University of Alberta

Regulation of Pituitary Somatotropes by Endogenous Somatostatins

by

Warren Keith Yunker ©

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

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"Who hears the fishes when they cry? It will not be forgotten by some memory that we were contemporaries."

H. D. Thoreau

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled Regulation of Pituitary Somatotropes by Endogenous Somatostatins submitted by Warren Keith Yunker in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Physiology and Cell Biology.

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Abstract

The regulation of pituitary somatotrope function in goldfish (*Carassius auratus*) by the somatostatin (SS) neuropeptide family was examined in the present study. Using reverse transcription-polymerase chain reaction and Southern blot analysis, mRNA for three different SS precursors (PSS-I, -II, and -III), which encode for the putative peptides SS_{14}, goldfish brain (gb)SS_{28} and [Pro^2]SS_{14}, respectively, were detected in goldfish hypothalamus. Interestingly, PSS-I and -II mRNA, but not PSS-III mRNA, were also detected in cultured pituitary cells. The effects of SS_{14}, gbSS_{28} and [Pro^2]SS_{14} on somatotrope signalling and growth hormone (GH) secretion were subsequently examined. SS_{14} and [Pro^2]SS_{14} were similar in their effects on somatotrope 3',5'-cyclic adenosine 5'-monophosphate levels and GH secretion but differed markedly from those of gbSS_{28}. This suggests that, in goldfish, different SS peptides may be responsible for selectively regulating different aspects of somatotrope function.

The ability of SS_{14} to inhibit basal and stimulated GH secretion was further investigated using single-cell Ca^{2+} imaging and time-matched column perifusion GH release experiments. As expected, application of various natural GH-releasing ligands, as well as pharmacological activators of their respective intracellular signalling cascades, stimulated GH release and increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) within single identified somatotropes. Furthermore, buffering these increases in $[Ca^{2+}]_i$ with a Ca^{2+} chelator impaired the corresponding GH responses. Surprisingly, SS_{14} reduced basal GH release without altering $[Ca^{2+}]_i$ in single identified somatotropes. Furthermore, stimulated increases in $[Ca^{2+}]_i$ persisted despite SS_{14} inhibition of stimulated GH

secretion. These results establish that, in goldfish, SS_{14} does not abolish stimulated Ca^{2+} signals as a means of inhibiting stimulated GH secretion. This type of regulatory mechanism would allow for the differential regulation of hormone release and other Ca^{2+}-dependent cellular processes by SS_{14}. The data also suggest that the cellular mechanisms underlying the observed effects of SS_{14} on Ca^{2+} signalling may be unrelated to those responsible for inhibiting GH release.

Overall, by comparing the effects of three closely related endogenous SS isoforms and subsequently examining, more closely, the intracellular mechanisms of one these peptides, this thesis provides evidence for the differential regulation of cellular functions at both the extracellular and intracellular levels.

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List of Nomenclature and Abbreviations

$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration
5-HT	Serotonin
A	Adenine
AA	Arachidonic acid
AC	Adenylate cyclase
AGH	Aminoguanidine hemisulfate
Ala	Alanine
ANOVA	Analysis of variance
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
ATP	Adenosine 5'-triphosphate
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BAPTA-AM	BAPTA-tetraacetoxymethyl ester
Bay K 8644	1,4-dihydro-2,6-dimethyl-5-nitro-4-[2′-(trifluoromethyl)phenyl]-3-pyridinecarboxylic acid methyl ester
BHQ	2,5-di(*tert*-butyl)-1,4-benzohydroquinone
bp	Base pairs
BSA	Bovine serum albumin
C	Cytosine
cADP	Cyclic adenosine diphosphate
CaM KII	CaM kinase II
CaM	Calmodulin
cAMP	3',5'-cyclic adenosine 5'-monophosphate
cDNA	Complementary DNA
cGMP	3',5'-cyclic guanosine 5'-monophosphate
cGnRH-II	Chicken GnRH-II
CICR	Ca^{2+}-induced Ca^{2+} release
CRE	cAMP response element
cSS_{22}	Catfish SS_{22}
CST	Cortistatin
CV	Coefficient of variation
Cys	Cysteine
D1	DA type 1 receptor
D2	DA type 2 receptor
DA	Dopamine
DAG	Diacylglycerol
dCTP	Deoxy-cytidine 5'-triphosphate
DIC	Differential interference contrast
DiC8	Dioctanoyl glycerol
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid

