am J Physiol 235:E97-E102

DeLeeuw R, Goos HJT, Peute J, Van Pelt AAM, Burzawa-Gerard E, Van Oordt PGWJ 1984 Isolation of gonadotrops from the pituitary of the African catfish, *Clarias*

lazera. Morphological and physiological characterization of purified cells. Cell Tissue Res 236:669-675

Iorio LC, Barnett A, Billard W, Gold EH 1986 Benzazepines : Structure-activity relationships between D1 receptor blockade and selected pharmacological effects. In: Breese GR, Creese I (eds) Neurobiology of Central D1-Dopamine Receptors, Plenum Press, New York, pp 1-14

Kah O, Dubourg P, Onteniente B, Geffard M, Calas A 1986 The dopaminergic innervation of the goldfish pituitary: An immunocytochemical study at the electron microscope level using antibodies against dopamine. Cell Tissue Res 244:577-582

Kah O, Dulka JG, Dubourg P, Thibault J, Peter RE 1987 Neuroanatomical substrate for the inhibition of gonadotrophin secretion in goldfish : Existence of a dopaminergic preoptico-hypophyseal pathway. Neuroendocrinology 45:451-458

Kaiser C 1983 Stereoisomeric probes of dopamine receptor. In : Kaiser C, Kebabian JW (eds) Dopamine Receptors. American Chemical Society, Washington DC, ACS Symposium Series 22:223-246

Kebabian JW, Beaulieu M, Itoh Y 1984 Pharmacological and biochemical evidence for the existence of two categories of dopamine receptor. Can J Neuro Sci 11:114-117

Marchant TA, Dulka JG, Peter RE 1989a Relationship between serum growth hormone levels and the brain and pituitary content of immunoreactive somatostatin in the goldfish, *Carassius auratus*. Gen Comp Endocrinol 73:458-468

Marchant TA, Chang JP, Nahorniak CS, Peter RE 1989b Evidence that gonadotropin-releasing hormone also function as a growth hormone-releasing factor in the goldfish. Endocrinology 124: 2509-2518

Munson PJ, Rodbard D 1980 LIGAND : A versatile computerized approach for characterization of ligand-binding system. Anal Biochem 107:220-239

Neumeyer JL, Baindur N, Bakthavachalam V, Yuan J, Madras BK, Kula NS, Campbell A, Baldessarini RJ 1992 Selective probes for characterization of dopamine D1 and D2 recep[illegible]. Neurochem Int [Suppl] 20:63S-68S

Niznik HB 1987 Dopamine receptors : Molecular structure and fu[illegible]. Mol Cell Endocrinol 54: 1-22

Ohlstein EH, Berkowitz BA 1985 SCH23390 and SK&F83566 [illegible]s at

vascular dopamine and serotonin receptors. Eur J Pharmacol 108: 205-208

Omeljaniuk RJ, Peter RE 1989 *In vitro* binding characteristics of [^{3}H]spiperone to the pituitary of the goldfish (*Carassius auratus*). Gen Comp Endocrinol 74:57-67

Peter RE, Yu KL, Marchant TA, Rosenblum PM 1990 Direct neural regulation of the teleost adenohypophysis. J Exp Zool [Suppl] 4:84-89

Reader TA, Crondin L, Monteuil B, Dewar KM 1989 Dopamine D1 receptors labelled with [^{3}H]SCH23390 in rabbit cerebral cortex and neostriatum : Equilibrium binding, kinetics and selectivity. Naunyn-Schmiedeberg's Arch Pharmacol 340:617-625

Seeman P, Grigoriadis D, George SR, Watanabe M, Ulpian C 1986 Functional states of dopamine receptors. In : Woodruff GN, Poat JH, Roberts RJ (eds) Dopaminergic Systems and their Regulation. VCH, London, pp 97-109

Seeman P, Ulpian C 1986 Dopamine D1 and D2 receptor selectivities of agonists and antagonists. In: Goldstein M, Fuxe K, Tabachnick I (eds) Central D1 Dopamine Receptors. Plenum Press, New York, pp 55-63

Seeman P, Ulpian C, Grigoriadis D, Pri-Bar I, Buchman O 1985 Conversion of dopamine D1 receptors from high to low affinity for dopamine. Biochem Pharmacol 34:151-154

Senogles SE, Benovic JL, Amlaiky N, Unson C, Milligan G, Vinitsky R, Spiegel AM, Caron M 1987 The D2-receptor of anterior pituitary is functionally associated with a pertussin toxin-sensitive guanine nucleotide binding protein. J Biol Chem 262:4860-4867

Sibley DR, DeLean A, Creese I 1982 Anterior pituitary dopamine receptors. Demonstration of interconvertible high and low affinity states of the D-2 dopamine receptor. J Biol Chem 257:6351-6361

Sibley DR, Mahan LC, Creese I 1983 Dopamine receptor binding on intact cells. Absence of a high-affinity agonist-receptor binding state. Mol Pharmacol 23:295-302

Sibley DR, Monsma FJ 1992 Molecular biology of dopamine receptors. Trends Pharmacol Sci 13:61-69

Sidhu A, Fishman PH 1990 Identification and characterization of functional D1

dopamine receptors in a human neuroblastoma cell line. Biochem Biophys Res Commun 166:574-579

Sokoloff P, Giros B, Martres P, Bouthenet ML, Schwartz JC 1990 Molecular cloning and characterization of a novel dopamine receptor (D3) as a target for neuroleptics. Nature 347:146–151

Stoof JC, Tilders FJH 1989 Dopamine receptors in the central nervous system. In : Fluckiger E, Muller EE, Thorner MO (eds) The Role of Brain Dopamine. Springer-Verlag, New York, Basic and Clinical Aspects of Neuroscience 3:31-43

Strange PG 1987 Isolation and molecular characterization of dopamine receptors. In : Creese I, Fraser CM (eds) Dopamine Receptors. Alan. R. Liss. Inc., Receptor Biochemistry and Methodology 8:29-43

Sunahara RK, Guan HC, O'Dowd BF, Seeman P, Laurier LG, Ng G, George SR, Torchia J, Van Tol HHM, Niznik HB 1991 Cloning of the gene for a human dopamine D5 receptor with higher affinity for dopamine than D1 receptor. Nature 350:614-619

Van Tol HHM, Bunzow JR, Guan HC, Sunahara RK, Seeman P, Niznik HB, Civelli O 1991 Cloning of the gene for a human dopamine D4 receptor with high affinity for the antipsychotic clozapine. Nature 350:610-614

Wong AOL, Chang JP, Peter RE 1992 Dopamine stimulates growth hormone release from the pituitary of goldfish, *Carassius auratus*, through the dopamine D1 receptors. Endocrinology 130:1201-1210

Wong AOL, Chang JP, Peter RE 1993 *In vitro* and *in vivo* evidence that dopamine exerts growth hormone releasing activity in the goldfish. Am J Physiol (in press)

Chapter 6

Cyclic 3':5'-Adenosine Monophosphate (cAMP) mediates Dopamine D1-stimulated Growth Hormone Release from Goldfish Pituitary Cells [1]

6.1 Introduction

In the goldfish, dopamine (DA) is stimulatory to growth hormone (GH) release both *in vivo* (Chang *et al.*, 1985) and *in vitro* (Peter *et al.*, 1990; Wong *et al.*, 1992). The stimulatory actions of DA are exerted directly at the pituitary cell level through activation of DA D1 receptors (Chang *et al.*, 1990b; Wong *et al.*, 1992; Wong et al., 1993a,b). Recently, we have characterized DA D1-specific binding sites in the goldfish pituitary. These D1 binding sites are saturable, displaceable, stereoselective, and specific to the binding of DA D1 ligands (Wong *et al.*, 1993c). Since DA D1 receptors have not been identified in the pituitary of other vertebrates, these newly characterized "D1" receptors in the goldfish pituitary represent a novel neuroendocrine model to study the actions of DA in the control of pituitary hormones.

In mammals, DA D1 receptors are positively coupled to the cAMP-producing enzyme adenylate cyclase via a stimulatory G protein (Gs) (Battaglia *et al.*, 1986a; Elazar *et al.*, 1989). Activation of D1 receptors by DA or the D1 agonists, such as SKF38393, often leads to a rapid accumulation of cAMP. This has been demonstrated in the rat striatal slices (Battaglia *et al.*, 1986a,b), human neuroblastoma (Sidhu and Fishman, 1990), and cultured cell lines, such as LLC-PK1 (Grenaner and Healy, 1991) and COS-1 cells (Steffey *et al.*, 1991). In lower vertebrates, especially in teleosts, the signal transduction mechanism mediating DA D1 actions is still largely unknown.

In the present study, we have investigated the role of cAMP in mediating DA D1-stimulated GH release in the goldfish. The GH-releasing activity of pharmacological

[1] A version of this chapter has been submitted for publication : Wong AOL, Van Der Kraak G, and Chang JP (1993) **Neuroendocrinology.**

agents acting on the cAMP-dependent pathway, such as forskolin, IBMX, and cAMP analogs, were tested using goldfish pituitary cells in static incubation. The actions of SKF38393 on cAMP production and GH release were examined using a cell preparation enriched with goldfish somatotrophs. The possible involvement of the cAMP-dependent enzyme protein kinase A (PKA) was also investigated.

6.2 Materials and Methods

Animals Goldfish of the common or comet varieties were purchased from Ozark Fisheries (Stoutland, MO, USA) or Grassyforks Fisheries (Martinsville, IN, USA), and maintained in flow-through aquaria (1,800 liters) at 17 °C under a simulated natural photoperiod (Edmonton, AB, Canada) for at least 3 weeks prior to any experiments. The fish were fed to satiation daily with Ewos trout pellets (Astra Chemicals Ltd., Mississauga, ON, Canada). Goldfish of both sexes, with body weight ranging from 20 g to 30 g, were used in the present study. Since the seasonality of DA D1-stimulated GH release in the goldfish is known to be closely associated with the reproductive cycle (Wong *et al.*, 1993a,b), gonadal conditions of the fish are reported separately for each individual experiment. Gonadal conditions were determined by the gonadosomatic index (GSI = weight of gonad / total body weight x 100 %) and morphological characteristics of the gonads (for details, see chapter 2).

Reagents and test substances Dopamine (DA), N6 2'-O-dibutyryladenosine 3':5'-cyclic mono-phosphate (db.cAMP), 8-bromoadenosine 3':5'-cyclic monophosphate (8Br.cAMP), and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma Chemical Company (St Louis, MO, USA). Forskolin, 1,9-dideoxyforskolin, pertussis toxin, cholera toxin, the protein kinase A inhibitor H89, and the protein kinase C (PKC) activator sn-1,2-dioctanoyl glycerol (DiC8) were purchased from Calbiochem Corporation (San Diego, CA, USA). The D1 agonist SKF38393 (1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine-7,8-diol hydrochloride) was purchased from Research Biochemicals Inc. (Wayland, MA, USA), and the D2 agonist LY171555 (quinpirole hydrochloride) was a generous gift from Eli Lilly & Co. (Indianapolis, IN, USA). SKF38393, LY171555, db.cAMP, 8Br.cAMP, forskolin, 1,9-dideoxyforskolin, and

DiC8 were first dissolved in a minimal amount of dimethyl sulfoxide (DMSO), and subsequently diluted to appropriate concentrations with medium M199. DMSO levels in the final solution were always less than 0.1 %, and were previously shown to have no effects on basal GH release from goldfish pituitary cells (Chang *et al.*, 1990b).

Preparation of dispersed pituitary cells Dispersed pituitary cells from the goldfish were prepared as described previously (Chang *et al.*, 1990a). In brief, goldfish pituitaries diced into fragments (≈0.5 mm in thickness) were exposed to controlled trypsin / DNAse treatment. Pituitary fragments were then mechanically dispersed in Ca^{2+}-deficient medium (M199 with Hank's salts prepared without adding $CaCl_2$, supplemented with 2.2 g/l sodium bicarbonate, 25 mM HEPES, 100,000 units penicillin/l, 100 mg streptomycin/l, and 0.3 % BSA; pH 7.2, GIBCO), and harvested by centrifugation at 200x g for 10 min at 4 °C. Cell yield of the dispersion procedures was about 0.5 million cells per pituitary, and the viability of pituitary cells was >94 % as assayed by the trypan blue exclusion test. Pituitary cells enriched with somatotrophs were prepared by a discontinuous density gradient as described by de Leeuw *et al.* (1984) with minor modifications (Chang and Jobin, 1993). Mixed populations of pituitary cells, which normally have about 20% of cells immunoreactive to GH antiserum, were gently loaded on the top of a discontinuous Percoll gradient (with 40, 50, 60, 70, and 80 % Percoll, Pharmacia) and centrifuged at 1400x g for 25 min at 17 °C. After centrifugation, pituitary cells collected at the interphase between 60 and 70 % Percoll were found to have the highest amount of cells immunoreactive to GH antiserum (≈70 %). Viability of this enriched somatotroph preparation after Percoll density gradient separation was always >92 % as indicated by the trypan blue exclusion test. Since the cell yield of enriched somatotophs was rather low (about 0.07 million cells per pituitary), mixed populations of goldfish pituitary cells were used in most of the experiments in the present study.

Static incubation of pituitary cells Mixed populations of pituitary cells or enriched somatotroph preparations were resuspended in plating medium (M199 with Earle's salts, supplemented with 2.2 g/l sodium bicarbonate, 25 mM HEPES, 100,000 units penicillin/l, 100 mg streptomycin/l, and 1 % horse serum; pH 7.2, GIBCO), and were

cultured in 24-well Falcon Primaria plates (Becton Dickinson & Company, NJ, USA) at a density of 0.25 million cells/ml/well. After overnight incubation (15-18 hr) at 28 °C under 5% CO2 and saturated humidity, the culture medium was replaced by testing medium (M199 with Hank's salts, supplemented with 2.2 g/l sodium bicarbonate, 25 mM HEPES, 100,000 units penicillin/l, 100 mg streptomycin/l, and 0.1 % BSA; pH 7.2, GIBCO). Drug treatment was initiated by adding testing medium with the appropriate concentrations of test substances. Following a further incubation of 2 hours, 500 µl of testing medium was carefully removed from individual culture wells, and stored at -25 °C until their GH contents were assayed by a radioimmunoassay (RIA) previously validated for the measurement of goldfish GH (Marchant *et al.*, 1989). Samples for cAMP measurement were pretreated in boiling water for 10 min to inactivate the cAMP-degrading enzyme phosphodiesterase before storage. Samples for cell contents of cAMP were prepared by lysing the cells with distilled water followed by rapid freezing and thawing for 2 times. cAMP contents were measured by RIA as described by Knecht *et al.* (1982). The antiserum for cAMP assay was a generous gift from Dr. K.J. Catt (ERRB, NICHD, NIH, Maryland, USA). All treatments were carried out in quadruplicate in the same experiment, and each individual experiment was repeated for at least 3 times. GH data were expressed as a percentage of the basal GH release in control wells without any drug treatment (referred as "% control"), and were pooled for statistical analysis.

Data analysis and statistics GH and cAMP data were analyzed by analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test. Differences were considered significant when $P < 0.05$. ED_{50} values and the maximal GH responses for the dose-response curves in the present study were estimated by the four-parameter logistic equation using ALLFIT computer program developed by DeLean *et al.* (1978).

6.3 Results

Dose-dependent actions of DA and SKF38393 on GH release The experiment was conducted using goldfish in the early stages of gonadal regression (i.e., in postspawning

stages). Consistent with our previous findings using perifused goldfish pituitary fragments (Wong *et al.*, 1992), DA (1 nM - 10 μM) and the D1 agonist SKF38393 (1 nM - 10 μM) stimulated GH release in a dose-dependent manner from goldfish pituitary cells in static incubation (Fig. 6.1). The ED_{50} values (mean ± SEM) for DA- and SKF38393-stimulated GH release were estimated to be 26 ± 7 nM and 4.5 ± 1.4 nM, respectively.

Effects of SKF38393 and LY171555 on GH and cAMP release To facilitate the measurement of cAMP release following stimulation with the DA agonists, pituitary cell populations enriched with somatotrophs were prepared from goldfish in the late stages of gonadal recrudescence. The D1 agonist SKF38393 from 1 nM to 10 μM concentrations stimulated the release of GH (Fig. 6.2A) and cAMP (Fig. 6.2B) in a dose-dependent manner; the D2 agonist LY171555 was not effective in this regard. The ED_{50} values for SKF38393-stimulated GH and cAMP release were estimated to be 73 ± 32 nM and 109 ± 53 nM, respectively. The cellular contents of cAMP in experiments with SKF38393 and LY171555 were also examined; in both cases, cAMP contents in the pituitary cells were not affected by SKF38393 nor LY171555 treatment (Fig. 6.2C).

Effects of cholera toxin and pertussis toxin on GH release The GH responses to bacterial toxins, which are known to activate adenylate cyclase indirectly via G proteins, were examined in dispersed pituitary cells prepared from goldfish in the late stages of gonadal recrudescence. Concentrations of cholera toxin from 0.1 to 1000 ng/ml (Fig. 6.3A) and pertussis toxin from 0.01 to 100 ng/ml (Fig. 6.3B) stimulated GH release in a dose-dependent manner. The ED_{50} values for the GH dose-response curves to cholera toxin and pertussis toxin were estimated to be 5.6 ± 1.2 ng/ml and 0.8 ± 0.3 ng/ml, respectively.

Effects of forskolin and dideoxyforskolin on GH release Pituitary cells prepared from goldfish in gonadal recrudescence were used to examine the actions of the adenylate cyclase activator forskolin on GH release (Fig. 6.4). The GH-releasing effects of dideoxyforskolin, a forskolin derivative inactive in stimulating adenylate cyclase, were

also tested as a negative control. Forskolin (0.1 - 40 μM) stimulated GH release in a dose-dependent manner with an ED_{50} of 98 ± 40 nM. However, the same doses of dideoxyforskolin had no effects on GH release.

Effects of membrane-permeant cAMP analogs on GH release Experiments with membrane-permeant cAMP analogs were conducted using pituitary cells prepared from goldfish in different reproductive stages throughout the year. The membrane-permeant cAMP analogs 8Br.cAMP (Fig. 6.5A) and db.cAMP (Fig. 6.5B) stimulated GH release in a dose-dependent manner with the ED_{50} values of 137 ± 83 μM and 206 ± 92 μM, respectively.

Effects of IBMX on GH release Effects of the phosphodiesterase inhibitor IBMX on GH release were studied using pituitary cells from goldfish in different reproductive stages throughout the year (Fig. 6.6a). IBMX stimulated GH release in a dose-dependent manner with an ED_{50} of 10.1 ± 2.5 μM. The interactions of IBMX with forskolin and 8Br.cAMP on GH release were also tested (Fig. 6.6b). Similar to the results of the preceding sections, IBMX (100 μM), forskolin (10 μM) and 8Br.cAMP (1 mM) stimulated GH release when the drugs were administered alone. In the presence of 100 μM IBMX, the GH responses to forskolin (10 μM) and 8Br.cAMP (1 mM) were not further enhanced (Fig. 6.6b). These results indicate that 10 μM forskolin and 1 mM 8Br.cAMP maximally stimulated the cAMP-dependent pathway involved in GH release in the goldfish.

Effects of 8Br.cAMP on the dose-dependence of SKF38393-stimulated GH release Dispersed pituitary cells were prepared from goldfish in the early stages of gonadal regression (i.e., in postspawning stages). In the absence of 8Br.cAMP, the D1 agonist SKF38393 stimulated GH release in a dose-dependent manner, with an ED_{50} and the maximal GH response of 11.2 ± 2.1 nM and 173 ± 6 % control, respectively (Fig. 6.7). However, the dose-dependence of SKF38393-stimulated GH release was abolished with simultaneous treatment of 1 mM 8Br.cAMP. The GH response to 1 mM 8Br.cAMP alone was 192 ± 18 % control, and was not significantly different from the maximal GH

response to SKF38393. These results indicate that the maximal GH-releasing actions of 8Br.cAMP and SKF38393 are not additive at pituitary cell level.

Effects of H89 on the GH responses to 8Br.cAMP, SKF38393, and DiC8 Pituitary cells prepared from goldfish in the late stages of gonadal recrudescence were used to examine the actions of increasing doses of the PKA inhibitor H89 on the GH response to 8Br.cAMP (Fig. 6.8). The PKA inhibitor H89 suppressed the GH response elicited by 8Br.cAMP (1 mM) in a dose-dependent manner. At high concentrations (1-10 μM), H89 also reduced basal GH secretion. In a separate study, the actions of H89 on the GH responses to the D1 agonist SKF38393 and the PKC activator DiC8 were examined using pituitary cells prepared from goldfish in gonadal regression (Fig. 6.9). The PKA inhibitor H89 at 10 μM dose abolished the GH responses to SKF38393 (1 μM) without affecting the GH-releasing actions of DiC8 (0.1 mM).

6.4 Discussion

In the present study, DA and the D1 agonist SKF38393, but not the D2 agonist LY171555, stimulated GH release from goldfish pituitary cells. This is consistent with our previous findings that DA stimulates GH release from the pituitary of goldfish through activation of D1 receptors (Chang *et al.*, 1990b; Wong *et al.*, 1992). In mammals, cAMP mediates the biological actions of DA D1 receptors (for review, see Seeman and Niznik, 1988). Increase in cAMP synthesis following DA D1 stimulation has been reported in bovine parathyroid cells (Brown and Hughes, 1983), rat striatal slices (Battaglia *et al.*, 1986a,b) and human neuroblastoma (Sidu and Fishman, 1990). In this study, GH release induced by SKF38393 was accompanied with a concurrent increase in cAMP production, indicating that the D1 receptors in goldfish pituitary cells are positively coupled with cAMP synthesis via activation of adenylate cyclase. It is likely that cAMP is involved in DA D1-stimulated GH release in the goldfish.

This hypothesis is supported by other results of the current investigations. Forskolin, an activator of adenylate cyclase, stimulated GH release from goldfish pituitiary cells. Elevation of GH secretion was also observed following treatment with

the phosphodiesterase inhibitor IBMX. Both forskolin and IBMX have been previously shown to increase cAMP production in goldfish pituitary cells (Chang *et al.*, 1992). In contrast, dideoxyforskolin, which is known to have no binding affinity for adenylate cyclase and does not activate cAMP synthesis, did not stimulate GH release (for a review on the pharmacology of forskolin and its derivatives, see Laurenza *et al.*, 1989). These results indicate that pharmacological agents capable of increasing intracellular cAMP levels can mimick the GH-releasing effects of DA and the D1 agonist SKF38393.

Similar to forskolin and IBMX, the membrane-permeant cAMP analogs, 8Br.cAMP and db.cAMP, also induced GH release from goldfish pituitary cells in a dose-dependent manner. These results further confirm the involvement of cAMP in mediating GH release in the goldfish. The role of cAMP as a second messenger mediating GH release has been documented in mammals (for review, see Frohman and Jansson, 1986). The GH-releasing effects of cAMP analogs has been previously demonstrated in ovine fetal and neonatal pituitary cells (Silverman *et al.*, 1989), mixed populations of rat pituitary cells (Bilezikjian and Vale, 1983; Cronin *et al.*, 1984) and purified somatotrophs of the rat (Kraicer and Spence, 1981; Kraicer and Chow, 1982).

If the synthesis of cAMP is an essential step mediating DA D1-stimulated GH release in the goldfish, it is likely that DA D1 stimulation would not further enhance GH secretion after the cAMP-dependent pathway is saturated by a high dose of cAMP analog. In the present study, 8Br.cAMP at 1 mM dose maximally stimulated the cAMP-dependent pathway involved in GH release from goldfish pituitary cells. Under this maximal stimulation by 8Br.cAMP, increasing doses of the D1 agonist SKF38393 failed to further stimulate GH release. This absence of additivity between the GH responses to SKF38393 and 8Br.cAMP is in agreement with the hypothesis that cAMP is a second messenger mediating DA D1-stimulated GH release in the goldfish. This idea is further supported by the observations that the maximal GH responses to 8Br.cAMP (190 ± 9 % control) and db.cAMP (175 ± 6 % control) were similar in magnitude to that of DA (180 ± 7 % control). In addition, the ED_{50} values of SKF38393-induced cAMP (109 ± 53 nM) and GH release (72 ± 32 nM) from enriched somatotroph preparations are also in the same dose range of the binding affinity of D1 receptors (K_d = 34 ± 9 nM) in mixed populations of goldfish pituitary cells (Wong *et al.*, 1993c; see chapter 5).

In mammals, adenylate cyclase can be coupled to membrane receptors through two regulatory G proteins, the stimulatory Gs and inhibitory Gi proteins (for reviews, see Stadel and Lefkowitz, 1989; Permont and Iyengar, 1990). The Gs protein can be activated by cholera toxin through ADP-ribosylation of Gsα subunit, leading to activation of adenylate cyclase (Moss and Vaughan, 1992). On the other hand, the function of the Gi protein can be suppressed by ADP-ribosylation of the Giαβγ complex using pertussis toxin. This removes the inhibitory control over adenylate cyclase, and results in an activation of the enzyme activity (Codina *et al.*, 1990). In the present study, both cholera toxin and pertussis toxin stimulated GH secretion from goldfish pituitary cells. These results are consistent with the involvement of the adenylate cyclase-cAMP pathway in mediating GH release in the goldfish, and also suggest that G proteins (Gs and Gi) are involved in the regulation of somatotroph functions. DA D1 receptors are known to couple with the Gs protein in mammalian D1 systems (Battaglia *et al.*, 1986; Elazar *et al.*, 1989). In the goldfish, activation of D1 receptors appears to induce GH release from pituitary cells through the production of cAMP (see earlier discussion). It is likely that DA D1 receptors in the somatotrophs of goldfish are also coupled to adenylate cyclase through the Gs protein. The role of pertussis toxin-sensitive Gi protein in mediating GH release in the goldfish is still not clear. In mammals, especially in the rat, pertussis toxin blocks the inhibitory actions of somatostatin (SRIF) on GH release (Cronin *et al.*, 1983) and cAMP synthesis (Koch *et al.*, 1985). In a recent study, a "Gi"-like protein has been co-purified with SRIF receptors in the rat brain (Murray-Whelan and Schlegel, 1992). In the goldfish, SRIF is known to inhibit basal (Cook and Peter, 1984; Marchant *et al.*, 1987) and DA D1-stimulated GH release (Wong *et al.*, 1993a,b,c). Therefore, the present demonstration of a pertussis toxin-sensitive component of GH release may indicate a possible involvement of the Gi protein in the mediation of somatostatin actions.

Biological actions of cAMP are known to be mediated primarily by the cAMP-dependent enzyme PKA (for review, see Oyen *et al.*, 1988). In the present study, the GH-releasing actions of 8Br-cAMP and the D1 agonist SKF38393 were blocked by the PKA inhibitor H89. This PKA inhibitor has been reported to be highly selective for PKA activities in mammals (Chijiwa *et al.*, 1990). In the goldfish, H89 suppressed

both basal and 8Br.cAMP-stimulated GH release without affecting the GH responses to the PKC activator DiC8. A similar dose of DiC8 has been previously shown to stimulate GH release from goldfish pituitary cells via the PKC-dependent pathway (Chang *et al.*, 1991). These results strongly indicate that H89 is selective to PKA in the goldfish and does not cross-react with PKC within the doses tested. Therefore, we conclude that PKA is involved in the maintenance of basal GH secretion as well as the mediation of DA D1-stimulated GH release in the goldfish.

In summary, the present study strongly suggests that cAMP functions as a second messenger mediating DA D1-stimulated GH release in the goldfish. The involvement of the adenylate cyclase-cAMP-PKA pathway in the DA D1 system of goldfish pituitary cells is quite comparable to that of mammals (see introduction). The similarity of the signal transduction mechanisms between the goldfish and mammalian D1 systems also supports our previous hypothesis (see chapters 2) that DA D1 receptors are highly conserved during vertebrate evolution.

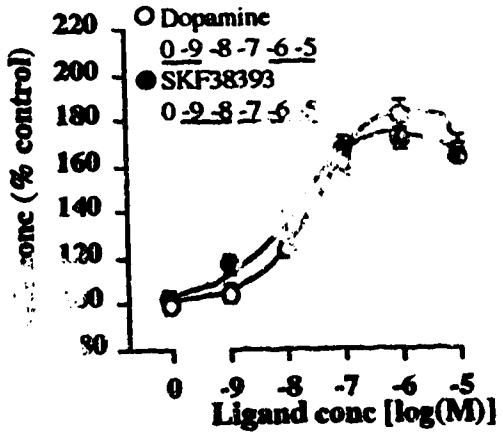


Fig. 6.1 Effects of dopamine and the D1 agonist SKF38393 on GH release from dispersed goldfish pituitary cells. Pooled data from three separate experiments (each with quadruplicate treatments) are presented (mean ± SEM, n = 12). Average GH level in the control wells was 718± 51 ng GH/ml. ED50s for the GH responses to DA and SKF38393 were estimated to be 26 ± 7 and 4.5 ± 1.4 nM, respectively. Concentrations of DA or SKF38393 giving a similar magnitude of GH responses are grouped within the same underscore (ANOVA followed by Fisher's LSD test, $P > 0.05$).

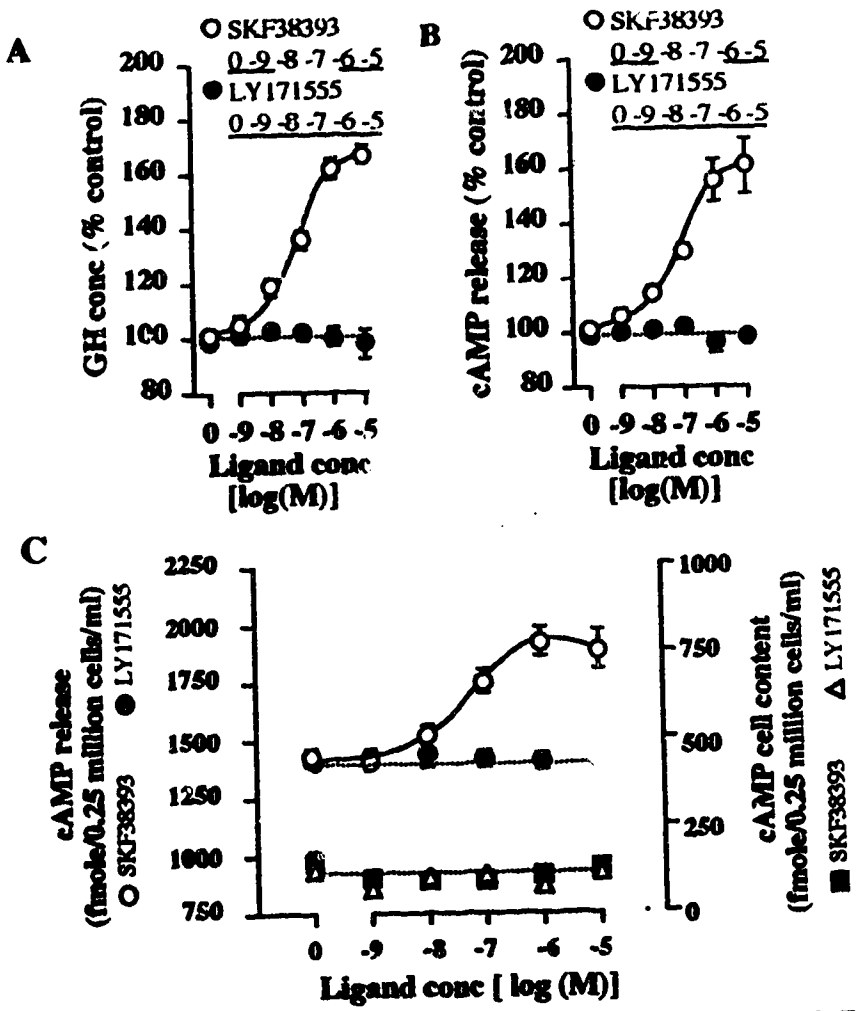


Fig. 6.2 Effects of the D1 agonist SKF38393 and D2 agonist LY171555 on GH (A) and cAMP release (B) from an enriched somatotroph preparation of the goldfish. Pooled data from three separate experiments (each with quadruplicate treatments) are presented (mean ± SEM, n = 12). cAMP data (in fmole cAMP/ml/0.25 million cells) from one of the three experiments are presented in (C) for the comparison of cAMP released versus the cellular cAMP contents in response to drug treatment (n = 4). Average GH and cAMP levels in the culture medium of control wells were 601 ± 45 ng GH/ml and 1286 ± 86 fmole cAMP/ml/0.25 million cells, respectively. ED50s for the responses of GH and cAMP to SKF38393 stimulation were estimated to be 73 ± 32 nM and 109 ± 53 nM, respectively. Concentrations of SKF38393 or LY171555 giving a similar magnitude of GH- or cAMP-release responses are grouped within the same underscore (ANOVA followed by Fisher's LSD test, $P > 0.05$).

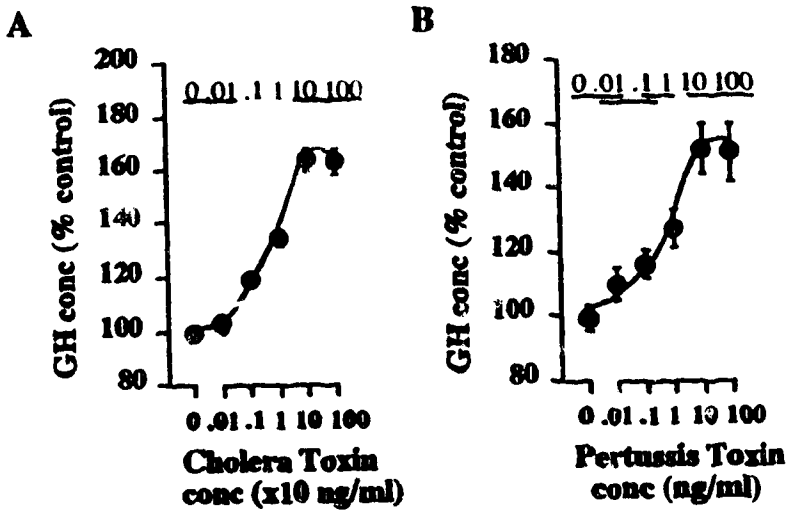


Fig. 6.3 Effects of cholera toxin (A) and pertussis toxin (B) on GH release from dispersed goldfish pituitary cells. Pooled data from five experiments for cholera toxin and four experiments for pertussis toxin (each experiment with quadruplicate treatments) are presented (mean ± SEM, n = 16 - 20). Average GH levels in the control wells for experiments with cholera toxin and pertussis toxin were 636 ± 15 and 501 ± 35 ng GH/ml, respectively. Doses of cholera toxin or pertussis toxin giving similar magnitude of GH responses are grouped within the same underscore (ANOVA followed by Fisher's LSD test, $P > 0.05$).

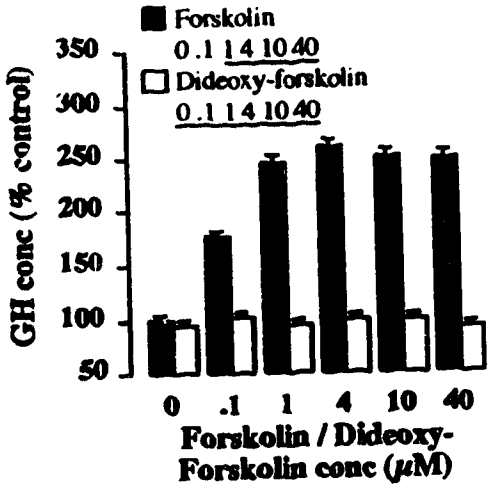


Fig. 6.4 Effects of forskolin and dideoxyforskolin on GH release from dispersed goldfish pituitary cells. Pooled data from three separate experiments (each with quadruplicate treatments) are presented (mean ± SEM, n = 12). Average GH levels in the control wells without drug treatment were 673 ± 36 ng GH/ml. Doses of forskolin or dideoxyforskolin giving similar magnitude of GH responses are grouped within the same underscore (ANOVA followed by Fisher's LSD test, $P > 0.05$).

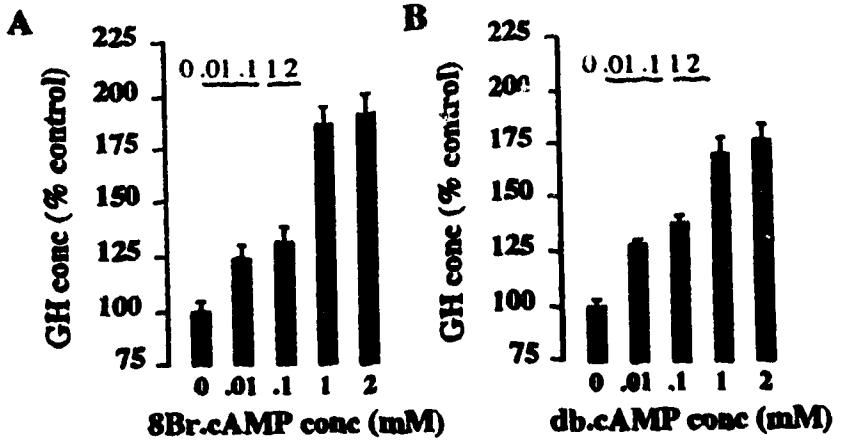


Fig. 6.5 Effects of 8Br.cAMP (A) and db.cAMP (B) on GH release from dispersed goldfish pituitary cells. Pooled data from at least three separate experiments are presented (mean ± SEM, n = 12 - 23). Average GH levels in the control wells for db.cAMP and 8Br.cAMP experiments were 536 ± 102 and 511 ± 68 ng GH/ml, respectively. Concentrations of db.cAMP or 8Br.cAMP giving similar magnitude of GH responses are grouped within the same underscore (ANOVA followed by Fisher's LSD test, $P > 0.05$)

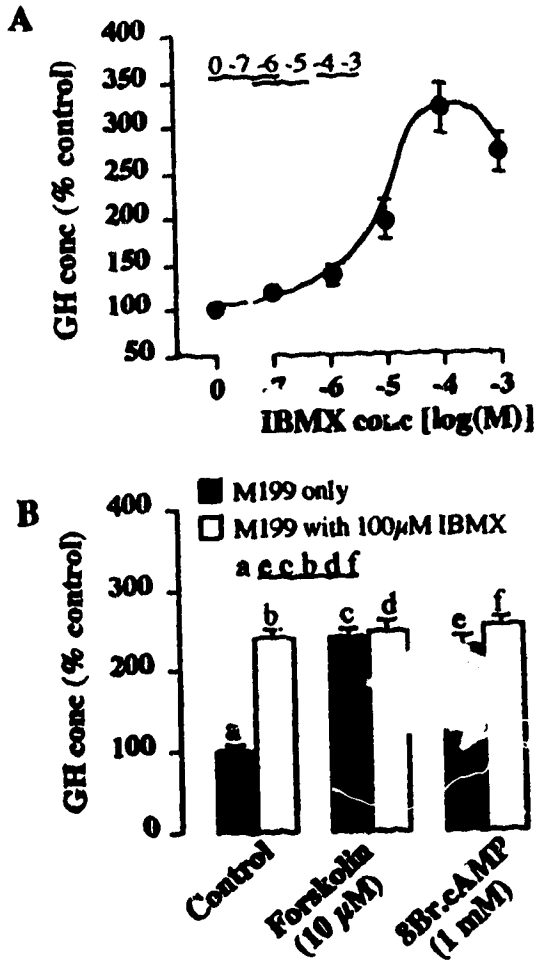


Fig. 6.6 Effects of IBMX on (A) basal GH release, and (B) the GH responses to forskolin and 8Br.cAMP in dispersed goldfish pituitary cells. Pooled data from at least three separate experiments are presented (mean ± SEM, n = 8 - 24). Average GH level in the control wells without drug treatment was 428 ± 48 ng GH/ml. Drug treatments giving similar magnitude of GH responses are grouped within the same underscore (ANOVA followed by Fisher's LSD test, $P > 0.05$).

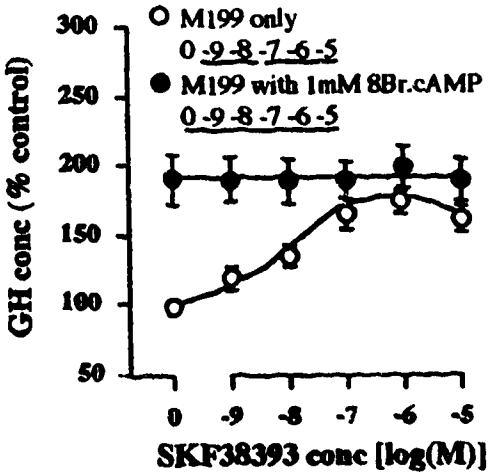


Fig. 6.7 Effects of 8Br.cAMP on the dose-dependence of SKF38393-stimulated GH release from dispersed goldfish pituitary cells. Pooled data from three separate experiments (each with quadruplicate treatments) are presented (mean ± SEM, n = 12). Average GH level in the control wells was 673 ± 59 ng GH/ml. Concentrations of SKF38393 giving a similar magnitude of GH responses are grouped within the same underscore (ANOVA followed by Fisher's LSD test, $P > 0.05$).

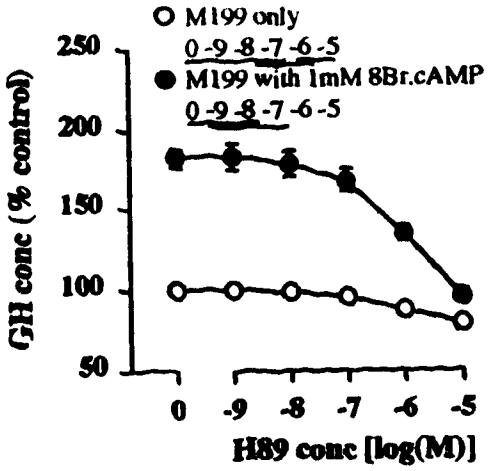


Fig. 6.8 Effects of increasing doses of H89 on 8Br.cAMP-stimulated GH release from dispersed goldfish pituitary cells. Pooled data from three separate experiments (each with quadruplicate treatments) are presented (mean ± SEM, n = 12). Average GH level in the control wells was 510 ± 18 ng GH/ml. Concentrations of H89 (with or without 8Br.cAMP) giving a similar magnitude of GH responses are grouped within the same underscore (ANOVA followed by Fisher's LSD test, $P > 0.05$).

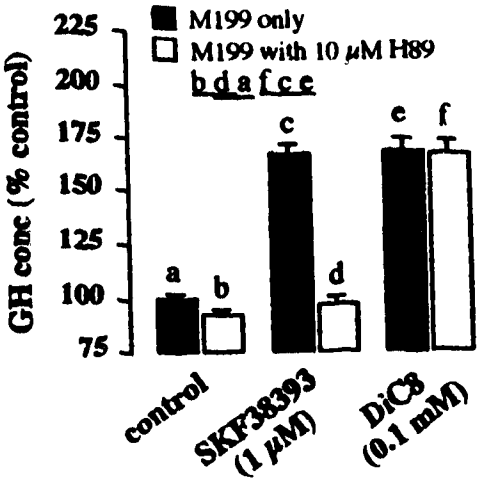


Fig. 6.9 Effects of H89 on the GH responses to SKF38393 and DiC8 using dispersed goldfish pituitary cells. Pooled data from four separate experiments (each with quadruplicate treatments) are presented (mean ± SEM, n = 16). Average GH level in the control wells was 1033 ± 89 ng GH/ml. Drug treatments giving a similar magnitude of GH responses are grouped within the same underscore (ANOVA followed by Fisher's LSD test, $P > 0.05$).

6.5 References

Battaglia G, Norman AB, Hess EJ, Creese I 1986a Forskolin potentiates the stimulation of rat striatal adenylate cyclase mediated by D1 dopamine receptors, guanine nucleotides, and sodium fluoride. J Neurochem 46:1180-1185

Battaglia G, Norman AB, Hess EJ, Creese I 1986b Functional recovery of D1 dopamine receptor-mediated stimulation of rat striatal adenylate cyclase activity following irreversible receptor modification by N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) : Evidence for spare receptors. Neurosci Let 46:1180-1185

Bilezikjian LM, Vale WW 1983 Stimulation of adenosine 3',5'-monophosphate production by growth hormone-releasing factor and its inhibition by somatostatin in anterior pituitary cells in vitro. Endocrinology 113:1726-1731

Brown EM, Hughes DB 1983 D-1 dopamine receptor-mediated activation of adenylate cyclase, cAMP accumulation, and PTH release in dispersed bovine parathyroid cells. In : Kaiser C, Kebabian JW (eds) Dopamine Receptors. American Chemical Society, Washington DC, ACS Symposium Series, 224:223-246

Chang JP, Cook H, Freedman GL, Wiggs AJ, Somoza GM, de Leeuw R, Peter RE 1990a Use of a pituitary cell dispersion method and primary culture system for the studies of gonadotropin-releasing hormone action in the goldfish, *Carassius auratus*. I. Initial morphological, static, and cell column perifusion studies. Gen Comp Endocrinol 77: 256-273

Chang JP, Jobin RM 1993 Teleost pituitary cells: Isolation, culture, and use. In: Hochachka PW, Mommsen TP (eds) Biochemistry and Molecular Biology of Fishes (vol.3): Analytical Techniques. Elsevier, Amsterdam (in press)

Chang JP, Jobin RM, de Leeuw R 1991 Possible involvement of protein kinase C in gonadotropin and growth hormone release from dispersed goldfish pituitary cells. Gen Comp Endocrinol 81:447-463

Chang JP, Marchant TA, Cook AF, Nahorniak CS, Peter RE 1985 Influences of catecholamines on growth hormone release in female goldfish, *Carassius auratus*. Neuroendocrinology 40:463-470

Chang JP, Wong AOL, Van Der Kraak G, Van Goor F 1992 Relationship between cyclic AMP-stimulated and native gonadotropin-releasing hormone-stimulated gonadotropin release in the goldfish. Gen Comp Endocrinol 86:359-377

Chang JP, Yu KL, Wong AOL, Peter RE 1990b Differential actions of dopamine receptor subtypes on gonadotropin and growth hormone release *in vitro* in goldfish. Neuroendocrinology 51:664-674

Chijiwa T, Mishima A, Hagiwara M, Sano M, Hayashi K, Inoue T, Naito K, Toshioka T, Hidaka H 1990 Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, N-[2-(p-bromocinnamylamino) ethyl]-5-isoquinoline sulfonamide (H89), of PC12D pheochromocytoma. J Biol Chem 265:5267-5272

Codina J, Yatani A, VanDongen AMJ, Padrell E, Carty D, Mattera R, Brown AM, Iyengar R, Birnbaumer L 1990 Receptor-effector coupling by pertussis toxin substrates: Studies with recombinant and native G-protein α subunits. In : Iyengar R, Birnbaumer L (eds) G Proteins. Academic Press Inc, pp 268-295

Cook AF, Peter RE 1984 The effects of somatostatin on serum growth hormone levels in the goldfish, *Carassius auratus*. Gen Comp Endocrinol 54:109-113

Cronin MJ, Hewlett EL, Evans WS,Thorner MO, Rogol AD 1984 Human pancreatic tumor growth hormone (GH)-releasing factor and cyclic adenosine 3',5'-monophosphate evoke GH release from anterior pituitary cells: The effects of pertussis toxin, cholera toxin, forskolin, and cycloheximide. Endocrinology 114:904-913

Cronin MJ, Rogol AD, Myers GA, Hewlett EL 1983 Pertussis toxin blocks the somatostatin-induced inhibition of growth hormone release and adenosine 3',5'-monophosphate accumulation. Endocrinology 113:209-218

DeLean A, Munson PJ, Rodbard D 1978 Simultaneous analysis of families of sigmoidal curves: application of bioassay, radioligand assay, and physiological dose-response curves. Am J Physiol 235:E97-E102

de Leeuw R, Goos HJT, Peute J, Van Pelt AAM, Burzawa-Gerard E, Van Oordt PGWJ 1984 Isolation of gonadotrops from the pituitary of the African catfish, *Clarias lazera*. Morphological and physiological characterization of purified cells. Cell

Tissue Res 236:669-675

Elazar Z, Siegel G, Fuchs S 1989 Association of two pertussis toxin-sensitive G-proteins with the D2-dopamine receptor from bovine striatum. EMBO J 8:2353-2357

Frohman LA, Jansson JO 1986 Growth hormone-releasing hormone. Endocr Rev 7:223-253

Grenader A, Healy DP 1991 Locally formed dopamine stimulates cAMP accumulation in LLC-PK1 cells via a DA1 dopamine receptor. Am J Physiol 260:F906-F912

Knecht M, Amsterdam A, Catt KJ 1982 Inhibition of granulosa cell differentiation by gonadotropin-releasing hormone. Endocrinology 110:865-872

Koch BD, Dorflinger LJ, Schonbrunn A 1985 Pertussis toxin blocks both cyclic AMP-mediated and cyclic AMP-independent actions of somatostatin. J Biol Chem 263:216-223

Kraicer J, Chow AEH 1982 Release of growth hormone from purified somatotrophs: Use of perifusion system to elucidate interrelations among Ca^{++}, adenosine 3',5'-monophosphate, and somatostatin. Endocrinology 111:1173-1180

Kraicer J, Spence JW 1981 Release of growth hormone from purified somatotrophs: Use of high K^+ and the ionophore A23187 to elucidate interrelations among Ca^{++}, adenosine 3',5'-monophosphate and somatostatin. Endocrinology 108:651-658

Laurenza A, Sutkowski EM, Seamon KB 1989 Forskolin : A specific stimulator of adenylyl cyclase or a diterpene with multiple sites of action. Trends pharmacol Sci 10:442-447

Marchant TA, Dulka JG, Peter RE 1989 Relationship between serum growth hormone levels and the brain and pituitary content of immunoreactive somatostatin in the goldfish, *Carassius auratus*. Gen Comp Endocrinol 73:458-468

Marchant TA, Fraser RA, Andrews PC, Peter RE 1987 The influence of mammalian and teleost somatostatins on the secretion of growth hormone from goldfish (*Carassius auratus L*) pituitary fragments *in vitro*. Regul Pept 17:41-52

Moss J, Vaughan M 1992 Activation of cholera toxin by ADP-ribosylation factors, 20-kDa guanine nucleotide-binding proteins. In : Stadtman ER, Chock PB, Levitzki A (eds) Current Topics in Cellular Regulation. Academic Press, 32:49-67

Murray-Whelan R, Schlegel W 1992 Brain somatostatin receptor-G protein interaction. $G\alpha$ C-terminal antibodies demonstrate coupling of the soluble receptor with $G_{i(1-3)}$ but not with G_o. J Biol Chem 267:2960-2965

Oyen O, Sandberg M, Eskild W, Levy FO, Knutsen G, Beeve S, Hansson V, Jahnsen T 1988 Differential regulation of messenger ribonucleic acids for specific subunits of cyclic adenosine 3',5'-monophosphate (cAMP)-dependent protein kinase by cAMP in rat sertoli cells. Endocrinology 122:2658-2666

Peter RE, Yu KL, Marchant TA, Rosenblum PM 1990 Direct neural regulation of the teleost adenohypophysis. J Exp Zool. [Suppl] 4:84-89

Permont RT, Iyengar R 1990 Adenylate cyclase and its regulation by Gs. In : Iyengar R, Birnbaumer L (eds) G Proteins. Academic Press Inc, pp 148-178

Seeman P, Niznik MB 1988 Dopamine D1 receptor pharmacology. ISI Atlas of Science : Pharmacology 2:161-170

Sidhu A, Fishman PH 1990 Identification and characterization of function D1 dopamine receptors in a human neuroblastoma cell line. Biochem Biophys Res Commun 166:574-579

Silverman BL, Bettendorf M, Kaplan SL, Grumbach MM, Miller WL 1989 Regulation of growth hormone (GH) secretion by GH-releasing factor, somatostatin, and insulin-like growth factor I in ovine fetal and neonatal pituitary cells *in vitro*. Endocrinology 124:84-89

Stadel JM, Lefkowitz RJ 1989 Dual regulation of adenylate cyclase by stimulatory and inhibitory pathways. In: DeGroot LJ (eds) Endocrinology (vol.1). W B Saunders, pp 75-93

Steffey ME, Synder GL, Barrett RW, Fink JS, Ackerman M, Adams P, Bhatt R, Gomez E, MacKenzie RG 1991 Dopamine D1 receptor stimulation of cyclic AMP accumulation in COS-1 cells. Eur J Pharmacol 207:311-317

Wong AOL, Chang JP, Peter RE 1992 Dopamine stimulates growth hormone release from the pituitary of goldfish, *Carassius auratus*, through the dopamine D1 receptors. Endocrinology 130:1201-1210

Wong AOL, Chang JP, Peter RE 1993a Dopamine functions as a growth hormone-

releasing factor in the goldfish, *Carassius auratus*. Fish Physiol Biochem (in press)

Wong AOL, Chang JP, Peter RE 1993b *In vitro* and *in vivo* evidence that dopamine exerts growth hormone releasing activity in the goldfish. Am J Physiol (in press)

Wong AOL, Chang JP, Peter RE 1993c Characterization of D1 receptors mediating dopamine-stimulated growth hormone release from pituitary cells of the goldfish, *Carassius auratus*. (submitted to Endocrinology)

Chapter 7

Entry of Extracellular Calcium mediates Dopamine D1-stimulated Growth Hormone Release from Goldfish Pituitary Cell [1]

7.1 Introduction

In the goldfish, the anterior pituitary is directly innervated by a preoptico-hypophyseal dopaminergic pathway (Kah *et al.*, 1987). Nerve terminals immumo-reactive to dopamine (DA) have been identified in close proximity to the somatotrophs (Kah *et al.*, 1986). These anatomical observations are consistent with our findings that DA is stimulatory to GH release in the goldfish, both *in vivo* (Chang *et al.*, 1985) and *in vitro* (Peter *et al.*, 1990; Wong *et al.*, 1992). Recently, we have demonstrated that the GH-releasing actions of DA are mediated through DA D1 receptors (Chang *et al.*, 1990b; Wong *et al.*, 1992). The GH responses to DA stimulation are seasonal (Wong *et al.*, 1993a) and can be blocked by the GH-release inhibitor somatostatin (Wong *et al.*, 1993b). Long-term treatment with the DA agonist apomorphine also stimulates the body growth of goldfish (Wong *et al.*, 1993a,b). These findings, taken together, strongly indicate that DA functions as a GH-releasing factor in the goldfish.

In mammals, GH release is mediated by both cAMP- as well as Ca^{2+}-dependent pathways (for review, see Frohman and Jansson, 1986). In the rat, elevation of cAMP synthesis preceding the GH release induced by GHRH has been reported (Bilezikjian and Vale, 1983; Sheppard *et al.*, 1985). The GH-releasing actions mediated by cAMP are also known to be Ca^{2+} dependent (Spence *et al.*, 1980; Kraicer and Chow, 1982), and increasing evidence suggests that the interactions between Ca^{2+} and cAMP form an integrated signal transduction system to mediate GH exocytosis (Ohlsson and Lindstrom, 1990; Lussier *et al.*, 1991a,b). In lower vertebrates, such as teleosts, the post-receptor

[1] A version of this chapter has been submitted for publication : Wong AOL, and Chang JP (1993) **Neuroendocrinology.**

mechanisms mediating GH release are still largely unknown. In the goldfish, basal GH release is known to be Ca^{2+}-dependent (Chang and de Leeuw, 1990; Jobin and Chang, 1992a). Using goldfish pituitary cells in static incubation, we have recently demonstrated that DA D1-stimulated GH release is mediated by the cAMP-dependent pathway (Wong *et al.*, 1993c). However, the possible involvement of Ca^{2+}-dependent pathways in this DA D1 action has not been addressed.

In the present study, we examined the role of extracellular Ca^{2+} ($[Ca^{2+}]e$) in the GH responses to DA D1 stimulation using dispersed goldfish pituitary cells. The possible involvement of voltage-sensitive Ca^{2+} channels (VSCC) in DA D1-stimulated GH release was studied by using the selective VSCC blockers nifedipine, nicardipine and verapamil, as well as by using the inorganic competitor of Ca^{2+} entry, $CoCl_2$. The GH-releasing actions of K^+-induced depolarization and pharmacological agents that can induce Ca^{2+} entry, such as A23187 and Bay K8644, were also investigated. To study the interactions between $[Ca^{2+}]e$ and cAMP in the mediation of DA D1-stimulated GH release, the GH responses to the membrane-permeant cAMP analog 8Br.cAMP and the adenylate cyclase activator forskolin were tested in Ca^{2+}-deficient incubation medium or in the presence of the VSCC blocker nifedipine.

7.2 Materials and Methods

Animals Goldfish of the common or comet varieties were purchased from Ozark Fisheries (Stoutland, MO, USA) or Grassyforks Fisheries (Martinsville, IN, USA), and maintained in flow-through aquaria (1,800 liters) at 17 °C under a simulated natural photoperiod (Edmonton, AB, Canada) for at least 3 weeks prior to any experiments. The fish were fed to satiation daily with Ewos trout pellets (Astra Chemicals Ltd., Mississauga, ONT, Canada). Goldfish of both sexes, with body weight ranging from 20 to 30 g, were used in the present study. Since the seasonality of DA D1-stimulated GH release in the goldfish is known to be closely associated with the reproductive cycle (Wong *et al.*, 1993a,b), gonadal conditions of the fish, determined by the gonado-somatic index (GSI = weight of gonad / total body weight x 100 %) and the morphological characteristics of the gonads, are reported separately for individual experiments (for details, see chapter 2).

Reagents and test substances Dopamine, nifedipine, nicardipine, verapamil, cobalt chloride, 8-bromoadenosine 3':5'-cyclic monophosphate (8Br.cAMP) were obtained from Sigma Chemical Company (St Louis, MO, USA). Forskolin, A23187, and (±)Bay K8644 were purchased from Calbiochem Corporation (San Diego, CA, USA). The D1 agonist SKF38393 was obtained from Research Biochemicals Inc. (Wayland, MA, USA). SKF38393, 8Br.cAMP, and forskolin were first dissolved in a minimal amount of dimethyl sulfoxide (DMSO), and subsequently diluted to appropriate concentrations with testing medium. Nifedipine, nicardipine, and verapamil were dissolved in ethanol. Ethanol and DMSO concentrations in the final solutions were always less than 0.1 %, and did not affect basal GH release from goldfish pituitary cells (Chang and de Leeuw, 1990).

Preparation of dispersed pituitary cells Dispersed pituitary cells from the goldfish were prepared as described previously (Chang *et al.*, 1990a). In brief, goldfish pituitaries diced into fragments (≈0.5 mm in thickness) were exposed to controlled trypsin / DNAse treatment. Pituitary fragments were then mechanically dispersed in Ca^{2+}-deficient medium (M199 with Hank's salts prepared without $CaCl_2$, supplemented with 2.2 g/l sodium bicarbonate, 25 mM HEPES, 100,000 units penicillin/l, 100 mg streptomycin/l, and 0.3 % BSA; pH 7.2, GIBCO), and harvested by centrifugation at 200x g for 10 min at 4 °C. Cell yield of the dispersion procedures was about 0.5 million cells per pituitary, and the viability of pituitary cells was >94 % as assayed by the trypan blue exclusion test. Pituitary cells enriched with somatotrophs were prepared by a discontinuous density gradient as described by de Leeuw *et al.* (1984) with minor modifications (Chang and Jobin, 1993). Mixed populations of dispersed pituitary cells, which normally have about 20% of cells immunoreactive to GH antiserum, were gently layered on the top of a discontinuous Percoll gradient (with 40, 50, 60, 70, and 80 % Percoll, Pharmacia) and centrifuged at 1400x g for 25 min at 17 °C. After centrifugation, pituitary cells collected at the interphase between 60 and 70 % Percoll were found to have the highest amount of cells immunoreactive to GH antiserum (≈70 %). Viability of this enriched somatotroph preparation after Percoll density gradient separation was always >92 % as indicated by

the trypan blue exclusion test. Since the cell yield of enriched somatotophs was rather low (about 0.07 million cells per pituitary), mixed populations of goldfish pituitary cells were used in most of the experiments in the present study.

Static incubation of pituitary cells Mixed populations of pituitary cells or cells from the enriched somatotroph preparation were resuspended in plating medium (M199 with Earle's salts, supplemented with 2.2 g/l sodium bicarbonate, 25 mM HEPES, 100,000 units penicillin/l, 100 mg streptomycin/l, and 1 % horse serum; pH 7.2, GIBCO), and cultured in 24-well Falcon Primaria plates (Becton Dickinson & Company, NJ, USA) at a density of 0.25 million cells/ml/well. After overnight incubation (15-18 hr) at 28 °C under 5 % CO2 and saturated humidity, the culture medium was replaced by testing medium (M199 with Hank's salts, supplemented with 2.2 g/l sodium bicarbonate, 25 mM HEPES, 100,000 units penicillin/l, 100 mg streptomycin/l, and 0.1 % BSA; pH 7.2, GIBCO). Drug treatment was initiated by adding testing medium with the appropriate concentrations of test substances. To test the effects of Ca^{2+}-free condition on GH release, Ca^{2+}-deficient medium (M199 with Hank's salts prepared without $CaCl_2$, containing 2.2 g/l sodium bicarbonate, 25 mM HEPES, 100,000 units penicillin/l, 100 mg streptomycin/l, and 0.1 % BSA; pH 7.2, GIBCO) supplemented with or without 0.1 mM EGTA was used in place of testing medium. Following a further incubation of 2 hours, 500 µl of testing medium was carefully removed from individual culture wells, and stored at -25 °C until their GH contents were assayed by a RIA previously validated for the measurement of goldfish GH (Marchant *et al.*, 1989b). All treatments were carried out in quadruplicate in the same experiment, and each experiment was repeated at least 3 times. Hormone data were expressed as a percentage of the basal hormone release in control wells without any drug treatment (as "% control"), and were pooled for statistical analysis.

Perifusion of pituitary cells enriched with somatotrophs Pituitary cells enriched with somatotrophs were resuspended in plating medium, and incubated with preswollen cytodex beads (Cytodex I, Sigma) at 28 °C under 5 % CO_2 and saturated humidity. Cell

attachment was >95 % after overnight incubation (15-18 hr). Cytodex beads with enriched somatotrophs attached were loaded into 0.5 ml micro-columns (≈1 million cells/ column; ACUSYST-S™, Endotronics Inc., Minneapolis, Minn., USA) as described previously (Chang *et al.*, 1990a). The total dead volume of the perifusion system was about 1.7 ml. Pituitary cells were then perifused with testing medium at a flow rate of 15 ml/hr. After 3 hours of perifusion, basal GH release from enriched somatotrophs remained relatively stable in the absence of any stimulation. Test substances were then added from a drug reservoir into the perifusion column through a 3-way stopcock. Perifusates from each column were collected in 5-min fractions, and stored at -25 °C until their GH contents were assayed. GH data were transformed into "% pretreatment" as defined previously (Wong *et al.*, 1992). In brief, GH data from each individual column were expressed as a percentage of the mean GH contents of the first 6 fractions of perifusates at the beginning of the perifusion experiment (i.e., prior to any drug treatment). This transformation was done to allow pooling of GH data from separate columns of the same experiment without distorting the profile of hormone release. GH responses were quantified by calculating the net change in GH release (i.e., area under the curve) after a particular drug treatment (for details, see chapter 2).

Data analysis and statistics GH data were analyzed by analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test. Differences were considered significant when $P<0.05$.

7.3 Results

Incubation with Ca^{2+}-deficient medium on the GH responses to A23187 and Bay K8644

Mixed populations of pituitary cells were prepared from goldfish undergoing gonadal recrudescence. Similar to the results of our previous studies using ionomycin (Chang and de Leeuw, 1990), the Ca^{2+} ionophore A23187 (10 μM) and the VSCC agonist Bay K8644 (1 μM) were effective in stimulating GH release from goldfish pituitary cells in the present study (Fig. 7.1). These GH responses to A23187 and Bay K8644 were blocked by incubation with Ca^{2+}-deficient medium. Incubation with Ca^{2+}-deficient medium also significantly reduced the basal GH release.

Increasing doses of KCl and NaCl on GH release Effects of K^+-induced membrane depolarization on GH release were examined using pituitary cells prepared from goldfish undergoing gonadal recrudescence. Increasing doses of KCl (5 - 15 mM) stimulated GH release in a dose-dependent manner (Fig. 7.2). However, the GH response was reduced when a high dose of KCl (20 mM) was used. Similar doses of NaCl (5 - 20 mM), on the other hand, were not effective in elevating GH release. When administered at 20 mM dose, NaCl caused a significant reduction in basal GH levels. This inhibitory action of NaCl was probably due to the osmotic effects of a high level of electrolytes.

Incubation with Ca^{2+}-deficient medium on the GH responses to DA and SKF38393 The influence of $[Ca^{2+}]_e$ on DA-stimulated GH release was studied using mixed populations of pituitary cells prepared from goldfish undergoing gonadal recrudescence (Fig. 7.3). In normal testing medium (containing 1.25 mM $CaCl_2$), DA from 1 nM to 10 μM doses stimulated GH release in a dose-dependent manner. However, the GH responses to DA were abolished in Ca^{2+}-deficient medium supplemented with or without 0.1 mM EGTA. No significant differences were found in the basal GH levels in experiments using normal testing medium, Ca^{2+}-deficient medium, or Ca^{2+}-deficient medium supplemented with 0.1 mM EGTA. In a separate study, the effects of Ca^{2+}-deficient medium on the D1 agonist SKF38393-stimulated GH release were examined using pituitary cells enriched with somatotrophs (Fig. 7.4). Enriched somatotrophs were prepared from goldfish in the late stages of gonadal recrudescence. SKF38393 at 1 μM dose induced a significant increase in GH release from enriched somatotrophs under static incubation. However, this GH response was blocked by incubation with Ca^{2+}-deficient medium. Similar to the results of the preceding study, no differences were observed in basal GH levels between experiments using normal testing medium and Ca^{2+}-deficient medium.

Blocking Ca^{2+} entry through VSCC on DA- and SKF38393-stimulated GH release Pituitary cells were prepared from goldfish in early stages of gonadal recrudescence. The GH responses to 1μM dose of DA (Fig. 7.5A) and SKF38393 (Fig. 7.6A) were

abolished by simultaneous treatments with the dihydropyridine VSCC blockers nefidipine (10 μM) and nicardipine (10 μM). Similarly, the phenylalkylamine VSCC blocker verapamil (10 μM) and the inorganic competitive inhibitor of Ca^{2+} entry $CoCl_2$ (5 mM) were also effective in blocking the GH-releasing actions of DA (Fig. 7.5B) and SKF38393 (Fig. 7.6B). Nifedipine, nicardipine and verapamil did not alter the basal GH release from goldfish pituitary cells at 10 μM dose. However, basal GH levels were significantly reduced in the presence of 5 mM $CoCl_2$.

Dose-dependence of SKF38393-stimulated GH release and actions of nifedipine on the GH responses to SKF38393 Enriched somatotrophs were prepared for perifusion experiments from goldfish in the late stages of gonadal recrudescence. Increasing concentrations (10 nM-1 μM) of the D1 agonist SKF38393 administered as 2-min pulses at 1 hour intervals stimulated GH release in a dose-dependent manner (Fig. 7.7A). The protocol for SKF38393 perifusion has been previously validated to have no potentiating or desensitizing effects on GH release within the doses tested (Wong *et al.*, 1992). Since salmon gonadotropin (GTH)-releasing hormone (sGnRH) is known to stimulate GH and GTH-II release in the goldfish (Marchant *et al.*, 1989a), 2-min pulses of sGnRH at 50 nM were given at the beginning and at the end of the perifusion experiment to serve as an internal control. In this experiment, the GH responses to the first and second sGnRH pulses were not significantly different form each other, indicating that the responsiveness and viability of perifused pituitary cells were maintained during the course of perifusion. The GH responses to 1 μM SKF38393 were greatly suppressed in the presence of 10 μM nifedipine (Fig. 7.7B). Continuous perifusion of 10 μM nifedipine or 0.1 % ethanol did not affect basal GH release from enriched somatotrophs.

Extracellular Ca^{2+} on the GH responses to 8Br.cAMP and forskolin Goldfish undergoing gonadal recrudescence were used to prepare dispersed pituitary cells for static incubation experiments. The membrane-permeant cAMP analog 8Br.cAMP (1 mM) and the adenylate cyclase activator forskolin (10 μM) were stimulatory to GH release; however, these GH responses were suppressed by incubation with Ca^{2+}-deficient

medium (Fig. 7.8). Similar to the results of the preceding studies, no significant differences in basal GH levels were found between experiments using normal testing medium and Ca^{2+}-deficient medium.

Effects of nifedipine on the GH responses to 8Br.cAMP and forskolin Pituitary cells were prepared from goldfish undergoing gonadal recrudescence. 8Br.cAMP (1 mM) and forskolin (10 μM) induced a significant increase in GH release from pituitary cells under static incubation (Fig. 7.9). These GH responses were suppressed in the presence of 10 μM nifedipine. Nifedipine alone did not affect basal GH release in the present study.

7.4 Discussion

The role of extracellular Ca^{2+} ($[Ca^{2+}]e$) in DA D1-stimulated GH release was examined in the present study using goldfish pituitary cells. The GH responses to DA and the D1 agonist SKF38393 were abolished by using Ca^{2+}-deficient medium (supplemented with or without 0.1 mM EGTA), indicating that the availability of $[Ca^{2+}]e$ is important for the GH-releasing actions of DA. The stimulatory actions of DA on GH release were mimicked by pharmacological agents capable of inducing Ca^{2+} entry into goldfish pituitary cells, such as the Ca^{2+} ionophore A23187 and the VSCC agonist Bay K8644. Similar to DA D1-stimulated GH release, the GH responses to A23187 and Bay K8644 were blocked by incubation with Ca^{2+}-deficient medium. Previously, we have demonstrated that the D1 agonist SKF38393 (1 μM) increases intracellular Ca^{2+} levels in goldfish pituitary cells (Chang *et al.*, 1993). In this study, the GH responses to DA and SKF38393 were abolished by blocking $[Ca^{2+}]e$ entry using a high dose of $CoCl_2$. These results strongly indicate that the GH-releasing actions of DA are mediated through Ca^{2+} influx from the extracellular sources. In mammals, the involvement of $[Ca^{2+}]e$ in GHRH-stimulated GH release has been well documented (for review, see Frohman and Jansson, 1986). In the rat, pharmacological agents capable of inducing Ca^{2+} entry, such as A23187 and Bay K8644, are known to stimulate GH release from mixed populations of pituitary cells (Drouva *et al.*, 1988) and from purified somatotrophs (Kraicer and Chow, 1982).

Administration of high doses of KCl is generally accepted to be a valid method to

activate VSCC by causing membrane depolarization (Stojikovic *et al.*, 1988). In the goldfish, a high dose of KCl (30 mM) increases the intracellular Ca^{2+} levels in mixed populations of dispersed pituitary cells (Jobin and Chang, 1992b), indicating that this treatment is also effective in inducing Ca^{2+} entry via VSCC in the goldfish. In the present study, millimolar doses of KCl (5 - 20 mM) were effective in stimulating GH release from goldfish pituitary cells, suggesting that VSCC is involved in mediating GH release in the goldfish. This idea is also in agreement with our findings that Bay K8644 was stimulatory to GH release from goldfish pituitary cells (see earlier discussion). Bay K8644 is a dihydropyridine derivative known to activate VSCC in mammals (for review, see Tsien *et al.*, 1991).

The possible involvement of VSCC in DA D1-stimulated GH release was also examined in the present study. Blockade of $[Ca^{2+}]e$ entry via VSCC by the selective Ca^{2+} channel blockers nifedipine, nicardipine and verapamil inhibited the GH responses to DA and the D1 agonist SKF38393. These observations strongly indicate that the GH-releasing actions of DA are mediated through activation of Ca^{2+} entry through the VSCC. In mammals, the dihydropyridine (e.g., nifedipine and nicardipine) and phenylalkylamine Ca^{2+} channel blockers (e.g., verapamil) are known to exert their inhibitory actions by binding directly to the "L"-type VSCC (for review, see Catterall and Striessnig, 1992). In the rat, "L"-type Ca^{2+} currents characterized by high voltage activation and slow inactivation have been identified in the somatotrophs (Lewis *et al.*, 1988). In the goldfish, Ca^{2+} currents that can be inhibited by nifedipine have been demonstrated in mixed populations of pituitary cells (Chang *et al.*, 1991). Although the inhibitory actions of nifedipine, nicardipine and verapamil on DA D1-stimulated GH release have been demonstrated in this study, the presence of "L"-type VSCC in the goldfish somatotrophs is still not clear and awaits further investigation.

In other studies, we have demonstrated that the cAMP-dependent pathway is involved in the mediation of DA D1-stimulated GH release (Wong *et al.*, 1993c). In the present study, the GH responses to the membrane-permeant cAMP analog 8Br.cAMP and the adenylate cyclase activator forskolin were suppressed by incubation with Ca^{2+}-deficient medium or with the dihydropyridine VSCC blocker nifedipine. Previously, we have shown that the GH responses to forskolin can be reduced by the phenylalkylamine

VSCC blocker verapamil (Chang *et al.*, 1993). These results indicate that the GH responses mediated by the cAMP-dependent pathway also involve the entry of $[Ca^{2+}]e$ through VSCC. In mammals, GH release induced by activation of the cAMP-dependent pathway is known to be Ca^{2+}-dependent (Spence *et al.*, 1980; Kraicer and Chow, 1982). In the rat, the GH-releasing effects of cAMP analogs, forskolin, and the phosphodiesterase inhibitor IBMX can be inhibited by limiting $[Ca^{2+}]e$ in the culture medium (Spence *et al.*, 1980) or by using VSCC blockers (Bilezikjian and Vale, 1983). Recently, cAMP analogs and forskolin have been shown to increase intracellular Ca^{2+} levels ($[Ca^{2+}]i$) in the rat somatotrophs (Holl *et al.*, 1989; Lussier *et al.*, 1991b). These Ca^{2+} responses can be abolished by lowering $[Ca^{2+}]e$ levels or by using VSCC blockers (Lussier *et al.*, 1991b). In the goldfish, we have demonstrated that the D1 agonist SKF38393 stimulates cAMP and GH release in a dose-dependent manner (Wong *et al.*, 1993c). We also reported that a micromolar dose of SKF38393 can elevate $[Ca^{2+}]i$ in mixed populations of goldfish pituitary cells (Chang *et al.*, 1993). It is likely that DA D1-stimulated GH release is mediated by cAMP, which subsequently activates VSCC to allow the entry of $[Ca^{2+}]e$. This idea is consistent with the recent findings that cyclic nucleotides, including cAMP, can activate membrane-bound ion channels either by protein phosphorylation via protein kinases or by binding directly to ion channels (for review, see Matthews, 1991).

In this study, we have also examined the role of VSCC in the acute GH response to DA D1 stimulation using perifused goldfish somatotrophs. In these experiments, a high dose (10 μM) of the VSCC blocker nifedipine could not completely abolish the GH-releasing action of the D1 agonist SKF38393. These results suggest that a component of the acute GH response to SKF38393 is independent of Ca^{2+} entry through VSCC. This VSCC-independent component was only apparent within the first 5 min after SKF38393 administration. In the rat, the entry of $[Ca^{2+}]e$ via VSCC has been previously reported in luteinizing hormone (LH) releasing hormone-stimulated LH release. Similarly, a VSCC-independent component was observed during the initial phase of LH release, which was subsequently confirmed to be the result of $[Ca^{2+}]i$ mobilization (for review, see Catt and Stojikovic, 1989). Whether the VSCC-independent component of DA D1-stimulated GH release was also the result of $[Ca^{2+}]i$ mobilization is still not clear. However, it is worth

mentioning that activation of DA D1 receptors is known to increase the production of Inositol 1,4,5-trisphosphate (IP_3) (Undie and Friedman, 1990), and IP_3 is stimulatory to Ca^{2+} release from intracellular stores (for review, see Catt *et al.*, 1991).

In conclusion, we have demonstrated that the entry of $[Ca^{2+}]e$ through VSCC is a part of the signal transduction mechanisms mediating DA D1-stimulated GH release from goldfish pituitary cells. Probably, this Ca^{2+} influx takes place after the production of cAMP. The present study also provides evidence that the post-receptor cascades leading to GH release from goldfish pituitary cells are similar to those observed in mammals. These observations strongly suggest that the signal transduction pathways mediating GH release from the somatotrophs may be highly conserved during vertebrate evolution.

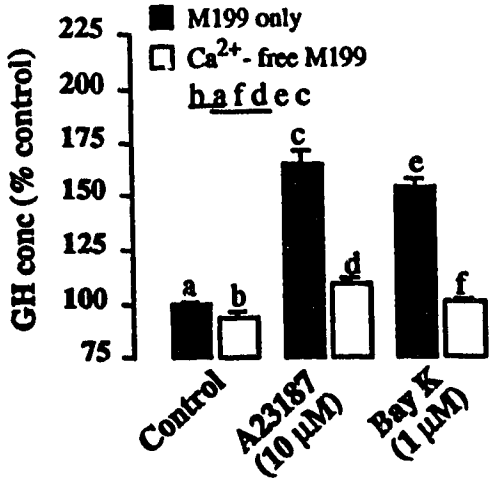


Fig. 7.1 Effects of extracellular Ca^{2+} on the GH responses to the Ca^{2+} ionophore A23187 and the Ca^{2+} channel agonist Bay K8644 using goldfish pituitary cells under static incubation. GH data were pooled from six separate experiments, each with quadruplicate treatments (mean ± SEM, n = 24). The average GH level in control wells was 513 ± 8 ng GH/ml. Drug treatments giving a similar magnitude of GH responses are grouped within the same underscore (ANOVA followed by Fisher's LSD test, $P > 0.05$).

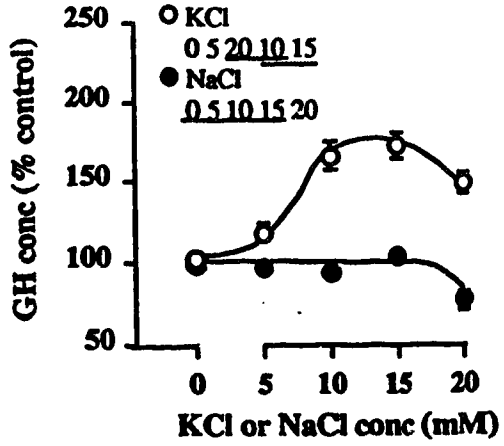


Fig. 7.2 Effects of increasing doses of KCl and NaCl on GH release from goldfish pituitary cells. GH data were pooled from three separate experiments, each with quadruplicate treatments (mean ± SEM, n = 12). The average GH level in control wells was 556 ± 24 ng GH/ml. Concentrations of KCl or NaCl giving a similar magnitude of GH responses are grouped within the same underscore (ANOVA followed by Fisher's LSD test, $P > 0.05$).

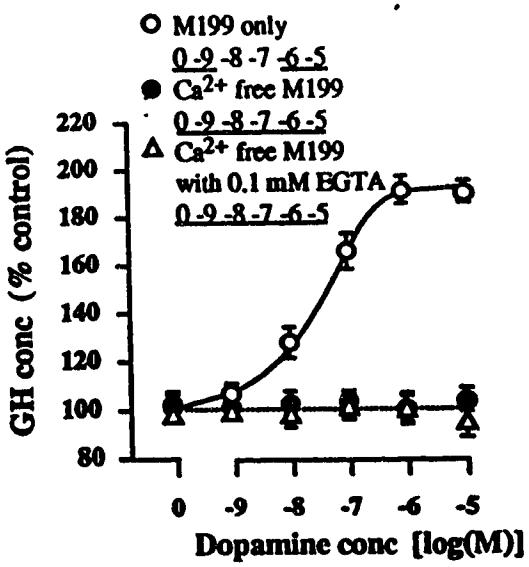


Fig. 7.3 Effects of extracellular Ca^{2+} on DA-stimulated GH release from goldfish pituitary cells. GH data were pooled from four separate experiments, each with quadruplicate treatments (mean ± SEM, n = 16). The average GH concentrations in control wells for experiments with normal M199, Ca^{2+}-free M199, and Ca^{2+}-free M199 with 0.1 mM EGTA were 630 ± 77, 677 ± 58, and 664 ± 67 ng GH/ml/0.25 million cells, respectively. In each of the incubation conditions (with or without Ca^{2+}), concentrations of DA giving a similar magnitude of GH responses are grouped within the same underscore (ANOVA followed by Fisher's LSD test, $P > 0.05$).

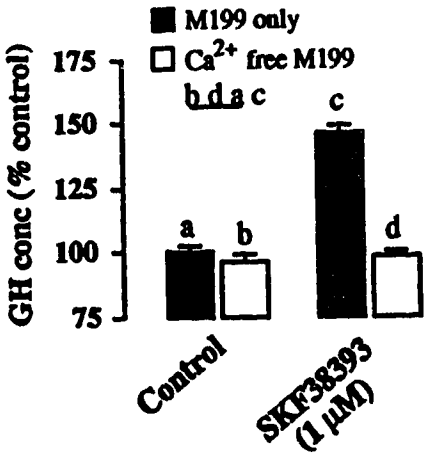


Fig. 7.4 Effects of extracellular Ca^{2+} on the D1 agonist SKF38393-stimulated GH release from goldfish pituitary cell preparations enriched with somatotrophs. GH data were pooled from three separate experiments, each with quadruplicate treatments (mean ± SEM, n = 12). The average GH level in control wells was 573 ± 60 ng GH/ml. Drug treatments giving a similar magnitude of GH responses are grouped within the same underscore (ANOVA followed by Fisher's LSD test, $P > 0.05$).

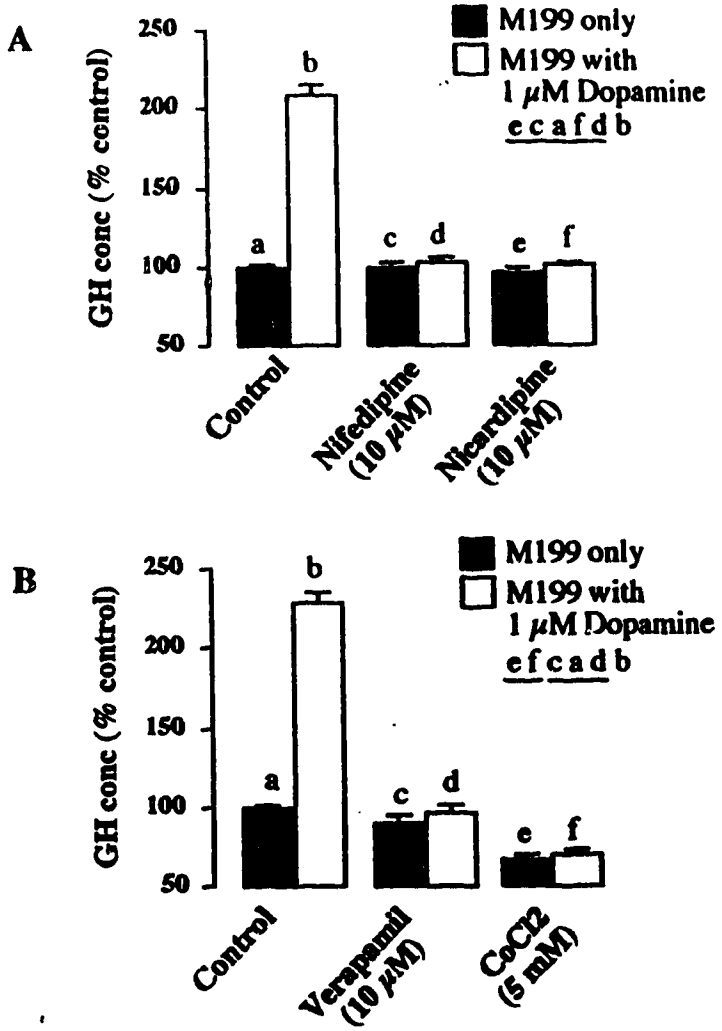


Fig. 7.5 Effects of blocking Ca^{2+} entry on DA-stimulated GH release from goldfish pituitary cells. The GH response to 1μM DA was examined in the presence or absence of (A) nifedipine (10 μM) and nicardipine (10 μM), and (B) verapamil (10 μM) and $CoCl_2$ (5 mM). GH data were pooled from four separate experiments, each with quadruplicate treatments (mean ± SEM, n = 16). The average GH level in control wells was 594 ± 22 ng GH/ml. Drug treatments giving a similar magnitude of GH responses are grouped within the same underscore (ANOVA followed by Fisher's LSD test, $P > 0.05$).

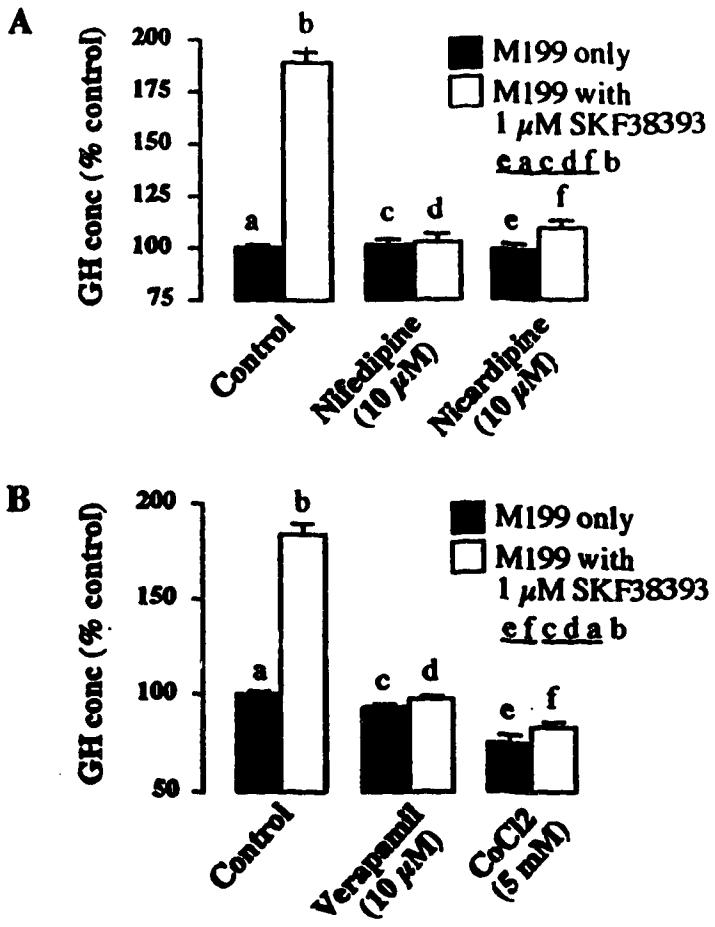


Fig. 7.6 Effects of blocking Ca^{2+} entry on the D1 agonist SKF38393-stimulated GH release from goldfish pituitary cells. The GH response to 1μM SKF38393 was examined in the presence or absence of (A) nifedipine(10 μM) and nicardipine (10 μM), and (B) verapamil (10 μM) and $CoCl_2$ (5 mM). GH data were pooled from four separate experiments, each with quadruplicate treatments (mean ± SEM, n = 16). The average GH level in control wells was 785± 23 ng GH/ml. Drug treatments giving a similar magnitude of GH responses are grouped within the same underscore (ANOVA followed by Fisher's LSD test, $P > 0.05$).

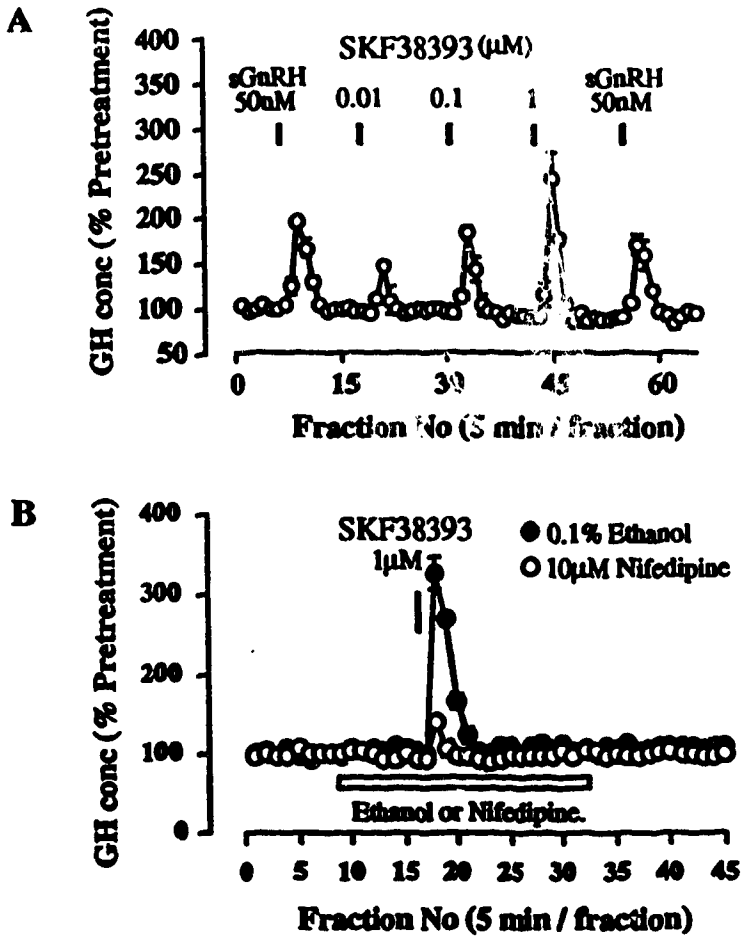


Fig. 7.7 Effects of the Ca^{2+} channel blocker nifedipine on SKF38393-stimulated GH release from pituitary cell preparations enriched with somatotrophs. (A) The D1 agonist SKF38393 from 0.01 to 1 μM doses was given as 2-min pulses at 1 hour intervals (x4 columns). 2-min pulses of salmon gonadotropin-releasing hormone (sGnRH) at 50 nM was given at the beginning and at the end of the perifusion to serve as an internal control. (B) 2-min pulses of 1 μM SKF38393 were given during the 1.5-hour continuous perifusion of 10 μM nifedipine (x4 columns). Ethanol at 0.1 %, the solvent for nifedipine, was used as the control treatment. The average pretreatment GH level for the experiments was 14 ± 2 ng GH/ml. All GH data are expressed as mean ± SEM (n = 4).

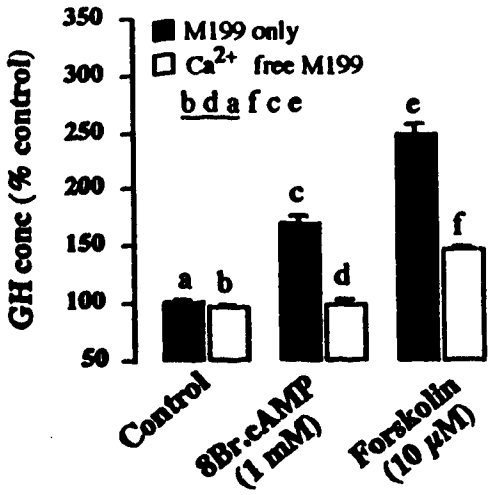


Fig. 7.8 Effects of extracellular Ca^{2+} on the GH responses to 8Br.cAMP and forskolin using goldfish pituitary cells under static incubation. GH data were pooled from four separate experiments, each with quadrupicate treatments (mean ± SEM, n = 16). The average GH level in control wells was 453 ± 21 ng GH/ml. Drug treatments giving a similar magnitude of GH responses are grouped within the same underscore (ANOVA followed by Fisher's LSD test, $P > 0.05$).

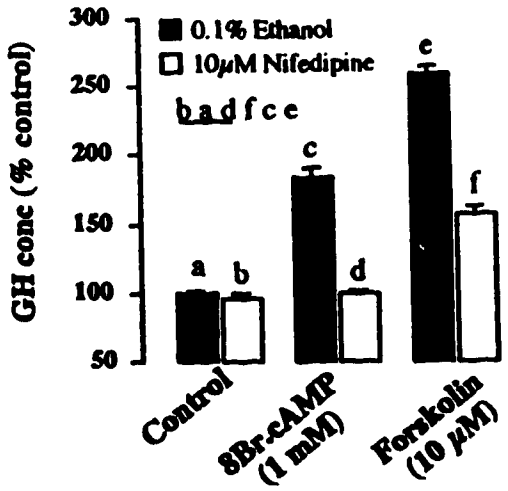


Fig. 7.9 Effects of the Ca^{2+} channel blocker nifedipine on the GH responses to 8Br.cAMP and forskolin using goldfish pituitary cells under static incubation. Ethanol at 0.1 %, the solvent for nifedipine, was used as the control treatment. GH data were pooled from three separate experiments, each with quadruplicate treatments (mean ± SEM, n = 12). The average GH level in control wells was 522 ± 27 ng GH/ml. Drug treatments giving a similar magnitude of GH responses are grouped within the same underscore (ANOVA followed by Fisher's LSD test, $P > 0.05$).

7.5 References

Bilezikjian LM, Vale WW 1983 Stimulation of adenosine 3',5'-monophosphate production by growth hormone-releasing factor and its inhibition by somatostatin in anterior pituitary cells *in vitro*. Endocrinology 113:1726-1731

Catt KJ, Hunyady L, Balla T 1991 Second messengers derived from inositol lipids. J Bioenerg Biomemb 23:7-27

Catt KJ, Stojikovic SS 1989 Calcium signaling and gonadotropin secretion. Trends Endocrinol Metab 1:15-20

Catterall WA, Striessnig J 1992 Receptor sites for Ca^{2+} channel antagonists. Trends Pharmacol Sci 13:256-262

Chang JP, Cook H, Freedman GL, Wiggs AJ, Somoza GM, de Leeuw R, Peter RE 1990a Use of a pituitary cell dispersion method and primary culture system for the studies of gonadotropin-releasing hormone action in the goldfish, *Carassius auratus*. I. Initial morphological, static, and cell column perifusion studies. Gen Comp Endocrinol 77:256-273

Chang JP, de Leeuw R 1990 *In vitro* goldfish growth hormone responses to gonadotropin-releasing hormone : Possible roles of extracellular calcium and arachidonic acid metabolism ? Gen Comp Endocrinol 80:155-164

Chang JP, Jobin RM 1993 Teleost pituitary cells: Isolation, culture, and use. In : Hochachka PW, Mommsen TP (eds) Biochemistry and Molecular Biology of Fishes (vol.3) : Analytical Techniques. Elsevier, Amsterdam (in press)

Chang JP, Jobin RM, Wong AOL 1993 Intracellular mechanisms mediating gonadotopin and growth hormone release in the goldfish, *Carassius auratus*. Fish Physiol Biochem (in press)

Chang JP, Marchant TA, Cook AF, Nahorniak CS, Peter RE 1985 Influences of catecholamines on growth hormone release in female goldfish, *Carassius auratus*. Neuroendocrinology 40:463-470

Chang JP, Price CJ, Jobin RM, Goldberg JI 1991 Ionic currents in primary cultures of dispersed goldfish pituitary cells. Soc NeuroSci Abstracts 17:114.5 (Abs)

Chang JP, Yu KL, Wong AOL, Peter RE 1990b Differential actions of dopamine

receptor subtypes on gonadotropin and growth hormone release *in vitro* in goldfish. Neuroendocrinology 51:664-674

de Leeuw R, Goos HJT, Peute J, Van Pelt AAM, Burzawa-Gerard E, Van Oordt PGWJ 1984 Isolation of gonadotrops from the pituitary of the African catfish, *Clarias lazera*. Morphological and physiological characterization of purified cells. Cell Tissue Res 236:669-675

Drouva SV, Rerat E, Bihoreau C, Laplante E, Rasolonjanahary R, Clauser H, Kordon C 1988 Dihydropyridine-sensitive calcium channel activity related to prolactin, growth hormone, and luteinizing hormone release from anterior pituitary cells in culture : Interactions with somatostatin, dopamine, and estrogens. Endocrinology 123:2762-2773

Frohman LA, Jansson JO 1986 Growth-hormone releasing hormone. Endocr Rev 7:223-254

Holl RW, Thorner MO, Leong DA 1989 Cytosolic free calcium in normal somatotropes: Effects of forskolin and phorbol ester. Am J Physiol 256:E375-E380

Jobin RM, Chang JP 1992a Differences in extracellular calcium involvement mediating the secretion of gonadotropin and growth hormone stimulated by two closely releated endogenous GnRH peptides in goldfish pitutiary cells. Neuroendocrinology 55:156-166

Jobin RM, Chang JP 1992b Actions of two native GnRHs and protein kinase C modulators on goldfish pituitary cells. Studies on intracellular calcium levels and gonadotropin release. Cell Calcium 13:531-540

Kah O, Dulka JG, Dubourg P, Thibault J, Peter RE 1987 Neuroanatomical substrate for the inhibition of gonadotropin secretion in goldfish : Existence of a dopaminergic preoptico-hypophyseal pathway. Neuroendocrinology 45:451-458

Kah O, Dubourg P, Onteniente B, Geffard M, Calas A 1986 The dopaminergic innervation of the goldfish pituitary : An immunocytochemical study at the electron-microscope level using antibodies against dopamine. Cell Tissue Res 244:577-582

Kraicer J, Chow AEH 1982 Release of growth hormone from purified somatotrophs : Use of perifusion system to elucidate interrelations among Ca^{++}, adenosine 3',5'-

monophosphate, and somatostatin. Endocrinology 111:1173-1180

Lewis DL, Goodman MB, St.John PA, Baker JL 1988 Calcium currents and Fura-2 signals in fluorescence-activated cell sorted lactotrophs and somatotrophs of rat anterior pituitary. Endocrinology 123:611-621

Lussier BT, French MB, Moor BC, Kraicer J 1991a Free intracellular Ca^{2+} concentration and growth hormone (GH) release from purified rat somatotrophs. I. GH-releasing factor-induced Ca^{2+} influx raises $[Ca^{2+}]i$. Endocrinology 128:570-582

Lussier BT, French MB, Moor BC, Kraicer J 1991b Free intracellular Ca^{2+} concentration and growth hormone (GH) release from purified rat somatotrophs. III. Mechanism of action of GH-releasing factor and somatostatin. Endocrinology 128:592-603

Marchant TA, Chang JP, Nahorniak CS, Peter RE 1989a Evidence that gonadotropin-releasing hormone also functions as a growth hormone-releasing factor in the goldfish. Endocrinology 124: 2509-2518

Marchant TA, Dulka JG, Peter RE 1989b Relationship between serum growth hormone levels and the brain and pituitary content of immunoreactive somatostatin in the goldfish, *Carassius auratus*. Gen Comp Endocrinol 73:458-468

Matthews G 1991 Ion channels that are directly activated by cyclic nucleotides. Trends Pharmacol Sci 12:245-247

Ohlsson L, Lindstrom P 1990 The correlation between calcium outflow and growth hormone release in perifused rat somatotrophs. Endocrinology 126:488-497

Peter RE, Yu KL, Marchant TA, Rosenblum PM 1990 Direct neural regulation of the teleost adenohypophysis. J Exp Zool [Suppl] 4:84-89

Sheppard MS, Moor BC, Kraicer J 1985 Release of growth hormone (GH) from purified somatotrophs : Interaction of GH-releasing factor and somatostatin and role of adenosine 3',5'-monophosphate. Endocrinology 117:2364-2370

Spence JW, Sheppard MS, Kraicer J 1980 Release of growth hormone from purified somatotrophs : Interrelation between Ca^{++} and adenosine 3',5'-monphosphate. Endocrinology 106:764-769

Stojikovic SS, Izumi SI, Catt K 1988 Participation of voltage-sensitive calcium

channels in pituitary hormone release. J Biol Chem 263:13054-13061

Tsien RW, Ellinor PT, Horne WA 1991 Molecular diversity of voltage-dependent Ca^{2+} channels. Trends Pharmacol Sci 12:349-354

Undie AS, Friedman E 1990 Stimulation of a dopamine D1 receptor enhances inositol phosphates formation in rat brain. J Pharmacol Exp Ther 253:987-992

Wong AOL, Chang JP, Peter RE 1992 Dopamine stimulates growth hormone release from the pituitary of goldfish, *Carassius auratus*, through the dopamine D1 receptors. Endocrinology 130:1201-1210

Wong AOL, Chang JP, Peter RE 1993a Dopamine functions as a growth hormone-releasing factor in the goldfish, *Carassius auratus*. Fish Physiol Biochem (in press)

Wong AOL, Chang JP, Peter RE 1993b *In vitro* and *in vivo* evidence that dopamine exerts growth hormone releasing activity in the goldfish. Am J Physiol (in press)

Wong AOL, Van Der Kraak G, Chang JP 1993c Cyclic 3',5'-adenosine monophosphate (cAMP) mediates dopamine D1-stimulated growth hormone release from goldfish pituitary cells. (submitted to Endocrinology)

Chapter 8

Independence of Dopamine D1-stimulated and Protein Kinase C-induced Growth Hormone Release from Goldfish Pituitary Cell [1]

8.1 Introduction

The release of growth hormone (GH) in teleosts is known to be under both stimulatory and inhibitory controls from the hypothalamus (for review, see Nishioka *et al.*, 1988). In the goldfish, GH release can be stimulated by salmon gonadotropin-releasing hormone (sGnRH) (Marchant *et al.*, 1989a) and inhibited by somatostatin (SRIF) (Marchant *et al.*, 1987). Recently, we have reported that the neurotransmitter dopamine (DA) stimulates GH release from the goldfish pituitary through activation of DA D1 receptors (Wong *et al.*, 1992). The GH responses to DA are additive to that of sGnRH (Chang *et al.*, 1990b; Wong *et al.*, 1993c) and can be suppressed by SRIF (Wong *et al.*, 1993a,b). The GH-releasing actions of DA have a distinct pattern of seasonality (Wong *et al.*, 1992a,b), and long-term treatment with the DA agonist apomorphine stimulates the body growth of goldfish (Wong *et al.*, 1993b). These findings strongly indicate that DA functions as a GH-releasing factor in the goldfish.

In mammals, especially in the rat, the protein kinase C (PKC) activators TPA and DiC8 stimulate GH release from mixed populations of pituitary cells (Negro-Vilar and Lapetina, 1985) and from purified somatotrophs (French *et al.*, 1989). Recently, PKC activity has been identified in the somatotrophs of the rat (French *et al.*, 1991), suggesting that PKC is involved in the mediation of GH release. In teleosts, the post-receptor mechanism mediating GH secretion is still largely unknown. Our recent studies have demonstrated that DA D1-stimulated GH release in the goldfish is mediated by the cAMP- (Wong *et al.*, 1993d) and Ca^{2+}-dependent pathways (Wong and Chang, 1993).

[1] A version of this chapter has been submitted for publication : Wong AOL, and Chang JP (1993) **Neuroendocrinology.**

The GH-releasing actions of cAMP in the goldfish are exerted mainly through the enzyme protein kinase A (Wong *et al.*, 1993d). Similar to the cases in mammals, the PKC activators TPA and DiC8 are stimulatory to GH secretion from goldfish pituitary cells (Chang *et al.*, 1991). However, the possible involvement of PKC in DA D1-stimulated GH release has not been addressed in these studies.

The present investigation examined the role of PKC in DA D1-stimulated GH release in the goldfish. Goldfish pituitary cells were desensitized by TPA pretreatment to deplete the endogenous PKC contents (Jobin and Chang, 1993; Jobin *et al.*, 1993). The GH-releasing actions of DA and the D1 agonist SKF38393 were then tested using these PKC desensitized cells. The involvement of PKC in this DA D1 action was also investigated by using the selective PKC inhibitor H7. The results based on PKC desensitization and inhibition by H7 were further confirmed by studying the possible additivity of the GH responses to PKC- and DA D1-stimulation. Since cAMP is known to be a second messenger for DA D1-stimulated GH release in the goldfish (Wong *et al.*, 1993d), the interactions between the cAMP- and PKC-dependent pathways in GH release were also examined.

8.2 Materials and Methods

Animals Goldfish of the common or comet varieties were purchased from Ozark Fisheries (Stoutland, MO, USA) or Grassyforks Fisheries (Martinsville, IN, USA), and maintained in flow-through aquaria (1,800 liters) at 17 °C under a simulated natural photoperiod (Edmonton, AB, Canada) for at least 3 weeks prior to any experiments. The fish were fed to satiation daily with Ewos trout pellets (Astra Chemicals Ltd., Mississauga, ONT, Canada). Goldfish of both sexes, with body weight ranging from 20 to 30 g, were used in the present study. Since the seasonality of DA D1-stimulated GH release in the goldfish is known to be closely associated with the reproductive cycle (Wong *et al.*, 1993a,b), gonadal conditions of the fish are reported separately for individual experiments. Gonadal conditions were determined by the gonadosomatic index (GSI = weight of gonad / total body weight x 100 %) and the morphological characteristics of the gonads (for details, see chapter 2).

Reagents and test substances Dopamine (DA) and 8-bromoadenosine 3':5'-cyclic monophosphate (8Br.cAMP) were obtained from Sigma Chemical Company (St Louis, MO, USA). Forskolin, sn-1,2-dioctanoyl glycerol (DiC8), and 12-O-tetradecanoyl 4β-phorbol-13-acetate (TPA) were purchased from Calbiochem Corporation (San Diego, CA, USA). SKF38393 was obtained from Research Biochemicals Inc. (Wayland, MA, USA). SKF38393, 8Br.cAMP, DiC8, TPA, and forskolin were first dissolved in a minimal amount of dimethyl sulfoxide (DMSO), and subsequently diluted to appropriate concentrations with medium M199. DMSO concentrations in the final solutions were always less than 0.1 %, and did not affect basal GH release from goldfish pituitary cells (Chang *et al.*, 1990b). Aliquots of salmon gonadotropin-releasing hormone (sGnRH) (Peninsula Laboratories Inc., Belmont, CA, USA) in 0.1 M acetic acid were frozen at -25 °C, and diluted to 100 nM solution with M199 immediately prior to use.

Preparation of dispersed pituitary cells Dispersed pituitary cells from the goldfish were prepared as described previously (Chang *et al.*, 1990a). In brief, goldfish pituitaries diced into fragments (≈0.5 mm in thickness) were exposed to a controlled trypsin / DNAse treatment. Pituitary fragments were then mechanically dispersed in Ca^{2+}-deficient medium (M199 with Hank's salts prepared without $CaCl_2$, supplemented with 2.2 g/l sodium bicarbonate, 25 mM HEPES, 100,000 units penicillin/l, 100 mg streptomycin/l, and 0.3 % BSA; pH 7.2, GIBCO), and harvested by centrifugation at 200x g for 10 min at 4 °C. Cell yield of the dispersion procedures was about 0.5 million cells per pituitary, and the viability of pituitary cells was >94 % as assayed by the trypan blue exclusion test. Pituitary cells enriched with somatotrophs were prepared by a discontinuous density gradient as described by de Leeuw *et al.* (1984) with minor modifications (Chang and Jobin, 1993). Mixed populations of dispersed pituitary cells, which normally have about 20% of cells immunoreactive to GH antiserum, were gently layered on the top of a discontinuous Percoll gradient (with 40, 50, 60, 70, and 80 % Percoll, Pharmacia) and centrifuged at 1400x g for 25 min at 17 °C. After centrifugation, pituitary cells collected at the interphase between 60 and 70 % Percoll were found to have the highest amount of cells immunoreactive to GH antiserum (≈70 %). Viability of this enriched somatotroph preparation after Percoll density gradient separation was always

>92 % as indicated by the trypan blue exclusion test. Since the cell yield of enriched somatotroph preparations was rather low (about 0.07 million cells per pituitary), mixed populations of goldifish pituitary cells were used in most of the experiments in the present study.

Static incubation of pituitary cells Mixed populations of pituitary cells or cells from the enriched somatotroph preparation were resuspended in plating medium (M199 with Earle's salts, supplemented with 2.2 g/l sodium bicarbonate, 25 mM HEPES, 100,000 units penicillin/l, 100 mg streptomycin/l, and 1 % horse serum; pH 7.2, GIBCO), and cultured in 24-well Falcon Primaria plates (Becton Dickinson & Company, NJ, USA) at a density of 0.25 million cells/ml/well. After overnight incubation (15-18 hr) at 28 °C under 5% CO_2 and saturated humidity, the culture medium was replaced by testing medium (M199 with Hank's salts, supplemented with 2.2 g/l sodium bicarbonate, 25 mM HEPES, 100,000 units penicillin/l, 100 mg streptomycin/l, and 0.1 % BSA; pH 7.2, GIBCO). Drug treatment was initiated by adding testing medium with the appropriate concentrations of test substances. Following a further incubation of 2 hours, 500 µl of testing medium was carefully removed from individual culture wells, and stored at -25 °C until their GH contents were assayed by a RIA previously validated for the measurement of goldfish GH (Marchant *et al.*, 1989b). All treatments were carried out in quadruplicate in the same experiment, and each experiment was repeated at least 3 times. For PKC-desensitization experiments, pituitary cells were exposed to TPA (10 nM) for 4 hours prior to drug treatments to deplete the cellular PKC contents as described by Jobin and Chang (1993). Pretreatment with 0.1 % DMSO, the solvent for TPA, for 4 hours was used as the control for these experiments. The procedures for PKC desensitization did not alter the viability of pituitary cells. GH data reported in the present study were expressed as a percentage of the basal GH release in control wells without any drug treatment (as "% control"), and were pooled for statistical analysis.

Perifusion of pituitary cells Mixed populations of pituitary cells were resuspended in plating medium, and incubated with preswollen cytodex beads (Cytodex I, Sigma) at 28

°C under 5 % CO_2 and saturated humidity. Cell attachment on cytodex beads was >95 % after overnight incubation (15-18 hr). To down-regulate cellular PKC contents, pituitary cells were incubated with 10 nM TPA for 4 hours prior to the perifusion experiments. Incubation with 0.1 % DMSO, the solvent for TPA, was used as the control for PKC desensitization. Following the pretreatment with TPA or DMSO, cytodex beads with pituitary cells attached were loaded into 0.5 ml micro-columns (≈2 million cells/ column; ACUSYST-S™, Endotronics Inc., Minneapolis, Minn., USA) as described previously (Chang *et al.*, 1990a). The total dead volume of the perifusion system was about 1.7 ml. Pituitary cells were then perifused with testing medium at a flow rate of 15 ml/hr. After 3 hours of perifusion, basal GH release from pituitary cells remained relatively stable in the absence of any stimulation. Test substances were then added from a drug reservoir into the perifusion column through a 3-way stopcock. Perifusates from each column were collected in 5-min fractions, and stored at -25 °C until their GH contents were assayed. GH data were transformed into "% pretreatment" as defined previously (Wong *et al.*, 1992). In brief, GH data from each individual column were expressed as a percentage of the mean GH contents of the first 6 fractions of perifusates at the beginning of the perifusion experiment (i.e., prior to the addition of test substances). This transformation was done to allow pooling of GH data from separate columns of the same experiment without distorting the profile of hormone release. GH responses were quantified by calculating the net change in GH release (i.e., area under the curve) after a particular drug treatment (for details, see chapter 2).

Data analysis and statistics GH data were analyzed by Student's *t* test, or by analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test. Differences were considered significant when $P<0.05$.

8.3 Results

GH-release responses of pituitary cells after PKC desensitization Pituitary cells for perifusion experiments were prepared from goldfish undergoing gonadal regression. TPA pretreatment significantly reduced the GH responses to subsequent stimulation by TPA (1 nM) and DiC8 (10 μM), but not that of forskolin (1 μM) (Fig. 8.1). The same

treatment was also effective in blocking the GH responses to sGnRH (100 nM); however, the GH-releasing actions of DA (1 μM) and the D1 agonist SKF38393 (1 μM) were not affected (Fig. 8.2). In these perifusion studies, TPA pretreatment did not alter the basal GH release (see legends of Fig. 8.1 and 8.2). Similar experiments were also conducted using goldfish pituitary cells under static incubation (Fig. 8.3). The pituitary cells for this study were prepared from goldfish in late stages of gonadal regression. Similar to the results of perifusion studies, TPA pretreatment suppressed the GH responses to TPA (10 nM) and DiC8 (0.1 mM), without affecting the GH release induced by DA (1 μM), SKF38393 (1 μM), 8Br.cAMP (1 mM) and forskolin (10 μM). In these experiments, basal GH levels were reduced by previous exposure to TPA.

Blocking PKC pathway by H7 on the GH-release responses of pituitary cells Pituitary cells were prepared from goldfish undergoing gonadal recrudescence. Using mixed populations of goldfish pituitary cells, the PKC inhibitor H7 at 20 μM dose suppressed the GH responses to DiC8 (0.1 mM) but not that of 8Br.cAMP (1 mM) (Fig. 8.4). In a separate study using an enriched somatotroph preparation, H7 at the same dose was not effective in blocking the GH responses to 1 μM SKF38393 (Fig. 8.5).

Additivity of the GH responses induced by DA agonists and PKC activation If PKC is involved in DA D1-stimulated GH release in the goldfish, it is likely that the GH responses to PKC activation and DA D1 stimulation will not be additive. To test this hypothesis, the additivity of SKF38393- and DiC8-stimulated GH release was examined using pituitary cells prepared from goldfish in gonadal regression (postspawning). The D1 agonist SKF38393 stimulated GH release in a dose-dependent manner (Fig. 8.6A). The GH responses to various doses of SKF38393 were enhanced in the presence of 0.1 mM DiC8 by an average of 88 ± 2 % control, which was not significantly different from the mean GH response to 0.1 mM DiC8 alone (83 ± 5 % control). In a reciprocal experiment, increasing concentrations of DiC8 stimulated GH release in a dose-related manner (Fig. 8.6B). In the presence of 1 μM SKF38393, the dose-response curve of DiC8-stimulated GH release shifted upward by an average of 63 ± 1 % control. Again, this enhancement of GH response was not different from that of 1 μM SKF38393 alone

(60 ± 7 % control). Similarly, in experiments using cell preparations enriched with somatotrophs (Fig. 8.5), the GH responses to 1 μM SKF38393 was also enhanced by 0.1 mM DiC8 in a magnitude (89 ± 15 % control) not different from the net GH response to DiC8 alone (90 ± 7 % control).

In a separate study, the GH releasing action of the DA agonist apomorphine (1 μM) was tested in the presence of forskolin (10 μM) and TPA (100 nM), respectively (Fig. 8.7). According to our previous dose-response studies (Chang *et al.*, 1991; Wong *et al.*, 1993d), these doses of forskolin and TPA are known to induce the maximal GH-release responses of the corresponding drugs. In the present study, apomorphine, forskolin and TPA are all effective in elevating basal GH release from goldfish pituitary cells. The GH response to apomorphine in the presence of 100 nM TPA was enhanced by an average of 76 ± 9 % control, which was not different from the net GH response to TPA alone (85 ± 25 % control). In contrast, apomorphine (1 μM) could not further stimulate GH release in the presence of 10 μM forskolin. These results, taken together, indicate that the GH-releasing actions of PKC- and DA D1-stimulation are independent and additive at the pituitary cell level.

Additivity of GH release mediated by the PKC- and cAMP-dependent pathways Since DA D1-stimulated GH release in the goldfish is mediated mainly by cAMP (Wong *et al.*, 1993d), the additivity of GH reponses to the PKC- and cAMP-dependent pathways was also examined (Fig. 8.8). The experiments were performed using pituitary cells prepared from goldfish at different reproductive stages throughout the year. Forskolin (10 μM) and 8Br.cAMP (1 mM), the stimulators for the cAMP-dependent pathway, were effective in stimulating GH release from goldfish pituitary cells. Their GH responses were further enhanced by simultaneous treatment with the PKC activators TPA (100 nM) and DiC8 (0.1 mM). The magnitude of these enhancements (199 ± 23 % control for TPA, 181 ± 16 % control for DiC8) was similar to the net GH responses to TPA or DiC8 alone (171 ± 37 and 166 ± 31 % control, respectively). These results indicate that the GH responses mediated by the PKC- and cAMP-dependent pathways are additive at the pituitary cell level. In this study, the stimulators for the PKC- (i.e., TPA and DiC8) and cAMP-dependent pathways (i.e., forskolin and 8Br.cAMP) were administered at doses known

to induce the respective maximal GH-release responses (Chang *et al.*, 1991; Wong *et al.*, 1993d).

8.4 Discussion

Previously, we have shown that activation of PKC stimulates GH release in the goldfish (Chang *et al.*, 1991). In the present study, TPA and DiC8, two known activators for PKC activity in mammals, were found to be stimulatory to GH release from goldfish pituitary cells. These results indicate that the PKC-dependent pathway is a part of the signal transduction system mediating GH release in the goldfish. The possible involvement of PKC in DA D1-stimulated GH release was then investigated using goldfish pituitary cells pretreated with TPA to deplete the endogenous PKC contents. TPA pretreatment using pituitary cells from mammals is known to cause a loss of responsiveness to exogenous PKC activators, a reduction of extractable PKC activity, and a drop in binding sites for phorbol esters (for review, see McArdle and Conn, 1989). Similar TPA pretreatment also reduces the amount of PKC immunoreactivity in goldfish pituitary cells (Jobin *et al.*, 1993). In this study, previous exposure to TPA blocked the GH responses to subsequent stimulation by TPA and DiC8, but not that of forskolin and 8Br.cAMP, indicating that TPA-induced down-regulation of PKC contents selectively affects the PKC- but not the cAMP-dependent pathway. Although the procedures for PKC desensitization have been shown to reduce GH contents in goldfish pituitary cells (Jobin and Chang, 1993), the presence of distinct GH responses to forskolin and 8Br.cAMP in TPA-pretreated cells strongly argues that the releasable pool of GH was not severely affected by the depletion of PKC. This is also in agreement with the general observation that GH release as a response to stimulation (e.g., sGnRH) only represents a small fraction (<0.5%) of the total cellular GH contents in goldfish pituitary cells (R.M. Jobin, personal communication).

In the goldfish, sGnRH is known to stimulate GH release via the PKC-dependent pathway (Chang *et al.*, 1991). This is confirmed by the present demonstration that PKC desensitization induced by TPA pretreatment blocked the GH responses to sGnRH. However, a similar TPA pretreatment had no effects on the GH responses to DA and the

D1 agonist SKF38393, suggesting that PKC may not be involved in the post-receptor mechanisms of this DA D1 action. This idea is supported by the present findings that SKF38393-stimulated GH release was not affected by the PKC inhibitor H7. Incubation with H7 suppressed the GH releasing actions of the PKC activator DiC8 but not that of the cAMP analog 8Br.cAMP, indicating that H7, at the dose used, is a selective inhibitor for the PKC- but not the cAMP-dependent pathway in the goldfish. The lack of PKC involvement in DA D1-stimulated GH release is also consistent with our demonstrations that the abilities of SKF38393 and DiC8 to induce GH release were additive at the pituitary cell level. Similar additivity was also observed between the GH-releasing actions of the DA agonist apomorphine and TPA. In the goldfish, apomorphine is known to stimulate GH release through activation of DA D1 receptors (Wong *et al.*, 1993b).

The additivity of GH responses to PKC- and DA D1-stimulation also suggests that GH release in the goldfish is mediated by at least two separate post-receptor mechanisms. Previously, we have shown that DA D1-stimulated GH release is mediated primarily by the cAMP-dependent pathway (Wong *et al.*, 1993d; see chapter 6). This is in agreement with the present demonstration that apomorphine failed to enhance GH release in the presence of a maximal stimulatory dose of forskolin. Therefore, we speculate that the GH-releasing action of the cAMP-dependent pathway, which is coupled to DA D1 receptors, acts in parallel with that of the PKC-dependent pathway to stimulate GH release. This hypothesis is supported by our findings that the GH responses to forskolin and 8Br.cAMP were additive to that of the PKC activators TPA and DiC8. Since the involvement of PKC in sGnRH-stimulated GH release has been confirmed in the present study (see earlier discussion), it is conceivable that stimulated GH release from goldfish pituitary cells is mediated by two independent pathways, the cAMP-dependent pathway coupled to DA D1 receptors and the PKC-dependent pathway coupled to sGnRH receptors. This idea is also consistent with our previous findings that the GH-releasing action of DA is independent and additive to that of sGnRH (Wong *et al.*, 1993c; see chapter 4).

Taken together, the present study demonstrated that PKC is not involved in DA D1-stimulated GH release in the goldfish. However, we have confirmed that PKC functions

as a second messenger mediating the GH response to sGnRH. The GH-releasing actions mediated by the cAMP- and PKC-dependent pathways in goldfish pituitary cells were found to be independent and additive to each other. These findings, as a whole, are consistent with our previous hypothesis (Wong *et al.*, 1993c; see chapter 4) that the independent actions of DA and sGnRH to stimulate GH release in the goldfish can be explained by two separate post-receptor mechanisms.

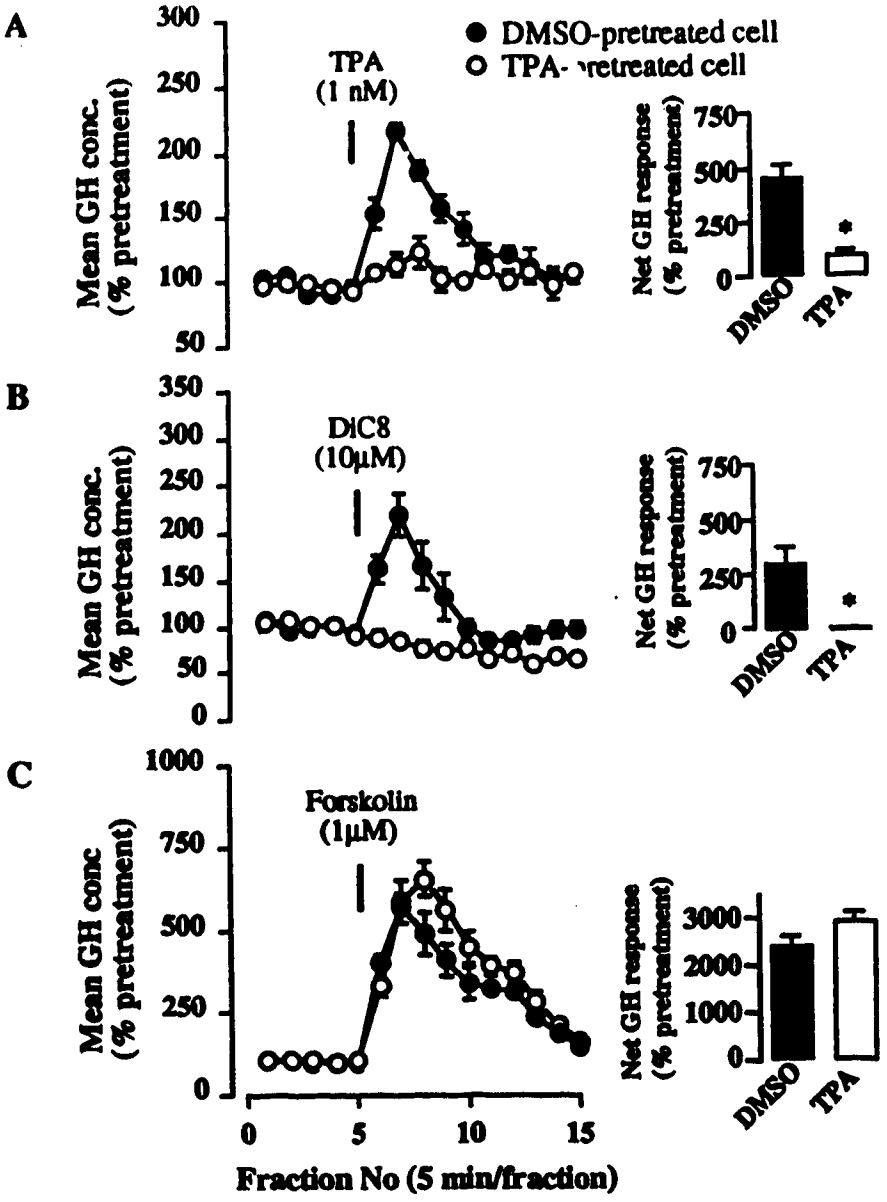


Fig. 8.1 Effects of PKC desensitization on the GH responses to 5-min pulses of TPA (A), DiC8 (B), and forskolin (C) using perifused goldfish pituitary cells. PKC desensitization was performed by pretreating the pituitary cells with 10 nM TPA for 4 hours. Similar treatment with 0.1 % DMSO was used as the control. GH data are expressed as % pretreatment, and pooled from four separate perifusion columns (mean ± SEM, n = 4). The average pretreatment GH levels for the TPA- and DMSO-pretreated groups were 12.0 ± 0.6 and 13.5 ± 0.6 ng/ml, respectively. The insets on the right represent the net GH responses, quantitated as area under the curve, after a particular drug treatment. (* : $P<0.05$, by Student's t test)

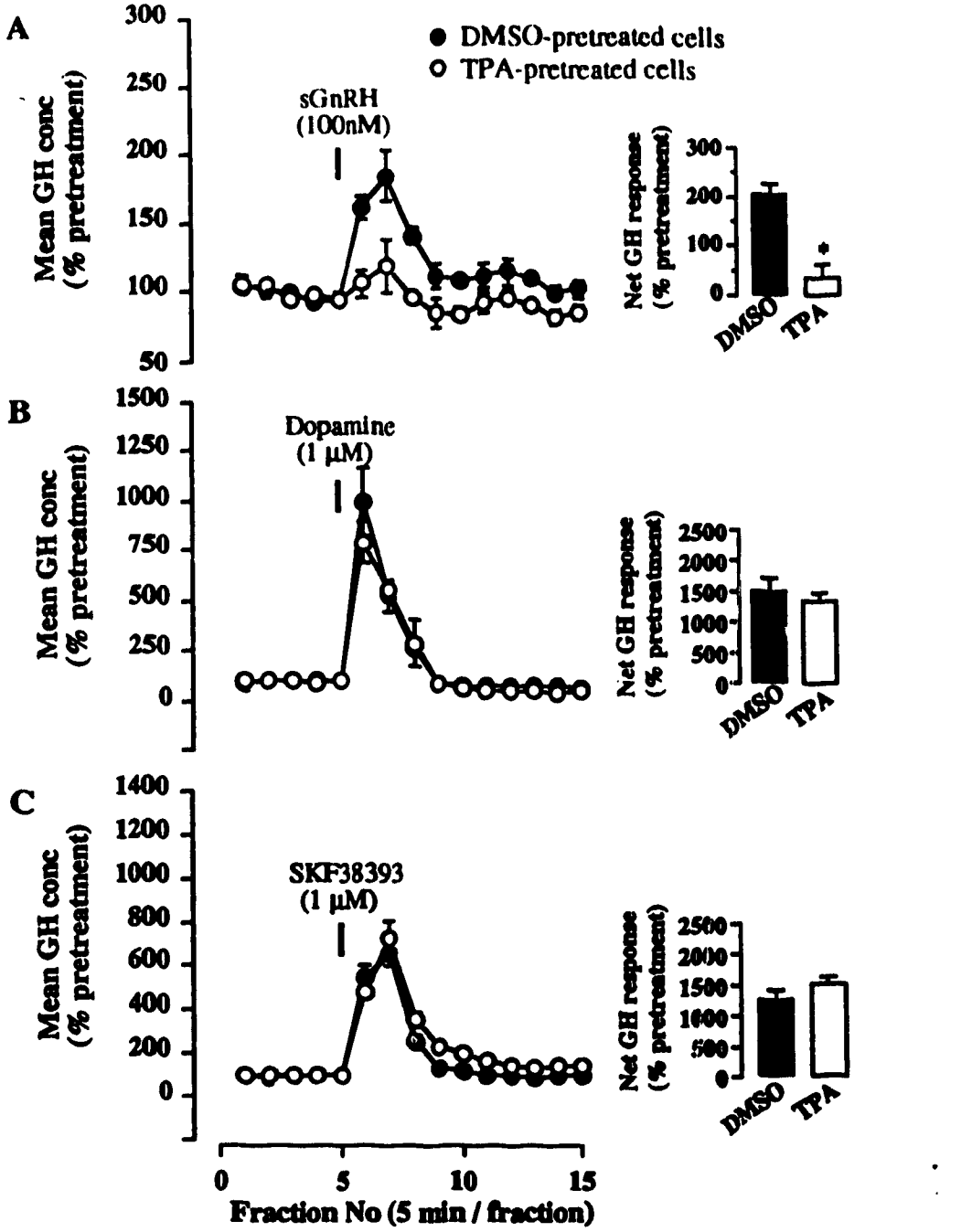
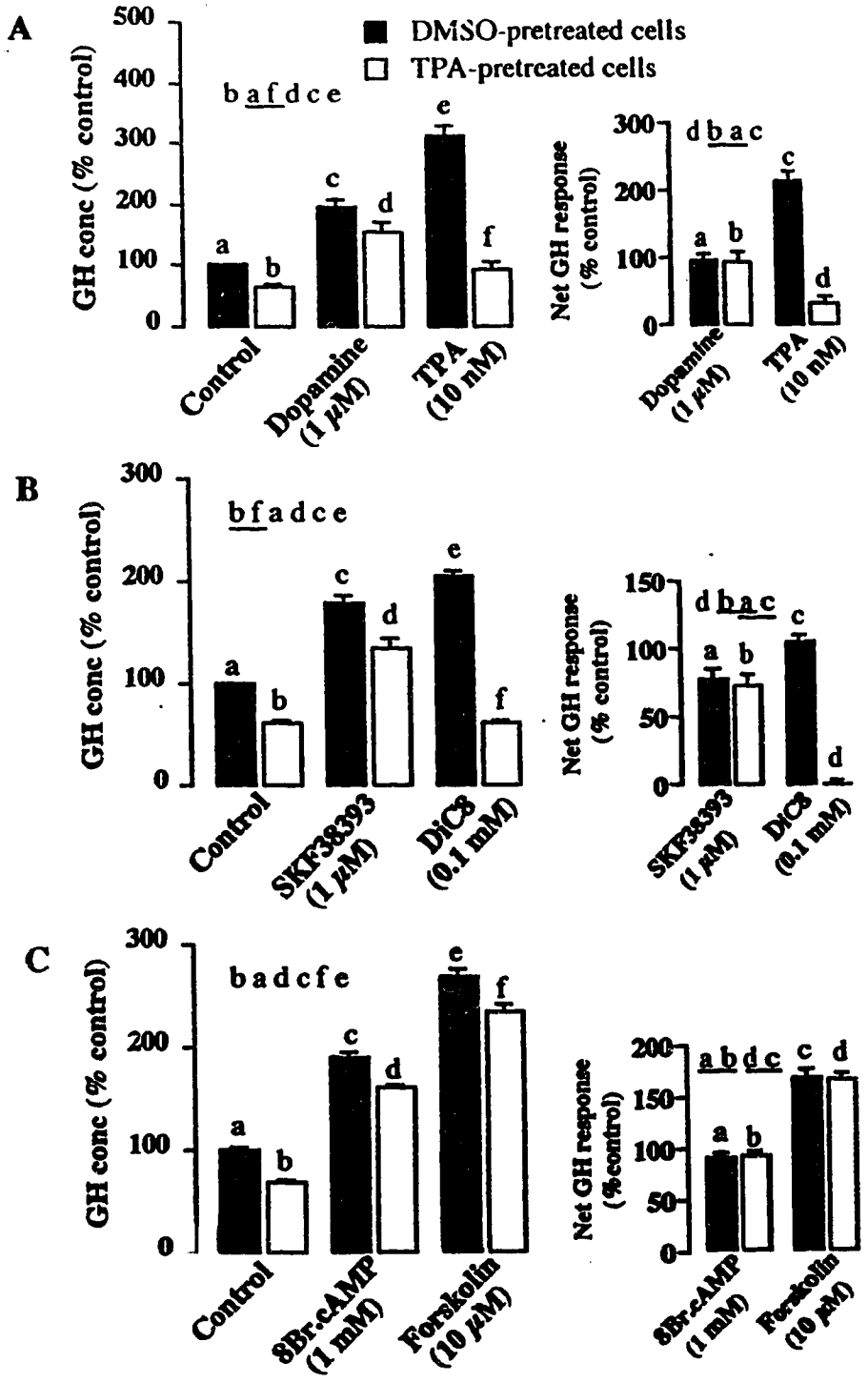


Fig. 8.2 Effects of PKC desensitization on the GH responses to 5-min pulses of sGnRH (A), dopamine (B), and the D1 agonist SKF38393 (C) using perifused goldfish pituitary cells. PKC desensitization was performed by pretreating the pituitary cells with 10 nM TPA for 4 hours. Similar treatment with 0.1 % DMSO was used as the control. GH data are expressed as % pretreatment, and pooled from four separate perifusion columns (mean ± SEM, n = 4). The average pretreatment GH levels for the TPA- and DMSO-pretreated groups were 6.6 ± 0.6 and 6.8 ± 0.3 ng/ml, respectively. The insets on the right represent the net GH responses, quantitated as area under the curve, after a particular drug treatment. (* : $P<0.05$, by Student's t test)

Fig. 8.3 PKC desensitization on the GH responses to dopamine and TPA (A), SKF38393 and DiC8 (B), and 8Br.cAMP and forskolin (C) using goldfish pituitary cells under static incubation. PKC desensitization was performed by pretreating the pituitary cells with 10 nM TPA for 4 hours. Similar treatment with 0.1 % DMSO was used as the control. GH data are expressed as % control, and pooled from three separate experiments (mean ± SEM, n = 12). The average GH levels in the "control wells" for the TPA- and DMSO-pretreated groups were 359 ± 28 and 504 ± 53 ng/ml, respectively. The insets on the right represent the net GH responses to drug treatments, quantified as the difference of the GH levels between the treatment group vs that of the corresponding "control" group. Drug treatments giving a similar magnitude of GH responses are grouped within the same underscore ($P>0.05$, ANOVA followed by Fisher's LSD test).


A
DMSO-pretreated cells
TPA-pretreated cells
GH conc (% control)
Net GH response (% control)
Control
Dopamine (1 μM)
TPA (10 nM)
B
SKF38393 (1 μM)
DiC8 (0.1 mM)
C
8Br-cAMP (1 mM)
Forskolin (10 μM)

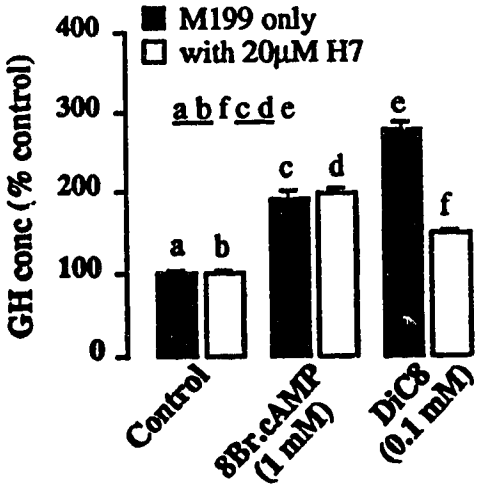


Fig. 8.4 Effects of the PKC inhibitor H7 on the GH responses to 8Br.cAMP and DiC8 using goldfish pituitary cells under static incubation. The GH data presented were transformed in % control as described in Materials & Methods, and are the pooled results from three separate experiments (mean ± SEM, n = 12). The average GH level in the control wells was 461 ± 20 ng/ml. Drug treatments giving a similar magnitude of GH responses are grouped within the same underscore (P>0.05, ANOVA followed by Fisher's LSD test).

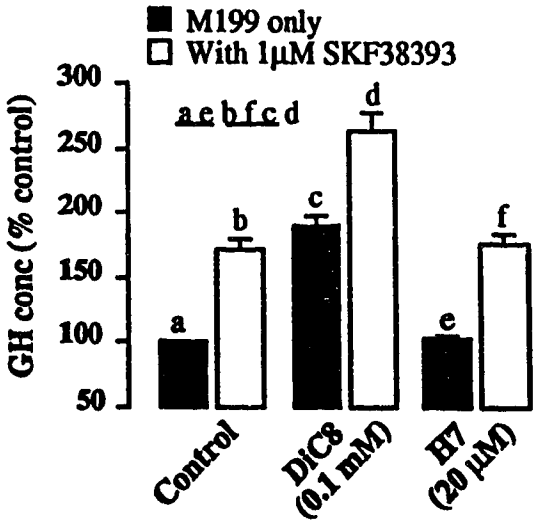


Fig. 8.5 Effects of DiC8 and H7 on the GH response to SKF38393 using goldfish pituitary cells enriched with somatotrophs under static incubation. GH data are expressed as % control, and pooled from three separate experiments (mean ± SE = 12). The average GH level in the control wells was 471 ± 58 ng. Drug treatments giving a similar magnitude of GH responses are grouped within the same underscore ($P>0.05$, ANOVA followed by Fisher's LSD test).

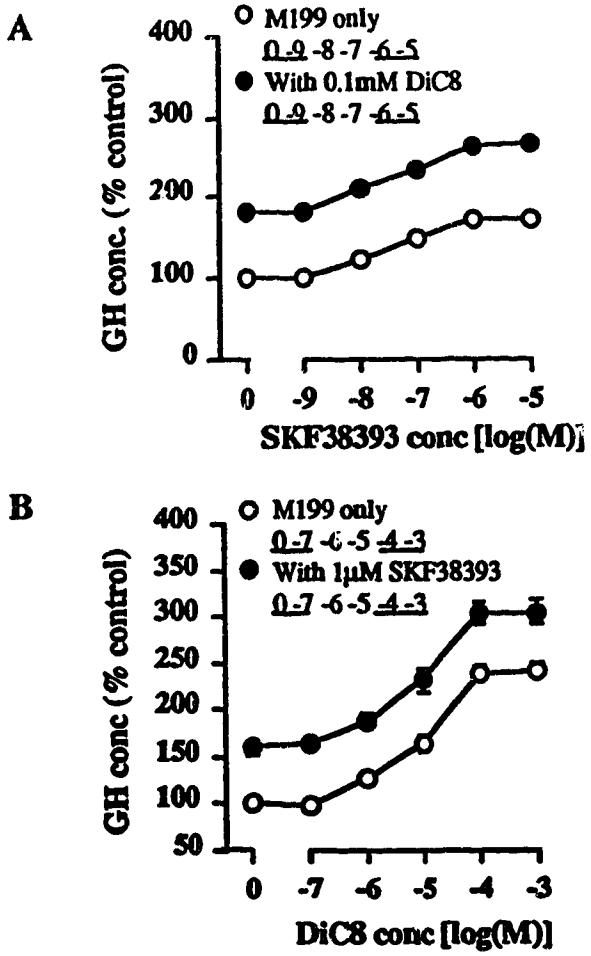


Fig. 8.6 Additivity of the D1 agonist SKF38393 and the PKC activator DiC8 on GH release from goldfish pituitary cells under static incubation. (A) The GH responses to increasing doses of SKF38393 (1 nM - 10 μM) were tested in the presence or absence of 0.1 mM DiC8. (B) The GH responses to increasing doses of DiC8 (0.1 μM to 1 mM) were tested in the presence or absence of 1 μM SKF38393. The average GH levels in the control wells of these two experiments were 702 ± 41 and 678 ± 45 ng/ml, respectively. GH data are expressed as % control, and pooled from four separate experiments (mean ± SEM, n = 16). Drug treatments giving a similar magnitude of GH responses are grouped within the same underscore (P>0.05, ANOVA followed by Fisher's LSD test).

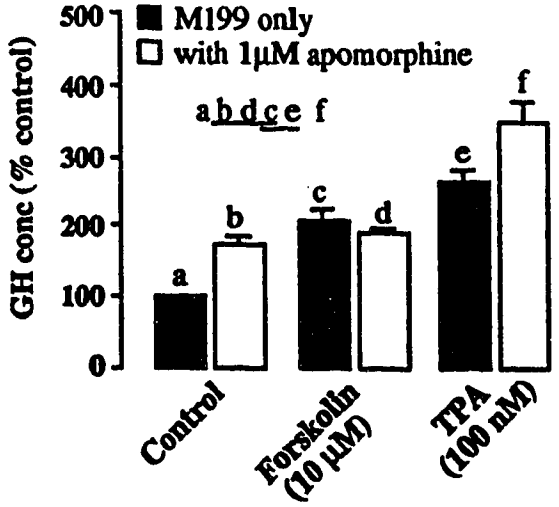


Fig. 8.7 Effects of forskolin and TPA on the GH response to the non-selective DA agonist apomorphine using goldfish pituitary cells under static incubation. The GH data presented were transformed into % control as described in Materials & Methods, and are the pooled results from three separate experiments (mean ± SEM, n = 12). The average GH level in the control wells was 609 ± 46 ng GH/ml. Drug treatments giving a similar magnitude of GH responses are grouped within the same underscore (P>0.05, ANOVA followed by Fisher's LSD test).

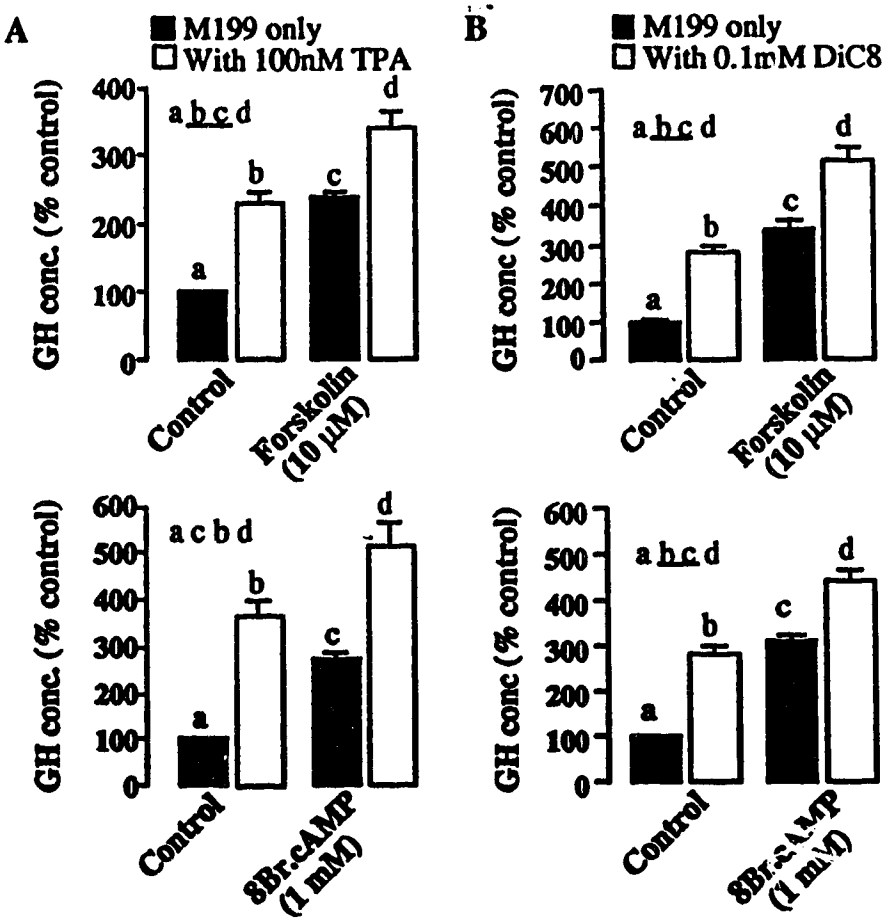


Fig. 8.8 Effects of TPA (A) and DiC8 (B) on the GH responses to forskolin (upper panels) and 8Br.cAMP (lower panels) using goldfish pituitary cells under static incubation. GH data are expressed as % control, and pooled from three separate experiments (mean ± SEM, n = 12). The average GH level in the control wells was 671 ± 42 ng/ml. Drug treatments giving a similar magnitude of GH responses are grouped within the same underscore (P>0.05, ANOVA followed by Fisher's LSD test).

8.5 References

Chang JP, Cook H, Freedman GL, Wiggs AJ, Somoza GM, de Leeuw R, Peter RE 1990a Use of a pituitary cell dispersion method and primary culture system for the studies of gonadotropin-releasing hormone action in the goldfish, *Carassius auratus*. I. Initial morphological, static, and cell column perifusion studies. Gen Comp Endocrinol 77:256-273

Chang JP, Jobin RM 1992 Teleost pituitary cells: Isolation, culture, and use. In : Hochachka PW, Mommsen TP (eds) Biochemistry and Molecular Biology of Fishes (vol.3) : Analytical Techniques. Elsevier, Amsterdam (in press)

Chang JP, Jobin RM, de Leeuw R 1991 Possible involvement of protein kinase C in gonadotropin and growth hormone release from dispersed goldfish pituitary cells. Gen Comp Endocrinol 81:447-463

Chang JP, Yu KL, Wong AOL, Peter RE 1990b Differential actions of dopamine receptor subtypes on gonadotropin and growth hormone release *in vitro* in goldfish. Neuroendocrinology 51:664-674

de Leeuw R, Goos HJT, Peute J, Van Pelt AAM, Burzawa-Gerard E, Van Oordt PGWJ 1984 Isolation of gonadotrops from the pituitary of the African catfish, *Clarias lazera*. Morphological and physiological characterization of purified cells. Cell Tissue Res 236:669-675

French MB, Moor BC, Lussier BT, Kraicer J 1989 Protein kinase C is not essential for growth hormone (GH)-releasing factor-induced GH release from rat somatotrophs. Endocrinology 124:2235-2244

French MB, Moor BC, Lussier BT, Kraicer J 1991 Growth hormone-releasing factor does not activate protein kinase C in somatotrophs. Mol Cell Endocrinol 79:139-146

Jobin RM, Chang JP 1993 Involvement of protein kinase C in the modulation of gonadotropin and growth hormone secretion from dispersed goldfish pituitary cells. Fish Physiol Biochem (in press)

Jobin RM, Ginsberg J, Matowe WC, Chang JP 1993 Down regulation of PKC levels leads to inhibition of GnRH-stimulated gonadotropin secretion from dispersed

pituitary cells of goldfish. Neuroendocrinology (in press)

Marchant TA, Chang JP, Nahorniak CS, Peter RE 1989a Evidence that gonadotropin-releasing hormone also functions as a growth hormone-releasing factor in the goldfish. Endocrinology 124:2509-2518

Marchant TA, Dulka JG, Peter RE 1989b Relationship between serum growth hormone levels and the brain and pituitary content of immunoreactive somatostatin in the goldfish, *Carassius auratus*. Gen Comp Endocrinol 73:458-468

Marchant TA, Fraser RA, Andrews PC, Peter RE 1987 The influence of mammalian and teleost somatostatins on the secretion of growth hormone from goldfish (*Carassius auratus L*) pituitary fragments *in vitro*. Regul Pept 17:41-52

McArdle CA, Conn PM 1989 Use of protein kinase C-depleted cells for investigation of the role of protein kinase C in stimulus-response coupling in the pituitary. In : Conn PM (ed) Methods in Enzymology, Academic Press, Inc., pp 287-301

Negro-Vilar A, Lapetina EG 1985 1,2-didecanoylglycerol and phorbol 12,13-dibutyrate enhance anterior pituitary hormone secretion *in vitro*. Endocrinology 117:1559-1564

Nishioka RS, Kelly KM, Bern HA 1988 Control of prolactin and growth hormone secretion in teleost fish. Zool Sci 5:267-278

Wong AOL, Chang JP, Peter RE 1992 Dopamine stimulates growth hormone release from the pituitary of goldfish, *Carassius auratus*, through the dopamine D1 receptors. Endocrinology 130:1201-1210

Wong AOL, Chang JP, Peter RE 1993a Dopamine functions as a growth hormone-releasing factor in the goldfish, *Carassius auratus*. Fish Physiol Biochem (in press)

Wong AOL, Chang JP, Peter RE 1993b *In vitro* and *in vivo* evidence that dopamine exerts growth hormone releasing activity in the goldfish. Am J Physiol (in press)

Wong AOL, Chang JP, Peter RE 1993c Interactions of somatostatin, gonadotropin-releasing hormone, and the gonads on dopamine-stimulated growth hormone release in the goldfish. (submitted to Gen Comp Endocrinol)

Wong AOL, Van Der Kraak G, Chang JP 1993d Cyclic 3':5'-adenosine monophosphate (cAMP) mediates dopamine D1-stimulated growth hormone release

from goldfish pituitary cells. (submitted to Endocrinology)

Wong AOL, Chang JP 1993 Entry of extracellular calcium mediates dopamine D1-stimulated growth hormone release from goldfish pituitary cells. (submitted to Endocrinology)

Chapter 9

General Discussion

In the present chapter, the major findings of this thesis are summarized. One of the novel observations of our current studies is the involvement of dopamine (DA) D1 receptors in the mediation of DA-stimulated growth hormone (GH) release from the goldfish pituitary. Using an *in vitro* perifusion system for goldfish pituitary fragments, we have provided extensive pharmacological evidence (see chapter 2) that DA stimulates GH release from the goldfish pituitary in a dose-dependent manner, and that this stimulatory action of DA can be mimicked by the D1 agonists SKF38393, SKF77434 and SKF82958, but not the D2 agonists bromocriptine and LY171555. The GH responses to DA and the D1 agonist SKF38393 can be blocked by the D1 antagonists SCH23390 and SKF83566; whereas the D2 antagonists domperidone and (-)sulpiride are not effective in this regard. A similar D1 specificity for DA-stimulated GH release in the goldfish was also demonstrated by *in vivo* studies using intraperitoneal (ip) injection of the non-selective DA agonist apomorphine and the D1 agonist SKF82958 (see chapter 3).

The stimulatory actions of DA on GH release in the goldfish are exerted directly at the pituitary cell level. This is supported by our findings that DA and the D1 agonist SKF38393, but not the D2 agonist LY171555, stimulate GH release from mixed populations of pituitary cells (see chapters 3, 5, 6 and 7) and cell preparations enriched with somatotrophs (see chapters 5 and 6). In the goldfish, DA is known to be a gonadotropin (GTH)-release inhibitor (see chapters 2, 3, and 4; for reviews, see Peter *et al.*, 1986; Peter *et al.*, 1990). Its inhibitory actions on basal and GTH-releasing hormone (GnRH)-stimulated GTH-II release are mediated through DA D2 receptors in the pituitary (Omeljaniuk *et al.*, 1987; Omeljaniuk *et al.*, 1989). The present demonstration of DA D1-stimulated GH release from goldfish pituitary cells provides a unique model for neuroendocrine regulation in vertebrates. In this model, the neurotransmitter DA differentially regulates the release of two distinct hormones, GH and GTH-II, in the same location (i.e., the anterior pituitary of the goldfish) through two

different receptors, the D1 and D2 receptors, respectively.

Previously, a preoptico-hypophyseal dopaminergic pathway has been identified in the goldfish (Kah *et al.*, 1986; Kah *et al.*, 1987), and DA nerve terminals are frequently recognized in close proximity to the somatotrophs (Kah *et al.*, 1984). These observations provide anatomical evidence for the involvement of DA in GH regulation of the goldfish. In the present study, the role of DA as a physiological GH-releasing factor in the goldfish was confirmed, for the first time, using both *in vivo* and *in vitro* approaches. Firstly, we have demonstrated that DA-stimulated GH release in the goldfish exhibits a distinct pattern of seasonality (see chapter 3 and Appendix I). In our *in vitro* perifusion studies, the sensitivity and responsiveness of goldfish pituitary fragments to DA stimulation were the highest in sexually regressed fish, intermediate in recrudescent fish, and the lowest in prespawning (or sexually mature) fish. Secondly, administration (ip) of the D1 antagonist SCH23390 suppressed the plasma GH levels in goldfish undergoing gonadal recrudescence (see chapter 3). These results indicate that DA D1 receptors are involved in the maintenence of basal GH release, at least during the period of sexual recrudescence. This is consistent with the previous findings that serum GH levels in the goldfish can be suppressed by treatment (ip) with the catecholaminergic neurotoxin, 6-hydroxydopamine, and the catecholamine synthesis inhibitor, α-methyl-*p*-tyrosine (Chang *et al.*, 1985). Thirdly, feeding goldfish in gonadal recrudescence with trout pellets pretreated with apomorphine increased plasma GH levels and the rate of body growth (see chapter 3). Using repeated injections (ip) of apomorphine (see Appendix I), similar results on GH release and the linear growth rate were also observed in goldfish undergoing gonadal regression. These observations are in agreement with our hypothesis that DA functions as a GH-releasing factor in the goldfish.

The physiological role of DA as a GH-releasing factor is further substantiated by studying the interactions of DA with other GH regulators in the goldfish. SRIF is known to inhibit basal and stimulated GH release in the goldfish, both *in vivo* (Cook and Peter, 1984; Marchant *et al.*, 1989b) and *in vitro* (Marchant *et al.*, 1987; Marchant *et al.*, 1989b). In the present study, the GH responses to DA, the DA agonist apomorphine, and the D1 agonist SKF38393 were blocked by simultaneous treatment with SRIF (see chapters 3, 4 and 5). This inhibitory action of SRIF was exerted directly at the pituitary

cell level, as SRIF attenuated both basal and DA-stimulated GH release from mixed populations of pituitary cells (see chapter 3) and pituitary cell preparations enriched with somatotrophs (see chapter 5). Besides SRIF, the neurotransmitters serotonin (5HT) and norepinephrine (NE) are also capable of inhibiting GH release from the goldfish pituitary (Chang *et al.*, 1985; Peter *et al.*, 1990; Somoza and Peter, 1991). Using perifused goldfish pituitary fragments and pituitary cells, we have demonstrated that 5HT and NE suppressed basal GH release and the GH responses to DA and the D1 agonist SKF38393 (see Appendix II), indicating that the inhibitory actions of 5HT and NE are exerted directly at the pituitary cell level. NE appears to be more effective than 5HT in inhibiting DA D1-stimulated GH release; NE at 5 μM dose completely abolished, whereas a similar dose of 5HT only partially reduced the GH responses to 1 μM SKF38393 (Appendix II).

Salmon GnRH (sGnRH) is stimulatory to GH release in the goldfish (Marchant *et al.*, 1989b; Habibi *et al.*, 1992). The GH response to sGnRH is also seasonal, being the highest in sexually mature fish, intermediate in recrudescent fish, and the lowest in sexually regressed fish (see Appendix III). In the current study, the GH-releasing actions of sGnRH and DA were found to be independent and additive to each other (see chapter 4). Our *in vitro* perifusion studies also indicate that the GH responses to DA and sGnRH are under the modulation of some unknown gonadal factors. In prespawning (or sexually mature) goldfish, the magnitude of GH responses to DA stimulation was significantly enhanced in both male and female fish after surgical removal of the gonads. However, the reverse was true for the GH responses to sGnRH (see chapter 4). It is likely that the gonads of goldfish, probably by the release of gonadal factors, exert differential actions on the GH-releasing effects of sGnRH and DA, being stimulatory to sGnRH and inhibitory to DA, respectively. This hypothesis is also consistent with the seasonality of DA- (see chapter 3) and sGnRH-stimulated GH release observed in the goldfish (see Appendix III). Taken together, the specific pattern of interactions between DA and other GH regulators in the goldfish, including SRIF, sGnRH, 5HT, NE and gonadal factor(s), stongly suggests that DA is an integral part of the complex GH neuroendocrine regulatory system.

Besides DA and sGnRH, other neuroendocrine factors, such as neuropeptide Y (NPY) and thyrotropin-releasing hormone (TRH), have been demonstrated to have GH-

releasing actions in the goldfish. NPY is more effective in stimulating GH release in sexually mature (=prespawning) goldfish, and is less effective in fish undergoing gonadal regression (Peng *et al.*, 1990). TRH stimulates GH release in both sexually mature and regressed goldfish, although the sensitivity to TRH stimulation is higher in sexually mature goldfish (Trudeau *et al.*, 1992). More recently, a GH-releasing hormone (GHRH) has been isolated from the common carp, and found to have GH-releasing activity in the goldfish (Vaughan *et al.*, 1992). This carp GHRH stimulates GH release in goldfish undergoing gonadal regression; however, it is not effective in stimulating GH release in goldfish undergoing gonadal recrudescence or maturation (C. Peng and R.E. Peter, unpublished results). Probably, these neuroendocrine factors, together with DA, sGnRH, SRIF and gonadal factors, interact in a time-specific manner throughout the reproductive cycle to regulate GH secretion in the goldfish (Fig. 9.1).

In the goldfish, serum GH levels increase during gonadal recrudescence in early spring, reach high levels throughout the spawning and post-spawning season from spring to early summer, and gradually return to low levels in late summer when the fish become sexually regressed (Marchant and Peter, 1986). SRIF contents in the hypothalamus, however, exhibit an inverse relationship with respect to the serum GH levels, being the highest in sexually regressed fish and the lowest in sexually mature (=prespawning) fish (Marchant *et al.*, 1989a). During the winter months, when the gonads are regressed, the goldfish pituitary is sensitive to the stimulation of DA, sGnRH, GHRH, and TRH. However, serum GH levels are relatively low, presumably because of a high SRIF inhibitory tone from the hypothalamus. Since the GH-releasing effects of DA and GHRH are the highest in sexually regressed goldfish (Wong *et al.*, 1993a,b; C. Peng and R.E. Peter, unpublished results), it is likely that these two GH-releasing factors are relatively important in maintaining the basal GH levels at this time. During the period of gonadal recrudescence (late winter to early spring), the combined effects of a high responsiveness to DA stimulation and a concurrent drop in SRIF inhibitory tone may be involved in the rise of serum GH levels observed in the goldfish at this stage. This is consistent with our previous findings that plasma GH levels in sexually recrudescent goldfish could be suppressed by the D1 antagonist SCH23390 (Wong *et al.*, 1993a; see

chapter 3). TRH may also contribute to this increase in GH levels, as the sensitivity of the goldfish pituitary to TRH stimulation is gradually increasing when the gonads become more sexually mature (Trudeau *et al.*, 1992). During the spawning season (from mid spring to early summer), when the goldfish is in the prespawning stages, the predominant role of DA as a GH-releasing factor is presumably replaced by sGnRH, NPY and TRH. This switch in functional predominance is under the influence of the gonads, which potentiates the GH-releasing effects of sGnRH, NPY and TRH, and at the same time attenuates the stimulatory actions of DA. These changes happen at the time when SRIF levels in the hypothalamus are low, and therefore high serum GH levels are observed. Since the magnitude of GH responses to TRH is in general smaller than that of sGnRH and NPY (Trudeau *et al.*, 1992; Peng *et al.*, 1990; Marchant *et al.*, 1989a), it is likely that sGnRH and NPY are the major factors maintaining the high GH levels found during the spawning season. In the late summer and autumn months, a build up of SRIF inhibitory tone in the hypothalamus, together with the decrease in gonadal influence due to the regression of post-spawning gonads, gradually returns the serum GH concentrations to a relatively low level.

The proposed model (Fig. 9.1) is intended to serve as a working model for future investigations of GH seasonality in the goldfish. In this model, two particular aspects have not been addressed. Firstly, the gonadal factor(s) responsible for the differential modulation of the GH-releasing activities by DA, TRH, NPY and sGnRH has not been included. In the goldfish, implantation with estradiol is known to increase basal GH levels (Trudeau *et al.*, 1992) and enhance the GH responses to NPY (C. Peng, personal communication), sGnRH and TRH (Trudeau *et al.*, 1992). Elevation of serum estradiol, testosterone and 17α,20β-dihydroxy-4-pregnen-3-one levels has been reported in sexually mature goldfish (Kobayashi *et al.*, 1986; Kobayashi *et al.*, 1987), and this increase of steroid levels occurs at a time when the GnRH receptor capacity in the pituitary is the highest (Habibi *et al.*, 1989). Whether sex steroids are the causative agents for the switch from DA to TRH, NPY and sGnRH as the major GH-releasing factors during the prespawning period still needs to be confirmed. Secondly, the temporal relationship of TRH, NPY and sGnRH as the functional GH-releasing factors during the spawning season has not been discussed in the model. In the goldfish,

elevation of serum estradiol levels occurs prior to that of testosterone during the prespawning period (Kobayashi *et al.*, 1986). In contrast to the potentiating effects of estradiol, testosterone is not effective in enhancing the GH responses to sGnRH and TRH (Trudeau *et al.*, 1992). However, testosterone implantation is effective in potentiating the GH responses to NPY (C. Peng, personal communication). Although the model proposed (Fig. 9.1) has been simplified by assuming that these 3 neuropeptides have a similar time course of actions on GH release throughout the reproductive cycle, we do not exclude the possibility that each of them may operate in an independent and time-specific manner during the spawning season. Although serotonin (Somoza and Peter, 1991) and norepinephrine (Chang *et al.*, 1985; Peter *et al.*, 1990) are known to inhibit GH secretion in the goldfish, the seasonality of these two factors on GH release has not been studied, and therefore, they are not included in the proposed model.

In the current study, we have demonstrated, for the first time, the presence of DA D1 receptor binding sites in the goldfish pituitary (see chapter 5). This is a novel finding in the field of comparative endocrinology, as DA D1 receptors have not previously been identified in the pituitary of vertebrates. In the goldfish, the distribution of D1 receptors overlaps with that of somatotrophs in the pars distalis (see chapter 5), and activation of D1 receptors stimulates GH release from pituitary fragments (see chapters 2, 3 and 4), mixed populations of pituitary cells (see chapters 3, 4 and 5), and cell preparations enriched with somatotrophs (see chapters 5 and 6). These results indicate that DA D1 receptors are present in the somatotrophs and mediate DA-stimulated GH release in the goldfish. Furthermore, the goldfish D1 receptors exhibit a binding affinity, stereoselectivity and rank order of ligand specificity comparable to that of the mammalian D1 systems, suggesting that DA D1 receptors are highly conserved during vertebrate evolution (for a review on the pharmacology of mammalian D1 receptors, see Neumeyer *et al.*, 1992).

The signal transduction mechanism involved in DA D1-stimulated GH release was also examined using dispersed goldfish pituitary cells. In mammals, especially in the rat, DA D1 receptors are known to be positively coupled with the cAMP-dependent pathway (Battaglia *et al.*, 1986; Monsma *et al.*, 1990). In the goldfish, the D1 agonist

SKF38393, but not the D2 agonist LY171555 stimulated GH and cAMP release from cell preparations enriched with somatotrophs (see chapter 6). Moreover, the GH-releasing actions of DA and SKF38393 could be mimicked by the adenylate cyclase activator forskolin, the phosphodiesterase inhibitor IBMX, as well as the membrane permeant cAMP analogs. These results indicate that an increase in the intracellular cAMP levels can initiate GH exocytosis from goldfish pituitary cells (see chapter 6). This is also supported by our findings that GH release could be induced by activating adenylate cyclase indirectly via the G proteins using pertussis toxin and cholera toxin (see chapter 6). Furthermore, the GH responses to the D1 agonist SKF38393 were abolished by the protein kinase A (PKA) inhibitor H89 as well as by saturating the cAMP-dependent pathway with the cAMP analog 8Br.cAMP (see chapter 6). These observations provide strong evidence that DA D1-stimulated GH release in the goldfish is mediated by the adenylate cyclase-cAMP-PKA pathway.

In the current study, the presence of a Ca^{2+}-dependent component in DA D1-stimulated GH release has been demonstrated. The GH-releasing actions of DA and the D1 agonist SKF38393 were completely abolished in a Ca^{2+}-deficient medium, indicating that the presence of extracellular Ca^{2+} is essential for DA D1-stimulated GH release (see chapter 7). Inducing Ca^{2+} entry by using the Ca^{2+} ionophore A23187, the voltage-sensitive Ca^{2+} channel (VSCC) agonist Bay K8644, and by activating VSCC directly using a high, depolarizing dose of K^+, are all stimulatory to GH release from goldfish pituitary cells (see chapter 7). This is consistent with the previous findings by Chang and de Leeuw (1990) that the entry of extracellular Ca^{2+} is a part of the second messenger system mediating GH secretion in the goldfish. The involvement of VSCC in DA D1 actions in the goldfish has also been indicated in the present study. The GH responses to DA and SKF38393 could be blocked by the VSCC blockers, such as nifedipine, nicardipine and verapamil, as well as by a high dose of $CoCl_2$, which is a known competitive inhibitor for Ca^{2+} entry through VSCC (see chapter 7). This is in agreement with the recent finding that SKF38393 stimulates an increase in intracellular Ca^{2+} levels in goldfish pituitary cells preloaded with Fura-2 (Chang *et al.*, 1993). In mammals, especially in the rat, interactions between the cAMP- and Ca^{2+}-dependent pathways in the

regulation of GH release have been reported (Narayanan *et al.*, 1989; Lussier *et al.*, 1990a,b,c). In the goldfish, it has been shown that the GH responses to forskolin and 8Br.cAMP could be suppressed by incubation with Ca^{2+}-deficient medium or by the VSCC blocker nifedipine (see chapter 7). These results strongly indicate that the entry of extracellular Ca^{2+} through VSCC is a part of the signal transduction mechanism mediating DA D1-stimulated GH release in the goldfish. Probably, this Ca^{2+} influx takes place after the activation of the cAMP-dependent pathway.

The possible involvement of the Ca^{2+}- and phospholipid-dependent enzyme protein kinase C (PKC) in DA D1-stimulated GH release was also investigated in the goldfish. Depleting endogenous PKC contents in goldfish pituitary cells by pretreatment with the phorbol ester TPA (Jobin *et al.*, 1993) significantly suppressed and totally abolished the GH responses to subsequent challenges by TPA and the diacylglycerol analog DiC8, respectively. However, a similar TPA pretreatment had no effects on the net GH responses to DA and the D1 agonist SKF38393 (see chapter 8). In the present study, the PKC inhibitor H7 was effective in blocking the GH releasing actions of the PKC activator DiC8, but not the GH responses to SKF38393. Furthermore, the GH-releasing actions of DiC8 were found to be additive to that of DA and SKF38393 (see chapter 8). These results, taken together, suggest that the PKC-dependent pathway is not a part of the second messenger system mediating DA D1-stimulated GH release in the goldfish.

The present study provides strong evidence that the second messenger cAMP, but not PKC, mediates the GH responses to DA D1-stimulation in the goldfish (see chapters 6 and 8). The additive effects of DiC8 and the D1 agonist SKF38393 on GH release (see chapter 8) also suggest that the cAMP- and PKC-dependent pathways may act independently in mediating GH secretion from the goldfish pituitary. This hypothesis is confirmed by the findings that the GH-releasing actions of forskolin and 8Br.cAMP, two known activators for the cAMP-dependent pathway, were additive to that of the PKC activators TPA and DiC8 (see chapter 8). Furthermore, the net GH responses to forskolin and 8Br.cAMP were not affected by TPA-induced PKC desensitization in goldfish pituitary cells (see chapter 8). This is in agreement with the recent report by Jobin and Chang (1993) that the GH-releasing actions of forskolin can not be reduced by depleting the PKC contents of goldfish pituitary cells.

In the current study, consistent with the previous findings by Chang *et al.* (1991a), TPA and DiC8 were found to be stimulatory to GH release from goldfish pituitary cells (see chapter 8). Although PKC is not a second messenger for DA D1 actions, these results suggest that PKC may be involved in the GH responses of another GH-releasing factor(s). One possible candidate for such a GH-releasing factor in the goldfish is GnRH. Two forms of GnRHs, namely sGnRH and chicken GnRH-II (cGnRH-II), have been identified in the hypothalamo-pituitary axis of the goldfish (Yu *et al.*, 1988). Both of them are stimulatory to GH release (Marchant *et al.*, 1989b; Habibi *et al.*, 1992) and competes for the same GnRH binding sites in goldfish somatotrophs (Cook *et al.*, 1991). In the present study, the GH-releasing actions of sGnRH were found to be independent and additive to that of DA (see chapter 4). Previously, it has been shown that the GH responses to prolonged stimulation (2 hr) of sGnRH can be blocked by the PKC inhibitor H7 (Chang *et al.*, 1991a) and by TPA-induced PKC down-regulation (Jobin and Chang, 1993). Similarly, in this study, the acute GH response to short-term stimulation (5 min) of sGnRH was abolished by prior depletion of cellular PKC contents (see chapter 8), confirming that PKC mediates sGnRH-stimulated GH release in the goldfish. Interestingly, our recent experiments have shown that sGnRH stimulation does not alter cAMP production in goldfish pituitary cells (Chang *et al.*, 1992), indicating that the cAMP-dependent pathway is not involved in the signal transduction mechanism of sGnRH. Based on these observations, a model (Fig. 9.2) is proposed in this chapter to describe the interactions of cAMP, PKC, and Ca^{2+} in mediating DA D1- and sGnRH-stimulated GH release in the goldfish.

In this model, GH release from goldfish somatotrophs can be mediated by at least two parallel pathways, the cAMP-dependent pathway coupled to DA D1 receptors and the PKC-dependent pathway coupled to GnRH receptors, as well as an interactive Ca^{2+} entry component mediating the GH-releasing actions of cAMP and PKC. DA stimulates cAMP synthesis by activating adenylate cyclase through D1 receptors. The coupling between D1 receptors and adenylate cyclase is probably mediated via the G proteins, since the bacteriotoxins, cholera toxin and pertussis toxin, were effective in stimulating GH release in the present study (see chapter 6). The subsequent stimulation of the cAMP-dependent enzyme PKA activates VSCC, allowing the entry of extracellular Ca^{2+}

into the somatotrophs to induce GH release. This accounts for both the cAMP- and Ca^{2+}-dependence of DA D1 action on GH release reported in the present study (see chapters 6 and 7). This is also consistent with the recent findings that cyclic nucleotides, including cAMP, can activate membrane ion channels by protein phosphorylation through the actions of protein kinases (for review, see Matthews, 1991). In our *in vitro* perifusion study using enriched somatotrophs from the goldfish, a high dose (10 μM) of the VSCC blocker nifedipine could not completely abolish the GH responses to the D1 agonist SKF38393 (see chapter 7). In contrast, the GH-releasing actions of SKF38393 were blocked totally by the PKA inhibitor H89 (see chapter 6). These observations suggest that PKA may also have direct stimulatory effects on GH release which are independent of its actions on VSCC.

sGnRH, on the other hand, activates PKC via the production of diacylglycerol. This is probably mediated through the activation of phospholipase C (PLC) coupled to the G proteins. In mammals, especially in the rat, it is well documented that PLC-induced hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce diacylglycerol and inositol 1,4,5-trisphosphate is essential for GnRH-stimulated LH release (for review, see Catt *et al.*, 1991). In the goldfish, GH exocytosis as a result of PKC activation is also mediated, at least in part, by Ca^{2+} entry through VSCC. This is supported by the recent findings that sGnRH-stimulated GH release from goldfish pituitary cells can be reduced by the VSCC blockers, such as nifedipine, nicardipine, and verapamil (Jobin and Chang, 1992a). Moreover, the GH responses to the PKC activator TPA can be inhibited by incubation with Ca^{2+}-deficient medium and by the VSCC blocker verapamil (Chang *et al.*, 1991a). The coupling of the PKC-dependent pathway with the entry of extracellular Ca^{2+} was also confirmed by Jobin and Chang (1992b) using fura-2 spectrofluorometry. The PKC activator TPA induces an increase in cytosolic free Ca^{2+} levels in goldfish pituitary cells, and these Ca^{2+} responses can be abolished by using Ca^{2+}-deficient medium (Jobin and Chang, 1992b). In the goldfish, PKC-mediated GH release may also have a component which is independent of Ca^{2+} entry via VSCC. This is indicated by the inability of verapamil and Ca^{2+}-deficient medium to completely abolish the GH responses to TPA (Chang *et al.*, 1991a).

In the goldfish, arachidonic acid is a part of the signal transduction pathways for

sGnRH-stimulated GTH-II release (Chang *et al.*, 1989; Chang *et al.*, 1991b). Arachidonic acid also stimulates GH release from goldfish pituitary cells, although it does not appear to mediate the GH responses to sGnRH stimulation (Chang and de Leeuw, 1990). Whether arachidonic acid is involved in DA D1 actions in the goldfish is still not clear. Therefore, we do not exclude the possibility that second messengers other than cAMP, such as arachidonic acid, may also participate in the GH responses to DA. Furthermore, the model proposed in this chapter (Fig. 9.2) has been simplified by assuming that both the cAMP- and PKC-dependent pathways act on the same population of VSCC. However, multiple forms of VSCC have been identified, and each of them is known to have its own characteristic electrophysiological properties (for a recent review on the classification of VSCC, see Tsien *et al.*, 1991). Therefore, it will be of interest in the future to examine whether cAMP and PKC are acting on the same population or different populations of VSCC in the goldfish somatotrophs.

To conclude, the present study has provided strong evidence that DA functions as a physiological GH-releasing factor in the goldfish. DA also interacts with other GH regulators to control the seasonality of GH release, and its actions are mediated through DA D1 receptors coupled to the cAMP-dependent signal transduction pathway. Results from the *in vivo* studies also demonstrated that oral administration of the DA agonist apomorphine was effective in increasing plasma GH levels and body growth in the goldfish. These findings may have potential applications in fish farming. In aquaculture, the productivity in many cases is limited by two factors : (1) the availability of fry for commercial fish farming, and (2) the growth rate of fish under captivity, which determine the overall cost-effectiveness of the aquaculture operation. Based on the earlier research in our laboratory, a fish spawning kit OVAPRIM (Syndel Laboratories, Vancouver, BC) has been developed and proven to be highly effective in a wide range of cultured fish. The results of the present study indicate that dietary supplement with long-lasting DA analogs may be a feasible way to enhance body growth and shorten the production cycle of commercial fish. The use of DA analogs is unique in a sense that DA has differential actions on GH and GTH-II release via the D1 and D2 receptors, respectively (see chapter 2). Non-selective DA agonists, at least theoretically, should be able to slow down the

reproductive activity of cultured fish by inhibiting GTH-II secretion, and at the same time channel the metabolic energy into body growth by stimulating GH release. Therefore, further investigations in the use of DA analogs to enhance body growth in teleosts may be of great potential value in fish farming.

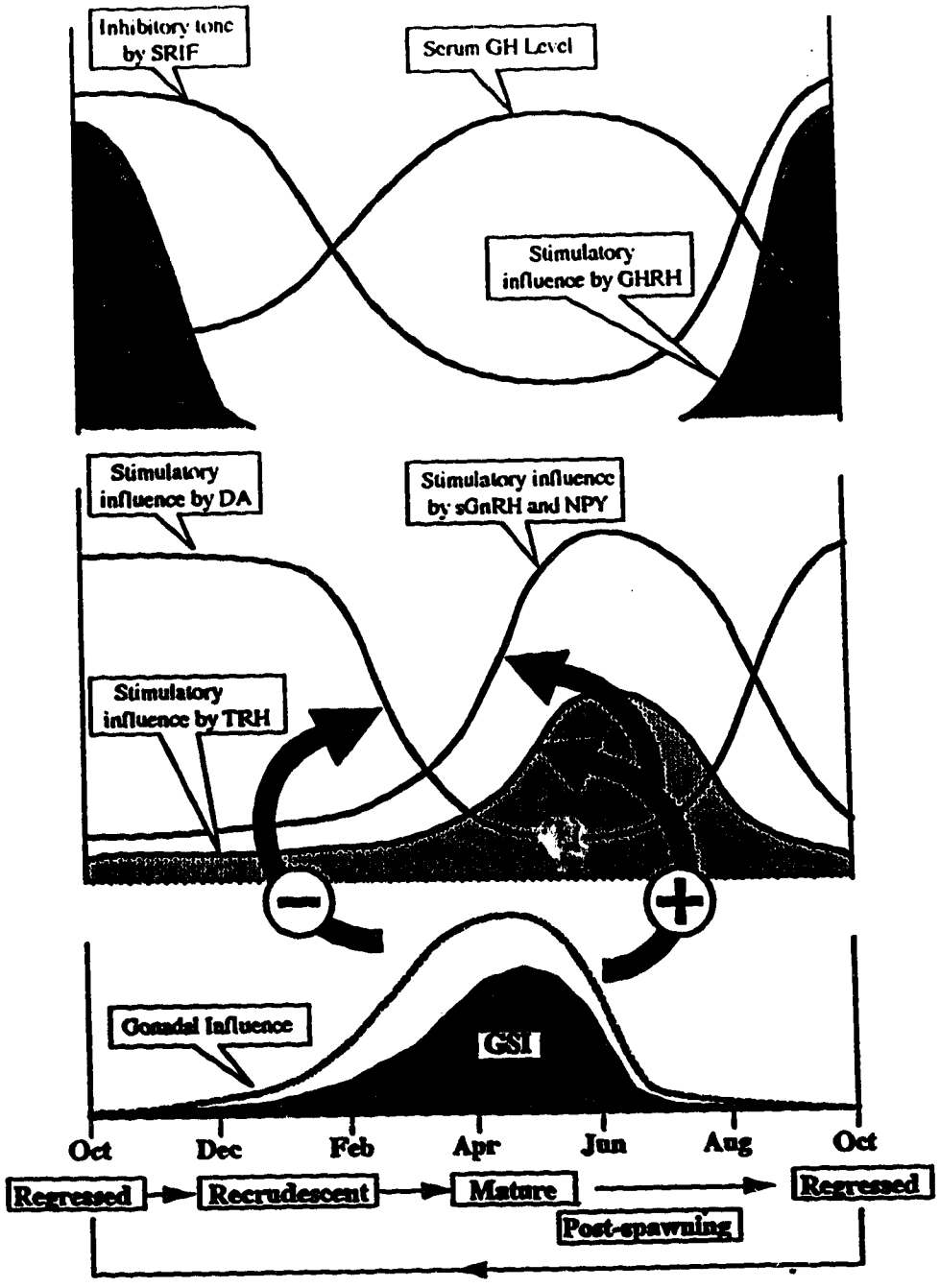


Fig. 9.1 A model for seasonal regulation of GH release in the goldfish. DA, sGnRH, SRIF, NPY, and TRH are the abbreviations for dopamine, salmon gonadotropin-releasing hormone, somatostatin, neuropeptide Y, and thyrotropin-releasing hormone, respectively. The positive and negative modulating effects of the gonads on GH release from the pituitary are represented as (+) and (–), respectively. Since the seasonal effects of norepinephrine and serotonin on GH release have not been studied in the goldfish, these two factors are not included in this model. (Refer to the text for details.)

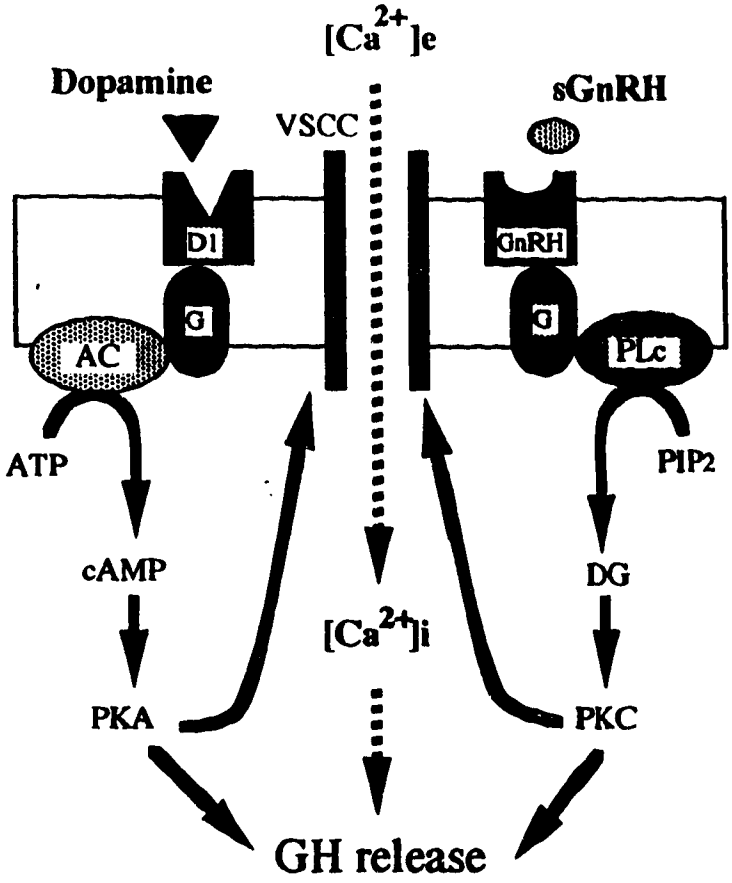


Fig 9.2 A model for the signal transduction pathways mediating DA- and sGnRH-stimulated GH release from goldfish pituitary cells. D1, GnRH, G, AC, PLc, DG, VSCC, $[Ca^{2+}]e$ and $[Ca^{2+}]i$ represent the D1 receptors, GnRH receptors, Gs proteins, membrane associated adenylate cyclase, phospholipase C, diacylglycerol, voltage-sensitive Ca^{2+} channels, extracellular and intracellular Ca^{2+}, respectively. (Refer to the text for details.)

References

Battaglia G, Norman AB, Hess EJ, Creese I 1986 Forskolin potentiates the stimulation of rat striatal adenylate cyclase mediated by D1 dopamine receptors, guanine nucleotides, and sodium fluoride. J Neurochem 46:1180-1185

Catt KJ, Hunyady L, Balla T 1991 Second messengers derived from inositol lipids. J Bioener Biomembr 23:7-27

Chang JP, de Leeuw R 1990 *In vitro* goldfish growth hormone responses to gonadotropin-releasing hormone. Possible roles of extracellular calcium and arachidonic acid metabolism ? Gen Comp Endocrinol 80:155-164

Chang JP, Freedman GL, de Leeuw R 1989 Participation of arachidonic acid metabolism in gonadotropin-releasing hormone stimulation of goldfish gonadotropin release. Gen Comp Endocrinol 76:2-11

Chang JP, Jobin RM, De Leeuw R 1991a Possible involvement of protein kinase C in gonadotropin and growth hormone release from dispersed goldfish pituitary cells. Gen.Comp.Endocrinol. 81:447-463

Chang JP, Marchant TA, Cook AF, Nahorniak CS, Peter RE 1985 Infuences of catecholamines on growth hormone release in female goldfish, *Carassius auratus.* Neuroendocrinology 40:463-470

Chang JP, Wildman B, Van Goor F 1991b Lack of involvement of arachidonic acid metabolism in chicken gonadotropin-releasing hormone II (cGnRH-II) stimulation of gonadotropin secretion in dispersed pituitary cells of goldfish, *Carassius auratus.* Identification of a major difference in salmon GnRH and chicken GnRH-II mechanisms of action. Mol Cell Endocrinol 79:75-83

Chang JP, Wong AOL, Van Der Kraak G, Van Goor F 1992 Relationship between cyclic AMP-stimulated and native gonadotropin-releasing hormone-stimulated gonadotropin release in the goldfish. Gen Comp Endocrinol 86:359-377

Chang JP, Yu KL, Wong ALO, Peter RE 1990 Differential actions of dopamine receptor subtypes on gonadotropin and growth hormone release *in vitro* in goldfish. Neuroendocrinology 51:664-674

Cook H, Berkenbosch JW, Fernhout MJ, Yu KL, Peter RE, Chang JP, Rivier JE 1991

Demonstration of gonadotropin releasing-hormone receptors on gonadotrophs and somatotrophs of the goldfish : An electron microscope study. Regul Pept 36:369-378

Cook AF, Peter RE 1984 The effects of somatostatin on serum growth hormone levels in the goldfish, *Carassius auratus*. Gen Comp Endocrinol 54:109-113

Habibi HR, De Leeuw R, Nahorniak CS, Goos HJT, Peter RE 1989 Pituitary gonadotropin-releasing hormone (GnRH) receptor activity in goldfish and catfish : Seasonal and gonadal effects. Fish Physiol Biochem 7:109-118

Habibi HR, Peter RE, Nahorniak CS, de L Milton RC, Millar RP 1992 Activity of vertebrate gonadotropin-releasing hormones and analogs with variant amino acid residues in positions 5, 7 and 8 in the goldfish pituitary. Regul Pept 37:271-284

Jobin RM, Chang JP 1993 Involvement of protein kinase C in the modulation of gonadotropin and growth hormone secretion from dispersed goldfish pituitary cells. Fish Physiol Biochem (in press)

Jobin RM, Chang JP 1992a Differences in extracellular calcium involvement mediating the secretion of gonadotropin and growth hormone stimulated by two closely related endogenous GnRH peptides in goldfish pituitary cells. Neuroendocrinology 55:156-166

Jobin RM, Chang JP 1992b Actions of two native GnRHs and protein kinase C modulators on goldfish pituitary cells. Studies on intracellular calcium levels and gonadotropin release. Cell Calcium 13:531-540

Jobin RM, Ginsberg J. Matowe WC, Chang JP 1993 Down regulation of PKC levels leads to inhibition of GnRH-stimulated gonadotropin secretion from dispersed pituitary cells of goldfish. Neuroendocrinology (in press)

Kah O, Dubourg P, Chambolle P, Calas A 1984 Ultrastructural identification of catecholaminergic fibers in the goldfish pituitary. A high-resolution radioautographic study after *in vitro* ^{3}H-dopamine administration. Cell Tissue Res 238:621-626

Kah O, Dubourg P, Onteniente B, Geffard M, Calas A 1986 The dopaminergic innervation of the goldfish pituitary : An immunocytochemical study at the electron microscope level using antibodies against dopamine. Cell Tissue Res 244:577-582

Kah O, Dulka JG, Dubourg P, Thibault J, Peter RE 1987 Neuroanatomical substrate for the inhibition of gonadotrophin secretion in goldfish : Existence of a dopaminergic preoptico-hypophyseal pathway. Neuroendocrinology 45:451-458

Kobayashi M, Aida K, Hanyu I 1986 Annual change in plasma levels of gonadotropin and steroid hormone in goldfish. Bull Japan Soc Sci Fish 52:1153-1158

Kobayashi M, Aida K, Hanyu I 1987 Hormone changes during ovulation and effects of steroid hormones on plasma gonadotropin levels and ovulation in goldfish. Gen Comp Endocrinol 67:24-32

Lussier BT, French MB, Moor BC, Kraicer J 1991a Free intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) and growth hormone release from purified rat somatotrophs. I. GH-releasing factor-induced Ca^{2+} influx raises $[Ca^{2+}]i$. Endocrinology 128:570-582

Lussier BT, French MB, Moor BC, Kraicer J 1991b Free intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) and growth hormone release from purified rat somatotrophs. II. Somatostatin lowers $[Ca^{2+}]i$ by inhibiting Ca^{2+} influx. Endocrinology 128:583-591

Lussier BT, French MB, Moor BC, Kraicer J 1991c Free intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) and growth hormone release from purified rat somatotrophs. III. Mechanism of action of GH-releasing factor and somatostatin. Endocrinology 128:592-603

Marchant TA, Chang JP, Nahorniak CS, Peter RE 1989b Evidence that gonadotropin-releasing hormone also functions as a growth hormone-releasing factor in the goldfish. Endocrinology 124:2509-2518

Marchant TA, Dulka JG, Peter RE 1989a Relationship between serum growth hormone levels and the brain and pituitary content of immunoreactive somatostatin in the goldfish, *Carassius auratus*. Gen Comp Endocrinol 73:458-468

Marchant TA, Fraser RA, Andrews PC, Peter RE 1987 The influence of mammalian and teleost somatostatins on the secretion of growth hormone from goldfish (*Carassius auratus L.*) pituitary fragments *in vitro*. Regul Pept 17:41-52

Marchant TA, Peter RE 1986 Seasonal variations in body growth rates and circulating levels of growth hormone in the goldfish, *Carassius auratus*. J Exp Zool 237:231-

Matthews G 1991 Ion channels that are directly activated by cyclic nucleotides. Trends Pharmacol Sci 12:245-247

Monsma FJ, Mahan LC, McVittie LD, Gerfen CR, Sibley DR 1990 Molecular cloning and expression of a D1 dopamine receptor linked to adenylyl cyclase activation. Proc Natl Acad Sci USA 87:6723-6727

Narayanan N, Lussier B, French M, Moor B, Kraicer J 1989 Growth hormone-releasing factor-sensitive adenylate cyclase system of purified somatotrophs : Effects of guanine nucleotides, somatostatin, calcium, and magnesium. Endocrinology 124:484-495

Neumeyer JL, Baindur N, Bakthavachalam V, Yuan J, Madras BK, Kula NS, Campbell A, Baldessarini RJ 1992 Selective probes for characterization of dopamine D1 and D2 receptors. Neurochem Int [Suppl] 20:63S-68S

Omeljaniuk RJ, Shih SH, Peter RE 1987 *In vivo* evaluation of dopamine receptor-mediated inhibition of gonadotropin secretion from the pituitary gland of the goldfish. J Endocrinol 114:449-458

Omeljaniuk RJ, Tonon MC, Peter RE 1989 Dopamine inhibition of gonadotropin and a melanocyte-stimulating hormone release *in vitro* from the pituitary of the goldfish (*Carassius auratus*). Gen Comp Endocrinol 74:451-467

Peng C, Huang YP, Peter RE 1990 Neuropeptide Y stimulates growth hormone and gonadotropin release from the goldfish pituitary *in vitro*. Neuroendocrinology 52:28-34

Peter, RE., Chang JP, Nahorniak CS, Omeljaniuk RJ, Sokolowska M, Shih SH, and Billard R 1986 Interactions of catecholamines and GnRH in regulation of gonadotropin secretion in teleost fish. Recent Prog Horm Res 42:513-548

Peter RE, Yu KL, Marchant TA, Rosenblum PM 1990 Direct neural regulation of the teleost adenohypophysis. J Exp Zool [Suppl] 4:84-89

Somoza GM, Peter RE 1991 Effects of serotonin on gonadotropin and growth hormone release from *in vitro* perifused goldfish pituitary fragments. Gen Comp Endocrinol 82:103-110

Trudeau VL, Sloley BD, Wong AOL, Peter RE 1993 Interactions of gonadal steroids with brain dopamine and gonadotropin-releasing hormone in the control of gonadotopin-II secretion in the goldfish. Gen Comp Endocrinol 89:39-50

Trudeau VL, Somoza GM, Nahorniak CS, Peter RE 1992 Interactions of estradiol with gonadotropin-releasing hormone and thyrotropin-releasing hormone in the control of growth hormone secretion in the goldfish. Neuroendocrinology 56:483-490

Tsien RW, Ellinor PT, Horne WA 1991 Molecular diversity of voltage-dependent Ca^{2+} channels. Trends Pharmacol Sci 12:349-354

Yu KL, Sherwood NM, Peter RE 1988 Differential distributions of two molecular forms of gonadotropin-releasing hormone in discrete brain areas of goldfish (*Carassius auratus*). Peptides 9:625-630

Appendix I

Dopamine functions as a Growth Hormone-Releasing Factor in the goldfish, Carassius auratus [1]

A1.1 Introduction

In mammals, dopamine (DA) is known to be a neuroendocrine regulator for the anterior pituitary hormones (Tuomisto and Mannisto, 1985). However, its role on growth hormone (GH) release is still controversial, as stimulatory, inhibitory, or no effects have been reported (for review, see Muller, 1987). In lower vertebrates, like the teleosts, even less is known about the actions of DA in GH regulation. In the goldfish, the anterior pituitary is directly innervated by a dopaminergic preoptico-hypophyseal pathway, and DA nerve terminals have been identified in the close proximity of somatotrophs (Kah *et al.*, 1987). These observations provide an anatomical basis for the possible involvement of DA in GH regulation in the goldfish. This hypothesis was confirmed by our findings that DA and its non-selective agonist apomorphine are stimulatory to GH release in the goldfish, both *in vivo* (Chang *et al.*, 1985) and *in vitro* (Peter *et al.*, 1990). More recently, we have demonstrated that the GH-releasing effect of DA is mediated through DA D1 receptors (Chang *et al.*, 1990b; Wong *et al.*, 1992a) (for reviews on DA receptors, see Niznik, 1987; Civelli *et al.*, 1991). These results strongly suggest that DA also functions as a GH-releasing factor in the goldfish, and possibly also in other teleosts.

In the present study, we provide further evidence to substantiate the role of DA as one of the GH-releasing factors in the goldfish. The seasonality and receptor specificity of the GH responses to DA were investigated using perifused pituitary fragments. The actions of somatostatin (SRIF), a known GH-release inhibitor in the goldfish (Marchant and Peter, 1989), on DA-stimulated GH release were examined using dispersed pituitary

[1] A version of this chapter has been accepted for publication : Wong AOL, Chang JP, and Peter RE (1993) **Fish Physiol Biochem.**

cells under static incubation. To further confirm the role of DA as a GH-releasing factor, an *in vivo* approach was used to test the influence of apomorphine, a non-selective DA agonist, on plasma GH levels and body growth of the goldfish.

A1.2 Materials and Methods

General Goldfish, obtained from Ozark Fisheries (Stoutland, MO, USA) or Grassyforks Fisheries (Martinsville, IN, USA), were maintained in flow-through aquaria (1,800 liters) at 17°C under a simulated photoperiod (Edmonton, Alberta, Canada) for at least 3 weeks before experiments. Gonadal conditions of the fish, determined by the gonadosomatic index (GSI = weight of gonad / total body weight x 100 %) and the morphological characteristics of the gonads (see chapter 2 for details), are reported separately for each individual experiment. DA and SRIF (Sigma, St Louis, MO, USA) were dissolved directly into the culture medium for *in vitro* studies. Apomorphine (Sigma) and the D1 antagonist (+)SCH23390 (Research Biochemicals Inc., Wayland, MA, USA) were first dissolved in a minimal amount of dimethyl sulfoxide, and subsequently diluted to appropriate concentrations using culture medium for *in vitro* perifusions, or propylene glycol for *in vivo* experiments. Aliquots of salmon gonadotropin (GTH)-releasing hormone (sGnRH) stock solution (Peninsula Laboratories Inc., Belmont, CA) in 0.1 M acetic acid were frozen at -25 °C, and diluted with perifusion medium to 50 nM concentration immediatedly prior to use.

In vitro experiments The seasonality and receptor specificity for DA-stimulated GH release were examined using a perifusion system for goldfish pituitary fragments (Chang *et al.*, 1984). Since sGnRH is known to be a stimulator for both GH and GTH-II release in the goldfish (Marchant *et al.*, 1989), two-minute pulses of 50 nM sGnRH were given as internal controls for individual perifusion columns. For the seasonality studies, goldfish at different reproductive stages, including sexual regression (November, 1989), recrudescence (February, 1990), and maturation (prespawning fish; April, 1990), were used. The GH responses to two-minute pulses of 10 nM to 1 μM DA were tested one hour after the first control 50 nM sGnRH pulses. The perifusion protocol was previously demonstrated to have no desensitizing nor potentiating effects

with the doses of DA tested (Wong *et al.*, 1992). For the studies of DA D1 receptor specificity in GH release, sixty-minute continuous perifusion of 5 μM (+)SCH23390 was initiated one hour after the first two-minute pulse of 500 nM DA, and a second 500 nM DA pulse was given half an hour later in the presence of (+)SCH23390. Perifusates were collected in five-minute fractions and stored at -25 °C until their GH contents were assayed using a radioimmunoassay previously validated for the measurement of goldfish GH (Marchant and Peter, 1989). Profiles of hormone release during the course of perifusion were presented as "% pretreatment" as defined previously (Wong *et al.*, 1992). This transformation was done to allow pooling of GH data from separate perifusion columns without distorting the profile of hormone release in the raw data. The GH responses to DA, calculated as the area under the curve, were expressed as the total amout of GH released.

The interactions of DA and SRIF on GH release were also investigated using a static incubation system for goldfish pituitary cells (Chang *et al.*, 1990a). The GH responses to 2-hour incubation of 1 μM DA were tested in the presence or absence of 10 nM SRIF. The GH contents in culture medium were assayed, and the data were expressed as a percentage of the basal hormone release in control wells without any drug treatments (referred to as "% control"). Since DA is known to inhibit GTH-II release in the goldfish via the DA D2 receptors (Omeljaniuk *et al.*, 1989), GTH-II contents were also measured (Peter *et al.*, 1984) to serve as a negative control. The antiserum for GTH radioimmunoassay is selective for the measurement of maturational GTH (or GTH-II) of the goldfish (Van Der Kraak *et al.*, 1991).

In vivo experiments The influence of DA on GH release in the goldfish *in vivo* was investigated by intraperitoneal (i.p.) injection of a non-selective DA agonist apomorphine at the doses of 2 and 20 μg/g body weight. Plasma samples were collected immediately before and at five hours after the drug treatment. Plasma GH and GTH-II contents were assayed as described in the preceding section. For each individual fish, the plasma hormone concentration after apomorphine treatment was expressed as a percentage of the hormone level before the drug administration (referred to as "% presample").

Effects of long-term administration of apomorphine on the body growth of goldfish

was also examined in the present study. Two groups of fish from the same stock were accomodated in separate tanks (1,800 liters) at 17°C and under 8L:16D photoperiod. The fish were fed to satiation daily with Ewos trout pellets (pellet size 5P; Astra Chemicals Ltd., Mississauga, Ontario, Canada) at a ration of 5 % body weight per day. The fish were fed in the morning, and the data for body weight and length were taken in the evening. The fish were tagged with numbered metal clips on the operculum for individual identication, and the initial body weight and length for each fish were recorded as described previously (Marchant *et al.*, 1989). The initial body weights and lengths for the control group and treatment group were 11.8 ± 0.5 g and 11.7 ± 0.8 g, and 6.5 ± 0.1 cm and 6.6 ± 0.2 cm, respectively (n = 9 - 10). The measurements were accurate up to the levels of 0.01 g body weight and 0.1 mm body length. After twelve days of acclimation, one group of fish received 20 μg/g body weight (i.p.) of apomorphine once every three days for another thirty days, while the other group serving as the control received vehicle injection over the same period of time. Body weight and length for individual fish were measured every three days and expressed as a percentage of the corresponding initial values.

Data analysis and statistics All the data presented are expressed as mean ± SEM. GH and GTH-II data were analyzed by analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test. Data for body growth were compared using Student's *t* test. Differences were considered significant when $P < 0.05$.

A1.3 Results

Seasonality of GH response to DA stimulation DA consistently stimulated GH release from perifused goldfish pituitary fragments in a dose-dependent manner, regardless of the reproductive stages of the donor fish (Fig. A1.1). The GH-releasing actions of DA were found to be the highest in sexually regressed fish, intermediate in sexually recrudescent fish, and the lowest in sexually mature (=prespawning) fish (Fig. A1.1, B).

DA receptor subtype mediating the GH-releasing actions of DA We have previously demonstrated that the D1 antagonist (+)SCH23390 abolished DA-stimulated GH release

in perifused pituitary fragments prepared from goldfish in late stages of gonadal recrudescence (Wong *et al.*, 1992). To confirm the receptor specificity of DA on GH release at different reproductive stages of the goldfish, a similar experiment was repeated using goldfish in sexual regression. Similar to our previous results, the GH responses to 0.5 μM DA were blocked by (+)SCH23390 at 5 μM dose (Fig. A1.2, A). In the same experiment, no significant differences were found between the GH responses to the first and second control 50 nM sGnRH pulses, indicating that the viability and responsiveness of pituitary fragments were maintained during the course of perifusion.

Actions of SRIF on DA-stimulated GH release Dispersed pituitary cells were prepared from goldfish undergoing gonadal recrudescence. DA at 1 μM concentration elevated the basal GH release (Fig. A1.2, B; left panel). SRIF at 10 nM dose reduced the basal GH levels and blocked the GH-releasing actions of DA. However, SRIF alone did not affect basal GTH-II release, and had no influence on the inhibitory effects of DA on GTH-II secretion (Fig. A1.2, B; right panel), demonstrating the specificity of SRIF actions on GH secretion.

Apomorphine on plasma GH levels and body growth Goldfish in gonadal regression were used to investigate the DA actions on plasma GH levels and body growth. Administration of apomorphine, a non-selective DA agonist, at 2 and 20 μg/g body weight (i.p.) induced a dose-related increase in plasma GH levels (Fig. A1.3, A; left panel). Meanwhile, the circulating levels of GTH-II were suppressed at a high dose of apomorphine (Fig. A1.3, A; right panel). Long-term treatment with apomorphine at 20 μg/g body weight (i.p.) promoted the linear body growth of the goldfish; fish that received apomorphine injections demonstrated a significant increase in body length compared to that of the vehicle-treated group (Fig. A1.3, B). However, no similar increases in body weight were observed during the treatment period (data not shown).

A1.4 Discussion

In the present study, the role of DA as a GH-releasing factor in the goldfish is

substantiated by the demonstration of a distinct seasonality for DA-stimulated GH release. The ability of DA to induce GH release was found to be the highest in sexually regressed fish, intermediate in recrudescent fish, and the lowest in sexually mature (=prespawning) fish. In a separate study using perifused pituitary fragments from ovariectomized goldfish in prespawning stages, the GH response to DA was significantly greater than that of the sham-operated fish (for details, see chapter 4), suggesting that the GH-releasing action of DA is under the negative modulation of gonadal factor(s). Effects of gonadal steroids on dopaminergic functions have been well documented in mammals. Sex steroids, e.g., estrogen and progesterone, are known to reduce DA D1 receptor capacity (Tonnaer *et al.*, 1989), decrease DA turnover (Dipaolo *et al.*, 1985), and modulate the excitability of DA neurons (Chiodo *et al.*, 1986). It is likely that the modulatory effects of the gonads on DA-stimulated GH release in the goldfish are also mediated by sex steroids.

SRIF has been identified in a variety of teleost species, including the goldfish (Kah *et al.*, 1982), and its physiological role as a GH-release inhibitor appears to be a common phenomenon among vertebrates (for review, see Hall *et al.*, 1986; Nishioka *et al.*, 1988). In the current study, SRIF blocked the GH response to DA by actions directly at the pituitary cell level. Together with the previous anatomical evidence that both SRIF (Kah *et al.*, 1982) and DA fibers (Kah *et al.*, 1987) are in close association with somatotrophs in the goldfish, it is apparent that DA and SRIF are a part of the complex GH regulatory mechanism in the goldfish.

The role of DA as a GH-releasing factor is also supported by our *in vivo* studies using the non-selective DA agonist apomorphine. Injection (i.p.) of apomorphine increased the circulating levels of GH in a dose-dependent manner. The GH releasing effect of apomorphine *in vivo* is known to be D1 specific, as this GH response can be blocked by the D1 antagonist SCH23390, but not the D2 antagonist pimozide (Wong *et al.*, 1993). This is also consistent with the results of the current *in vitro* perifusion studies, in which the GH response to DA was abolished by (+)SCH23390 (for a discussion on the involvement of D1 receptors in DA-stimulated GH release in the goldfish, see Wong *et al.*, 1992). The elevation of plasma GH levels induced by apomorphine was accompanied by a significant increase in body length of the goldfish.

This *in vivo* experiment (Nov - Dec, 1989) was conducted using sexually regressed goldfish which normally do not show significant body growth in the winter months (Marchant and Peter, 1986). Administration (i.p.) of apomorphine induced a 3.5 % increase in the body length of goldfish during the first 9 days of the experiment, and then the body length reached a plateau for the rest of the treatment period. The linear growth rate (as defined by Ricker, 1979) during the first 9 days of apomorphine treatment was about 0.3 % net increase in body length per day, which is almost three to four times the normal linear growth rate reported in sexually regressed goldfish (< 0.1 % net increase in body length per day) (Marchant and Peter, 1986), indicating that apomorphine is effective in stimulating body growth in the goldfish. The gradual loss of body growth response during the latter half of the experiment, however, was likely the result of handling stress caused by repeated injection. In a recent *in vivo* experiment using oral administration of apomorphine in sexually recrudescent goldfish, the stimulatory action of apormorphine on body growth was also confirmed. Feeding the sexually recrudescent goldfish with apomorphine-treated trout pellets (20 mg apomorphine / g pellet, at a ration of 5 % body weight per day) significantly increased the plasma GH levels and enhanced the rates of increase in both body weight and length (Wong *et al.*, 1993). These observations strongly suggest that the growth-promoting actions of apomorphine can be correlated with its ability to stimulate GH release from the goldfish pituitary. Although dopamine is also known to influence feeding behavior in mammals (for review, see Morley, 1987), the possibility of the body growth response observed in the present study being the result of an indirect action of apomorphine on feeding seems unlikely, as dopamine generally exerts an inhibitory effect on feeding behavior (Bednar *et al.*, 1991).

In summary, we have confirmed that the DA D1 receptor subtype mediates the GH response to DA in the goldfish. The GH-releasing effect of DA was seasonal, being the highest in sexually regressed fish, intermediate in recrudescent fish, and the lowest in sexually mature (=prespawning) fish. SRIF, a physiological GH-release inhibitor in the goldfish, blocked the DA-stimulated GH release at the pituitary cell level. *In vivo* treatment with apomorphine, a non-selective DA agonist, significantly elevated the plasma GH levels and enhanced the linear body growth of goldfish. These results strongly suggest that DA, besides its well-established role as a GTH-release inhibitor in

teleosts (for review, see Peter *et al.*, 1986), also functions as a GH-releasing factor in the goldfish.

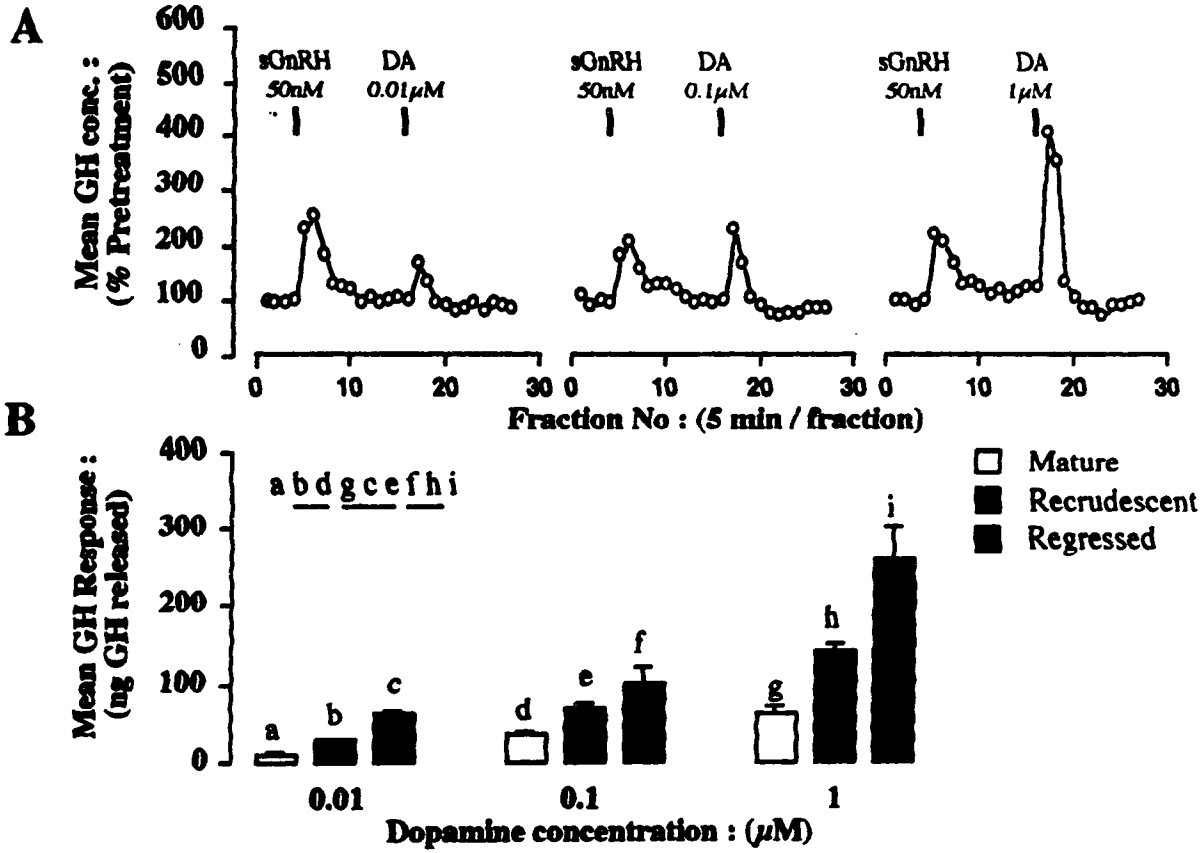


Fig. A1.1 Seasonality of GH responses to increasing concentrations of DA in perifused goldfish pituitary fragments. (A) is the GH-release profiles in representative columns using goldfish in sexual recrudescence. The average pretreatment GH level for the perifusion studies was 31.8 ± 2.6 ng GH/ml. Comparisions of the GH responses (mean ± SEM, n = 3) to 0.01, 0.1 and 1μM DA using goldfish at different reproductive stages are shown in (B). DA treatments giving similar GH-release responses were grouped within the same underscore after analysis with ANOVA followed by Fischer's LSD test ($P > 0.05$).

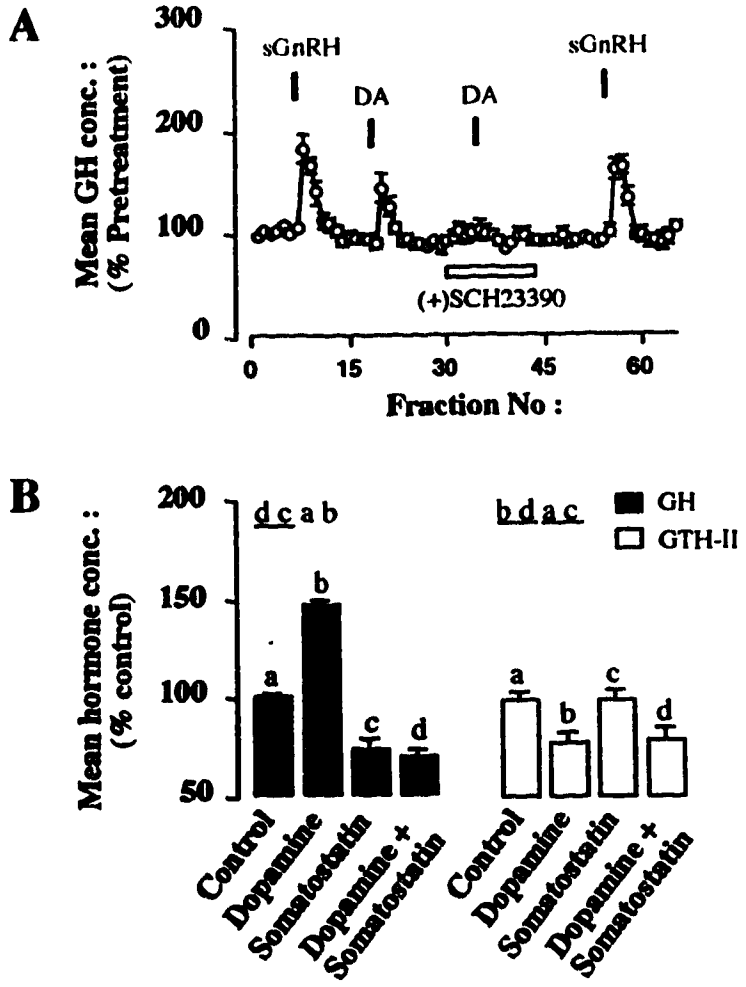


Fig. A1.2 Inhibitory effects of A) the D1 specific antagonist (+)SCH23390 and B) SRIF on DA-stimulated GH release in the goldfish. Perifused pituitary fragments were used for the study of (+)SCH23390, and the mean pretreatment GH level for the perifusion was 32.1 ± 1.9 ng GH/ml. Static incubation of dispersed pituitary cells was used for the study of SRIF, and the average GH and GTH-II. levels in the control wells of the SRIF experiments were 429 ± 35 ng GH/ml and 69.2 ± 9.2 ng GTH-II./ml, respectively. Drug treatments giving similar hormone-release responses were grouped within the same underscore after analysis with ANOVA followed by Fischer's LSD test ($P > 0.05$). All data are presented as mean ± SEM ($n = 4$). (For the doses of drug treatments, refer to Materials and Methods)

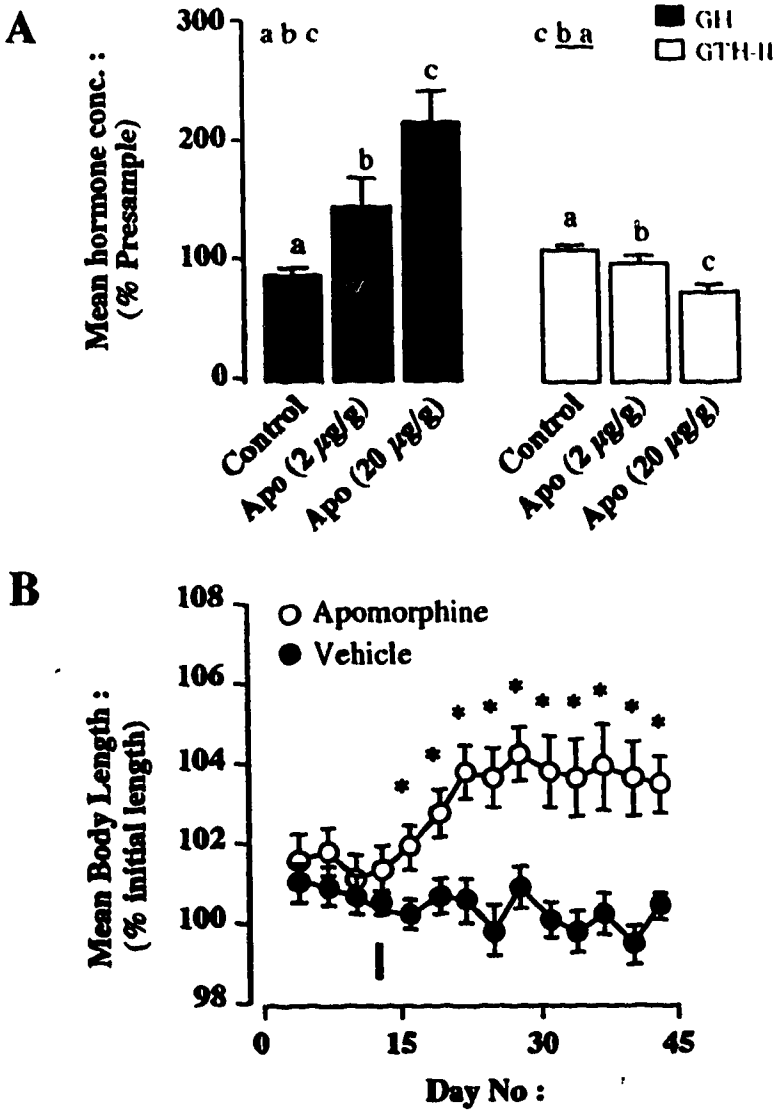


Fig. A1.3 Effects of apomorphine (Apo), a non-selective dopamine agonist, on (A) plasma GH and GTH-II levels, and (B) body growth in the goldfish. GH and GTH-II data were transformed into "% presample" as described in the text. The average presample GH and GTH-II. levels were 70.4 ± 15.3 ng GH/ml and 2.7 ± 0.1 ng GTH-II./ml, respectively. Doses of Apo giving similar hormone responses were grouped within the same underscore after analysis with ANOVA followed by Fischer's LSD test ($P > 0.05$). The average length of the goldfish at the beginning of the body growth experiment for the control and apomorphine-treated groups were 6.5 ± 0.1 cm and 6.6 ± 0.2 cm, respectively. Body length data of the Apo-treated group were compared to that of the vehicle-treated group at the same time point using Student's t test (*, $P < 0.05$). The bar in (B) represents the beginning of drug treatment. All data are expressed as mean ± SEM (n = 9 -10).

A1.5 References

Bednar I, Forsberg G, Linden A, Qureshi GA, Sodersten P 1991 Involvement of dopamine in inhibition of food intake by cholecystokinin octapeptide in male rats. J Neuroendocrinol 3:491-496

Chang JP, Cook H, Freedman GL, Wiggs AJ, Somoza GM, de Leeuw R, Peter RE 1990a Use of a pituitary cell dispersion method and primary culture system for the studies of gonadotropin-releasing hormone action in the goldfish, *Carassius auratus*. I. Initial morphological, static, and cell column perifusion studies. Gen Comp Endocrinol 77:256-273

Chang JP, Marchant TA, Cook AF, Nahorniak CS, Peter RE 1985 Influences of catecholamines on growth hormone release in female goldfish, *Carassius auratus*. Neuroendocrinology 40:463-470

Chang JP, MacKenzie DS, Gould DR, Peter RE 1984 Effects of dopamine and norepinephrine on *in vitro* spontaneous and gonadotropin-releasing hormone induced gonadotropin release by dispersed cells or fragments of the goldfish pituitary. Life Sci 35:2027-2033

Chang JP, Yu KL, Wong AOL, Peter RE 1990b Differential actions of dopamine receptor subtypes on gonadotropin and growth hormone release *in vitro* in goldfish. Neuroendocrinology 51:664-674

Chiodo LA, Glazer WM, Bunney BS 1986 Midbrain dopamine neurons : Electrophysiological studies on the acute effects of estrogen. In : Woodruff GN, Poat JA, Roberts PJ (eds) Dopaminergic System and their Regulation. V.C.H., London, pp 303-311

Civelli O, Bunzow JR, Grandy DK, Zhou QY, Van Tol HHM 1991 Molecular biology of the dopamine receptors. Eur J Pharmacol 207:277-286

DiPaolo T, Rouillard C, Bedard P 1985 17ß-Estradiol at a physiological dose acutely increases dopamine turnover in rat brain. Eur J Pharmacol 117:197-203

Hall TR, Harvey S, Scanes CG 1986 Control of growth hormone secretion in the vertebrates : A comparative survey. Comp Biochem Physiol 84A:231-253

Kah O, Chambolle P, Dubourg P, Dubois MP 1982 Localisation immunocytochimique

de la somatostatine dans le cerveau anterieur et l'hypophyse de deux Teleosteens, le Cyprin (*Carassius auratus*) et *Gambusia* sp. CR Acad Sci 294:519-524

Kah O, Dulka JG, Dubourg P, Thibault J, Peter RE 1987 Neuroanatomical substrate for the inhibition of gonadotrophin secretion in goldfish : Existence of a dopaminergic preoptico-hypophyseal pathway. Neuroendocrinology 45:451-458

Marchant TA, Chang JP, Nahorniak CS, Peter RE 1989 Evidence that gonadotropin-releasing hormone also functions as a growth hormone-releasing hormone in the goldfish. Endocrinology 124:2509-2518

Marchant TA, Peter RE 1989 Hypothalamic peptides influencing growth hormone secretion in the goldfish, *Carassius auratus*. Fish Physiol Biochem 7:133-139

Marchant TA, Peter RE 1986 Seasonal variations in body growth rates and circulating levels of growth hormone in the goldfish, *Carassius auratus*. J Exp Zool 237:231–239.

Morley JE 1987 Neuropeptide regulation of appetite and weight. Endocr Rev 8:256–287

Muller EE 1987 Neural control of somatotropic function. Physiol Rev 67:962-1053

Nishioka RS, Kelly KM, Bern HA 1988 Control of prolactin and growth hormone secretion in teleost fishes. Zool Sci 5:267-278.

Niznik HB 1987 Dopamine receptors : Molecular structure and function. Mol Cell Endocrinol 54:1-22

Omeljaniuk RJ, Tonon MC, Peter RE 1989 Dopamine inhibition of gonadotropin and a melanocyte-stimulating hormone release *in vitro* from the pituitary of the goldfish (*Carassius auratus*). Gen Comp Endocrinol 74:451-467

Peter RE, Chang JP, Nahorniak CS, Omeljaniuk RJ, Sokolowska M, Shih SH, Billard R 1986 Interactions of catecholamines and GnRH in regulation of gonadotropin secretion in teleost fish. Recent Prog Horm Res 42:513-548

Peter RE, Nahorniak CS, Chang JP, Crim LW 1984 Gonadotropin release from the pars distalis of the goldfish, *Carassius auratus*, transplanted beside the brain or into the brain ventricles : Additional evidence for a gonadotropin release-inhibitory factor. Gen Comp Endocrinol 55:337-346

Peter RE, Yu KL, Marchant TA, Rosenblum PM 1990 Direct neural regulation of the

teleost adenohypophysis. J Exp Zool (suppl) 4:84-89

Ricker WE 1979 Growth rates and models. In : Hoar WS, Randall DJ, Brett JR (eds) Fish Physiology, vol. VIII. Bioenergetics and Growth. New York, Academic Press, pp 677-743

Tonnaer JADM, Leinders L, Van Delft AML 1989 Ovariectomy and subchronic estradiol-17ß administration decrease dopamine D1 and D2 receptors in rat striatum. Psychoneuroendocrinology 14:469-476

Tuomisto J, Mannisto P 1985 Neurotransmitter regulation of anterior pituitary hormones. Pharmac Rev 37:249-332

Van Der Kraak G, Suzuki K, Peter RE, Itoh H, Kawauchi H 1992 Properties of common carp gonadotropin I and gonadotropin II. Gen Comp Endocrinol 85:217–229.

Wong AOL, Chang JP, Peter RE 1992 Dopamine stimulates growth hormone release from the pituitary of goldfish, *Carassius auratus*, through the dopamine D1 receptors. Endocrinology 130:1201-1210

Wong AOL, Chang JP, Peter RE 1993 *In vitro* and *in vivo* evidence that dopamine exerts growth hormone releasing activity in the goldfish. Am J Physiol (in press)

Appendix II

Effects of Norepinephrine and Serotonin on the Growth Hormone-Releasing Actions of Dopamine and Gonadotropin-Releasing Hormone in the goldfish

A2.1 Introduction

In teleosts, the anterior pituitary is directly innervated by two different types of nerve fibers from the hypothalamus, the aminergic and peptidergic fibers (for review, see Ball, 1981). In the goldfish, nerve fibers for somatostatin (SRIF) (Kah *et al.*, 1982), salmon gonadotropin (GTH)-releasing hormone (sGnRH) (Kah *et al.*, 1986), serotonin (5HT) (Kah and Chambolle, 1983), and catecholamines (Kah *et al.*, 1984) have been identified in close proximity to the somatotrophs, suggesting that these neuroendocrine factors may be involved in GH regulation at the pituitary level. This hypothesis is confirmed by the subsequent findings that sGnRH is stimulatory (Marchant *et al.*, 1989a; Habibi *et al.*, 1992), whereas SRIF (Cook and Peter, 1984; Marchant *et al.*, 1987), 5HT (Somoza and Peter, 1991) and norepinephrine (NE) (Chang *et al.*, 1985; Peter *et al.*, 1990) are inhibitory to GH release in the goldfish. SRIF and sGnRH are known to act directly at the pituitary cell level to regulate GH release (Chang *et al.*, 1990b; Wong *et al.*, 1993a,b; and Appendix I); however, the sites of action for NE and 5HT have not been clearly identified.

Recently, we have demonstrated that dopamine (DA), besides its role as a GTH-release inhibitor (for review, see Peter *et al.*, 1986), also functions as a GH-releasing factor in the goldfish (Chang *et al.*, 1990b; Wong *et al.*, 1992; Wong *et al.*, 1993a,b). The GH-releasing actions of DA are mediated through DA D1 receptors in the goldfish pituitary (see chapter 5; Wong *et al.*, 1992; Wong *et al.*, 1993a). In the present study, the effects of NE and 5HT on the GH-releasing actions of DA and sGnRH were investigated using perifused goldfish pituitary fragments. To test whether NE and 5HT act directly at the pituitary cell level, the actions of these two neurotransmitters on basal

GH release were also examined in experiments using goldfish pituitary cells.

A2.2 Materials and Methods

Animal Goldfish of the common or comet varieties were purchased from Ozark Fisheries (Stoutland, MO, USA) or Grassyforks Fisheries (Martinsville, IN, USA), and maintained in flow-through aquaria (1,800 liters) at 17 °C under a simulated natural photoperiod (Edmonton, AB, Canada) for at least 3 weeks prior to any experiments. Goldfish of both sexes, with body weight ranging from 20 g to 30 g, were used in the present study. Since a distinct seasonality of DA-stimulated GH release has been reported in the goldfish (Wong *et al.*, 1993a,b), gonadal conditions of the fish are reported separately for individual experiments. Morphological characteristics of the gonads as well as the gonadosomatic index (GSI = weight of gonad / total body weight x 100 %) were used to determine the reproductive status of the goldfish used in these experiments (for details, see chapter 2).

Perifusion of pituitary cells and pituitary fragments To test for the direct actions of NE and 5HT on GH release at the pituitary cell level, mixed populations of goldfish pituitary cells were prepared and perifused as described by Chang *et al.* (1990a). In these experiments, NE (1 μM) and 5HT (1 μM) were perifused continuously for 30 min. To examine the effects of NE and 5HT on the GH-releasing actions of DA and sGnRH, a perifusion system for goldfish pituitary fragments was used (MacKenzie *et al.*, 1984; Marchant *et al.*, 1989a). For most of the experiments, stimulants for GH release (i.e., DA at 0.5 μM, SKF38393 at 0.5 μM, or sGnRH at 50 nM) were administered as 2-min pulses before, during, and after the 1 hr continuous perifusion of test substances (i.e., NE and 5HT at 5 μM dose). For these *in vitro* perifusion studies using pituitary cells and pituitary fragments, perifusates from each column were collected in 5-min fractions, and stored at -25 °C until their hormone contents were assayed by a radioimmunoassay previously validated for the measurement of goldfish GH (Marchant *et al.*, 1989b). GH data were transformed into "% pretreatment" as defined previously (Wong *et al.*, 1992), and the GH responses were quantified by calculating the net change in hormone release (i.e., area under the curve) after a particular drug treatment (for details, see chapter 2).

Static incubation of pituitary cells Populations of pituitary cells enriched with goldfish somatotrophs were prepared as described by de Leeuw *et al.* (1984) with minor modifications (Chang and Jobin, 1993). These pituitary cells were seeded onto the bottom of 24-well culture plates for static incubation as described previously (Chang *et al.*, 1990a). Drug treatment was initiated by adding test medium with the D1 agonist SKF38393 (1 μM) in the presence or absence of NE (5 μM) and 5HT (5 μM). After incubation for 2 hours, 500 μl of test medium was carefully removed from individual culture wells, and stored at -25 °C until their GH contents were assayed. GH data were expressed as a percentage of the basal GH release in the control wells without any drug treatment (as "% control").

2.3 Results and Discussion

In the goldfish, administration (i.p.) of NE induces a dose-dependent suppression in serum GH levels (Chang *et al.*, 1985). More recently, NE (Peter *et al.* 1990) and 5HT (Somoza and Peter, 1991) were shown to inhibit basal GH release from perifused goldfish pituitary fragments. Pituitary fragments prepared from the goldfish are known to contain both pituitary cells as well as nerve terminals of the hypothalamic fibers (Chang *et al.*, 1990a). Therefore, it is not clear whether the inhibitory actions of these two neurotransmitters are exerted directly at the pituitary cell level or indirectly through the nerve terminals present in the goldfish pituitary fragments. In the current study, both NE (1 μM) and 5HT (1 μM) inhibited basal GH release from perifused goldfish pituitary cells (Fig. A2.1). In both cases, basal GH levels were significantly suppressed within 10 min after the initiation of drug treatment. Recovery to the pretreatment GH levels was observed within 30 min following the termination of drug treatment. These results strongly indicate that NE and 5HT can exert a rapid and reversible inhibitory action on basal GH release directly at the pituitary cell level.

In the goldfish, DA (Chang *et al.*, 1990b; Wong *et al.*, 1993a,b) and sGnRH (Marchant *et al.*, 1989a; Habibi *et al.*, 1991) are known to stimulate GH release, both *in vivo* and *in vitro*. It has been shown that the GH-releasing actions of DA are

mediated through DA D1 receptors in the goldfish pituitary cells (Wong *et al.*, 1992; and see chapter 5). In the present study, the actions of NE and 5HT on the GH-releasing effects of DA and sGnRH have also been examined. In perifusion experiments using goldfish pituitary fragments, NE (5 μM) inhibited basal GH release and abolished the GH-releasing actions of DA (0.5 μM) and sGnRH (50 nM) (Fig. A2.2). 5HT (5 μM) also suppressed basal GH release; however, it reduced, but did not totally abolish the GH responses to sGnRH (50 nM) , DA (0.5 μM), and the DA D1 agonist SKF38393 (0.5 μM) (Fig. A2.3 and A2.4). The inhibitory action of NE and 5HT on DA D1-stimulated GH release was further investigated using static incubation of a cell preparation enriched with goldfish somatotrophs (Fig. A2.5). In this experiment, both 5HT (5 μM) and NE (5 μM) significantly suppressed basal GH release from the enriched somatotrophs. Similar to the results of our perifusion studies, the GH responses to the D1 agonist SKF38393 (1 μM) were only partially inhibited by 5HT (5 μM) but totally abolished by the same dose of NE. Taken together, these observations indicate that in the goldfish the neurotransmitters NE and 5HT inhibit basal GH release and the GH responses to sGnRH and DA D1 action directly at the pituitary cell level. Moreover, the inhibitory action of NE on stimulated GH release in the goldfish appears to be more effective than that of 5HT. The physiological role of NE and 5HT in the regulation of GH release clearly warrants further investigation.

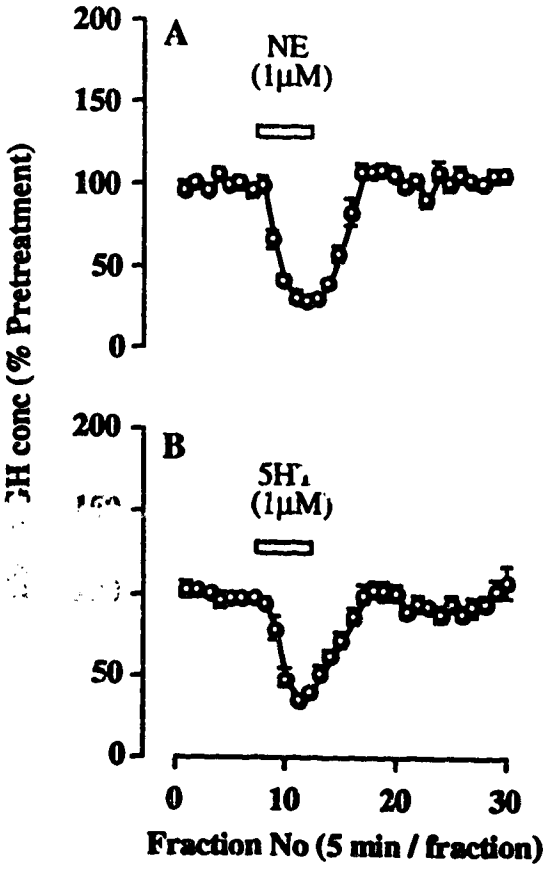


Fig. A2.1 Effects of norepinephrine (A) and serotonin (B) on GH release from perifused goldfish pituitary cells. Mixed populations of pituitary cells were prepared from goldfish in early stages of gonadal recrudescence. Norepinephrine (NE) and serotonin (5HT) at 1 μM dose were perifused continuously for 30 min (x6 columns each). The average pretreatment GH level for perifusion experiments was 37.5 ± 1.4 ng GH/ml. All GH data are expressed as mean ± SEM (n = 6).

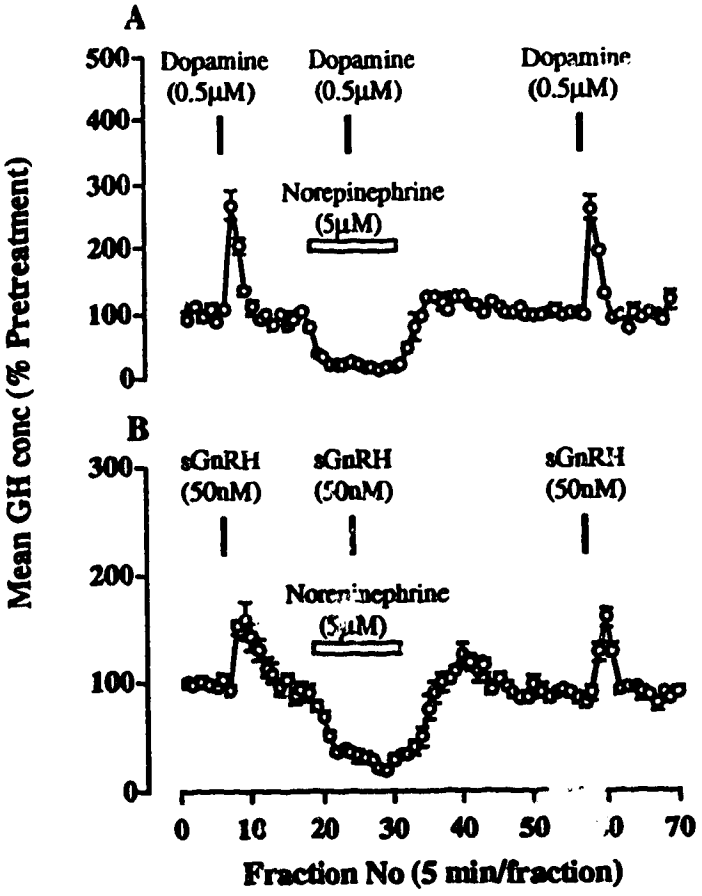


Fig. A2.2 Effects of norepinephrine on (A) dopamine- and (B) sGnRH-stimulated GH release from perifused goldfish pituitary fragments. Pituitary fragments were prepared from sexually regressed goldfish. Dopamine (0.5 μM) and sGnRH (50 nM) were administered as 2-min pulses before, during, and after the 1 hr continuous perifusion of norepinephrine (5 μM) (x4 columns each). The average pretreatment GH level for perifusion experiments was 30.6 ± 2.4 ng GH/ml. All GH data are expressed as mean ± SEM (n = 4).

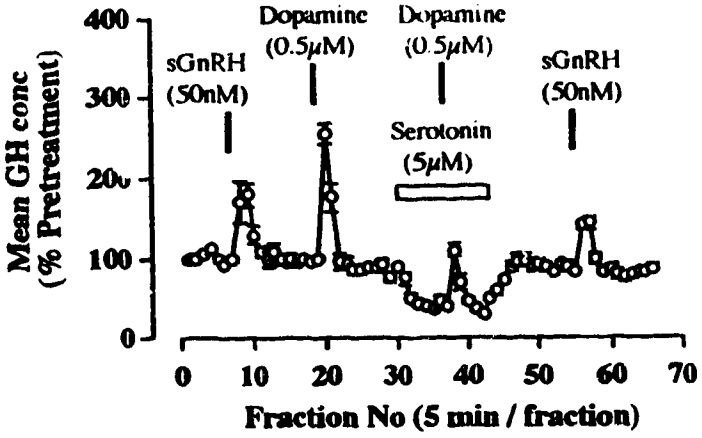


Fig. A2.3 Effects of serotonin on dopamine-stimulated GH release from perifused goldfish pituitary fragments. Pituitary fragments were prepared from goldfish undergoing gonadal recrudescence. 2-min pulses of sGnRH (50 nM) were given at the beginning and at the end of the perifusion experiment to serve as an internal control. Dopamine (0.5 μM) was administered as 2-min pulses before and during the 1 hr continuous perifusion of serotonin (5 μM) (x4 columns). The average pretreatment GH level for the experiment was 26.7 ± 0.9 ng GH/ml. All GH data are expressed as mean ± SEM (n = 4).

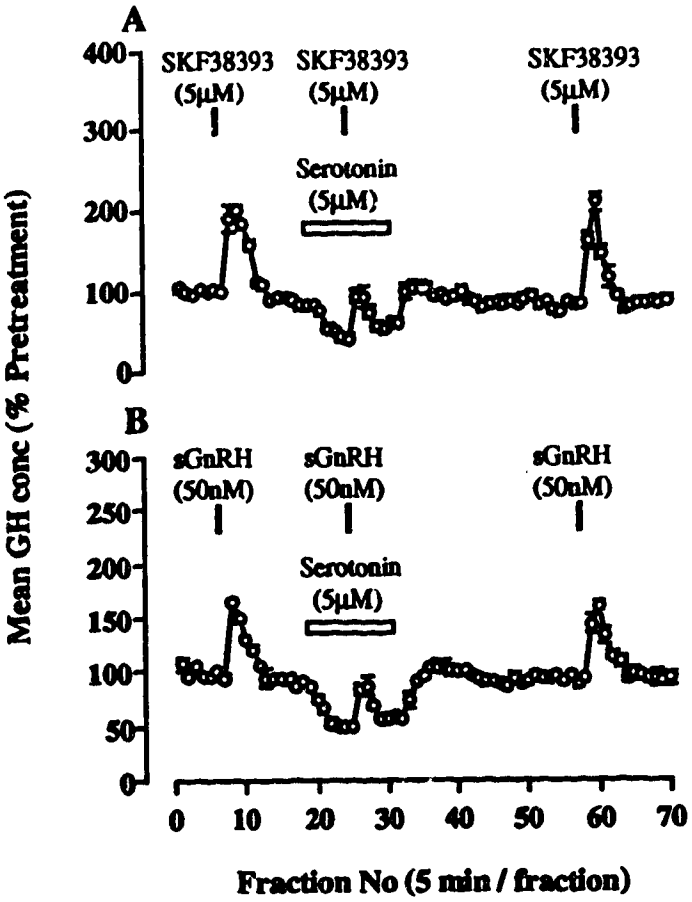


Fig. A2.4 Effects of serotonin on (A) the D1 agonist SKF38393- and (B) sGnRH-stimulated GH release from perifused goldfish pituitary fragments. Pituitary fragments were prepared from goldfish undergoing gonadal recrudescence. SKF38393 (0.5 μM) and sGnRH (50 nM) were administrated as 2-min pulses before, during, and after the 1 hr continuous perifusion of serotonin (5 μM) (x4 columns each). The average pretreatment GH level for these two perifusion experiment was 36.8 ± 2.1 ng GH/ml. All GH data are expressed as mean ± SEM (n = 4).

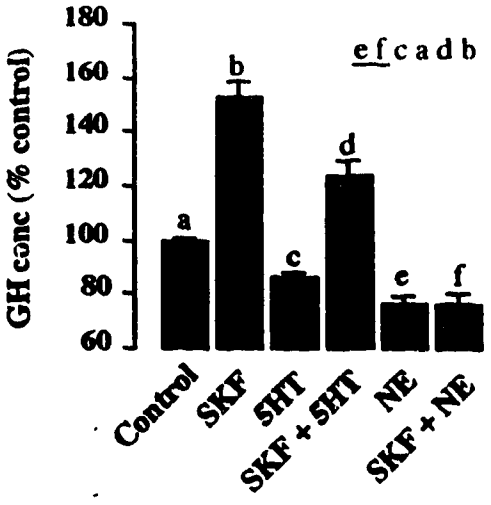


Fig. A2.5 Effects of norepinephine (NE) and serotonin (5HT) on the DA D1 agonist SKF38393 (SKF)-stimulated GH release from goldfish pituitary cells enriched with somatotrophs. Populations of pituitary cells enriched with somatotrophs were prepared from goldfish in late stages of gonadal recrudescence. SKF (1 μM) was applied in the presence or absence of NE (5 μM) and 5HT (5 μM). The average GH level in the control wells without any drug treatments was 531.3 ± 84.0 ng GH/ml. Pooled data from two separate experiments (each with quadruplicate treatments) are presented, and all GH data are expressed as mean ± SEM (n = 8). Drug treatment giving similar magnitude of GH responses are grouped with the same underscore (ANOVA followed by Fisher's LSD test, P>0.05).

2.4 References

Ball JN 1981 Hypothalamic control of the pars distalis in fishes. Gen Comp Endocrinol 44:135-170

Chang JP, Cook H, Freedman GL, Wiggs AJ, Somoza GM, de Leeuw R, Peter RE 1990a Use of a pituitary cell dispersion method and primary culture system for the studies of gonadotropin-releasing hormone action in the goldfish, *Carassius auratus*. I. Initial morphological, static, and cell column perifusion studies. Gen Comp Endocrinol 77:256-273

Chang JP, Jobin RM 1993 Teleost pituitary cells : Isolation, culture, and use. In : Hochachka PW, Mommsen TP (eds) Biochemistry and Molecular Biology of Fishes (vol.3) : Analytical Techniques. Elsevier, Amsterdam (in press)

Chang JP, Marchant TA, Cook AF, Nahorniak CS, Peter RE 1985 Influences of catecholamines on growth hormone release in female goldfish, *Carassius auratus*. Neuroendocrinology 40:463-470

Chang JP, Yu KL, Wong ALO, Peter RE Differential actions of dopamine receptor subtypes on gonadotropin and growth hormone release *in vitro* in goldfish. Neuroendocrinology 51:664-674

Cook AF, Peter RE 1984 The effects of somatostatin on serum growth hormone levels in the goldfish, *Carassius auratus*. Gen Comp Endocrinol 54:109-113

de Leeuw R, Goos HJT, Peute J, Van Pelt AAM, Burzawa-Gerard E, Van Oordt PGWJ 1984 Isolation of gonadotrops from the pituitary of the African catfish, *Clarias lazera*. Morphological and physiological characterization of purified cells. Cell Tissue Res 236:669-675

Habibi HR, Peter RE, Nahorniak CS, Raymond C, Milton DL, Millar RP 1992 Activity of vertebrate gonadotropin-releasing hormones and analogs with variant amino acid residues in positions 5,7 and 8 in the goldfish pituitary. Regul Pept 37:271-284

Kah O, Breton B, Dulka JG, Nunez-Rodriguez J, Peter RE, Corrigan A, Rivier JE, Vale WW 1986 A re-investigation of the GnRH (gonadotrophin-releasing hormone) systems in the goldfish brain using antibodies to salmon GnRH. Cell Tissue Res 244:327-337

Kah O, Chambolle P 1983 Serotonin in the brain of the goldfish, *Carassius auratus*. An immunocytochemical study. Cell Tissue Res 234:319-333

Kah O, Chambolle P, Dubourg P, Dubois MP 1982 Localisation immunocytochimique de la somatostatine dans le cerveau anterieur et l'hypophyse de deux Teleosteens, le Cyprin (*Carassius auratus*) et *Gambusia* sp. C R Acad Sci 294:519-524

Kah O, Dubourg P, Chambolle P, Calas A 1984 Ultrastructural identification of catecholaminergic fibers in the goldfish pituitary. Cell Tissue Res 238:621-626

MacKenzie DS, Gould DR, Peter RE, Rivier JE, Vale WW 1984 Response of superfused goldfish pituitary fragments to mammalian and salmon gonadotropin-releasing hormone. Life Sci 35:2019-2026

Marchant TA, Chang JP, Nahorniak CS, Peter RE 1989a Evidence that gonadotropin-releasing hormone also functions as a growth hormone-releasing factor in the goldfish. Endocrinology 124:2509-2518

Marchant TA, Dulka JG, Peter RE 1989b Relationship between serum growth hormone levels and the brain and pituitary content of immunoreactive somatostatin in the goldfish, *Carassius auratus*. Gen Comp Endocrinol 73:458-468

Marchant TA, Fraser RA, Andrews PC, Peter RE 1987 The influence of mammalian and teleost somatostatins on the secretion of growth hormone from goldfish (*Carassius auratus L*) pituitary fragments *in vitro*. Regul Pept 17:41-52

Peter RE, Chang JP, Nahorniak CS, Omeljaniuk RJ, Sokolowska M, Shih SH, Billard R 1986 Interactions of catecholamines and GnRH in regulation of gonadotropin secretion in teleost fish. Recent Prog Horm Res 42:513-548

Peter RE, Yu KL, Marchant TA, Rosenblum PM 1990 Direct neural regulation of the teleost adenohypophysis. J Exp Zool [Suppl] 4:84-89

Somoza GM, Peter RE 1991 Effects of serotonin on gonadotopin and growth hormone release from *in vitro* perifused goldfish pituitary fragments. Gen Comp Endocrinol 82:103-110

Wong AOL, Chang JP, Peter RE 1992 Dopamine stimulates growth hormone release from the pituitary of goldfish, *Carassius auratus*, through the dopamine-1 receptors. Endocrinology 130:1201-1210

Wong AOL, Chang JP, Peter RE 1993a Dopamine functions as a growth hormone-releasing factor in the goldfish, Carassius auratus. Fish Physiol Biochem (in press)

Wong AOL, Chang JP, Peter RE 1993b *In vitro* and *in vivo* evidence that dopamine exerts growth hormone-releasing activity in the goldfish. Am J Phy (press)

Appendix III

Seasonality of Salmon Gonadotropin-Releasing Hormone-stimulated Growth Hormone Release from Perifused Goldfish Pituitary Fragments

A3.1 Introduction

In the goldfish, growth hormone (GH) levels in circulation exhibit a seasonal pattern closely associated with the reproductive cycle; serum GH levels increase rapidly during gonadal recrudescence, remain at high levels throughout the spawning and post-spawning season, and gradually return to low levels when the fish become sexually regressed (Marchant and Peter, 1986). Somatostatin (SRIF) is a known GH-release inhibitor in the goldfish (Cook and Peter, 1984; Marchant *et al.*, 1987). SRIF contents in the goldfish hypothalamus exhibit an inverse relationship with respect to the serum GH levels, being the highest in sexually regressed and the lowest in sexually mature (=prespawning) fish (Marchant *et al.*, 1989a). These results indicate that the seasonal variation of serum GH levels in the goldfish is regulated by neuroendocrine factors from the hypothalamus.

Based on the earlier research in our laboratory, salmon gonadotropin (GTH)-releasing hormone (sGnRH) was shown to be stimulatory to GH and GTH-II release in the goldfish, both *in vivo* and *in vitro* (Marchant *et al.*, 1989a). More recently, GnRH receptors have been identified in the goldfish somatotrophs (Cook *et al.*, 1991), suggesting that sGnRH also functions as a physiological GH-releasing facor in the goldfish. By using an *in vitro* perifusion system for goldfish pituitary fragments, the seasonality of sGnRH-stimulated GH release was examined in the present study.

A3.2 Materials and Methods

Goldfish of the common or comet varieties were purchased from Ozark Fisheries (Stoutland, MO, USA) or Grassyforks Fisheries (Martinsville, IN, USA), and maintained in flow-through aquaria (1,800 liters) at 17 °C under a simulated natural

photoperiod (Edmonton, AB, Canada) for at least 3 weeks prior to any experiments. The fish were fed to satiation daily with Ewos trout pellets (Astra Chemicals Ltd., Mississauga, ONT, Canada). Goldfish of both sexes, with body weight ranging from 20 g to 30 g, were used in the present study. Gonadal conditions of the fish, determined by the gonadosomatic index (GSI = weight of gonad / total body weight x 100 %) as well as by the morphological characteristics of the gonad (for details, see chapter 2), are reported separately for the individual experiments. An *in vitro* perifusion system for goldfish pituitary fragments described by Mackenzie *et al.* (1984) was used to examine the responsiveness of the goldfish pituitary to sGnRH stimulation. sGnRH (Peninsula Laboratories Inc., Belmont, CA, USA) at 50 nM dose was administered as 2-min pulses to stimulate GH release from pituitary fragments prepared from the goldfish at different reproductive stages. Perifusates were collected in 5-min fractions, and stored at -25 °C until their hormone contents were assayed by a GH radioimmunoassay (Marchant *et al.*, 1989b). The GH data were transformed into "% pretreatment" as defined previously (Wong *et al.*, 1992), and the GH responses were quantified by calculating the net change in GH release (i.e., area under the curve) after the sGnRH treatment.

A3.3 Results and Discussion

Pituitary fragments from goldfish in different reproductive stages, including sexual regression (x6 columns), recrudescence (x4 columns), and sexual maturation (=prespawning fish; x4 columns), were used to investigate the seasonality of sGnRH-stimulated GH release (Fig. A3.1). sGnRH at 50 nM dose was previously validated to exert a maximal stimulatory effect on GH release from perifused goldfish pituitary fragments (Marchant *et al.*, 1989a). In the present study, the GH responses to 50 nM sGnRH were found to be the highest in sexually mature, intermediate in recrudescent, and the lowest in sexually regressed goldfish. These results demonstrate that the gradual maturation of the gonad can be correlated with an enhancement in GH responses to sGnRH stimulation, suggesting that sGnRH-stimulated GH release in the goldfish may be under the positive modulation of gonadal factor(s). This hypothesis is also in agreement with our previous castration studies using prespawning goldfish (see chapter 4), in which surgical removal of the gonads significantly reduced the GH responses to

sGnRH. In a separate study by Habibi *et al.* (1989), it was shown that the GnRH receptor binding capacity in the goldfish pituitary is at the lowest in sexually regressed and the highest in sexually mature (=prespawning) fish. Furthermore, no significant changes in GnRH receptor affinity can be observed throughout the reproductive cycle. Therefore, the high responsiveness to sGnRH on GH release in prespawning goldfish is likely due to an increase in GnRH receptor capacity in the somatotrophs during the period of sexual maturation.

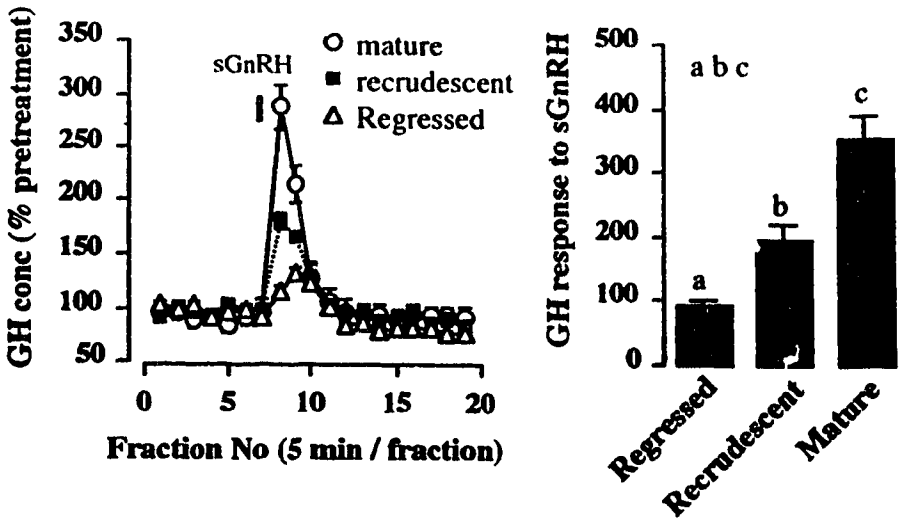


Fig. A3.1 Effects of sGnRH on GH release from perifused pituitary fragments of goldfish in sexual regression (x6 columns), recrudescence (x4 columns) and maturation (x4 columns). sGnRH at 50 nM dose was administered as 2-min pulses (as indicated by the black bar). The average pretreatment GH levels for perifusion were 42 ± 2 ng/ml for sexually regressed fish, 38 ± 3 ng/ml for recrudescent fish, and 42 ± 4 ng/ml for sexually mature (prespawning) fish, respectively. All GH data are expressed as mean ± SEM (n = 4 - 6). GH responses, quantified as area under the curve, are presented in the right panel. GH responses with similar magnitude were grouped within the same underscore (P>0.05, ANOVA followed by Fisher's LSD test).

A3.4 References

Cook H, Berkenbosch JW, Fernhout MJ, Yu KL, Peter RE, Chang JP, Rivier JE 1991 Demonstration of gonadotropin releasing-hormone receptors on gonadotrophs and somatotrophs of the goldfish: an electron microscope study. Regul Pept 36:369-378

Cook AF, Peter RE 1984 The effects of somatostatin on serum growth hormone levels in the goldfish, *Carassius auratus*. Gen Comp Endocrinol 54:109-113

Habibi HR, de Leeuw R, Nahorniak CS, Goos HJTh, Peter RE 1989 Pituitary gonadotropin-releasing hormone (GnRH) receptor activity in goldfish and catfish : Seasonal and gonadal effects Fish Physiol Biochem 7:109-118

MacKenzie DS, Gould DR, Peter RE, Rivier JE, Vale WW 1984 Response of superfused goldfish pituitary fragments to mammalian and salmon gonadotropin-releasing hormone. Life Sci 35:2019-2026

Marchant TA, Chang JP, Nahorniak CS, Peter RE 1989a Evidence that gonadotropin-releasing hormone also functions as a growth hormone-releasing factor in the goldfish. Endocrinology 124:2509-2518

Marchant TA, Dulka JG, Peter RE 1989b Relationship between serum growth hormone levels and the brain and pituitary content of immunoreactive somatostatin in the goldfish, *Carassius auratus*. Gen Comp Endocrinol 73:458-468

Marchant TA, Fraser RA, Andrews PC, Peter RE 1987 The influence of mammalian and teleost somatostatins on the secretion of growth hormone from goldfish (*Carassius auratus L*) pituitary fragments *in vitro*. Regul Pept 17:41-52

Marchant TA, Peter RE 1986 Seasonal variations in body growth rates and circulating levels of growth hormone in the goldfish, *Carassius auratus*. J Exp Zool 237:231-239

Wong AOL, Chang JP, Peter RE 1992 Dopamine stimulates growth hormone release from the pituitary of goldfish, *Carassius auratus*, through the dopamine D1 receptors. Endocrinology 130:1201-1210