EDTA	Ethylenediamine tetra-acetate
EGTA	Ethyleneglycol-bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid
Forskolin	7β-Acetoxy-8,13-epoxy-1α,6β,9α-trihydroxylabd-14-en-11-one
Fura-2 AM	5-(2-(2-(bis(2- ((acetyloxy)methoxy)-2-oxoethyl)amino)- 5-methylphenoxy)ethoxy)-2-benzofuranyl)- , (acetyloxy)methyl ester
G	Guanine
$gbSS_{28}$	Goldfish brain SS_{28}
gfsst	Goldfish sst
GH	Growth hormone
GHRH	GH-releasing hormone
GHRP	GH-releasing peptide
$giSS_{28}$	Goldfish intestine SS_{28}
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
GnRH	Gonadotropin-releasing hormone
G-protein	Guanosine 5'-triphosphate-binding protein
H89	N-[2-((*p*-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide
$hCST_{17}$	Human CST_{17}
$hCST_{29}$	Human CST_{29}
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid
hsst	Human sst
IC_{50}	Median inhibitory concentration
IP_3	Inositol (1,4,5)-trisphosphate
IP_3R	IP_3 receptor
irNOS	Immunoreactive NOS
irPACAP	Immunoreactive PACAP
irSS	Immunoreactive SS
K_{ir}	Inwardly rectifying K^+ channel
Leu	Leucine
LSD	Least significant difference
Lys	Lysine
Met	Methionine
mRNA	Messenger RNA
mSS_{28}	Mammalian SS_{28}
NE	Norepinephrine
NOS	NO synthase
NO	Nitric oxide
P	Probability
PAC_1	PACAP type 1 receptor
PACAP	Pituitary adenylate cyclase-activating polypeptide
PCR	Polymerase chain reaction
Phe	Phenylalanine
PKA	Protein kinase A
PKC	Protein kinase C

PLA_2	Phospholipase A_2
PLC	Phospholipase C
PPSS	PreproSS
Pro	Proline
PSS	ProSS
$rCST_{14}$	Rat CST_{14}
$rCST_{29}$	Rat CST_{29}
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-PCR
Ry	Ryanodine
RyR	Ry-receptor
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
Ser	Serine
SERCA	Sarcoplasmic reticulum/endoplasmic reticulum Ca^{2+}-ATPases
PMCA	Plasma membrane Ca^{2+}-ATPases
Tg	Thapsigargin
sGnRH	Salmon GnRH
SKF-38393	(±)-1-phenyl-2,3,4,5-tetrahydro-(1*H*)-3-benzazepine-7,8-diolhydrochloride
SNARE	Soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor
SNP	Sodium nitroprusside
SS	Somatostatin
sSS_{25}	Salmon SS_{25}
sst	SS receptor
T	Thymine
Thr	Threonine
TMB-8	8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate
TPA	Tetradecanoyl phorbol acetate
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine
VSCC	Voltage-sensitive Ca^{2+} channel

Chapter 1 – General Introduction

1.1 Introduction

The tetradecapeptide somatostatin (SS_{14}) was originally isolated from ovine hypothalamus by Brazeau et al. in 1973 (1) on the basis of its ability to inhibit growth hormone (GH) secretion. Today, the term somatostatin (SS) comprises a multigene peptide family that spans the vertebrate classes (reviewed in (2-4)). Surprisingly, immunoreactive and/or biologically active SS has also been identified in plants (5, 6) and even a ciliated protozoan (7). This latter finding suggests that the SS gene(s) may have evolved prior to the appearance of multicellular organisms (3).

The physiological role of SS_{14} as a hypothalamic regulator of somatotrope GH release has been extensively studied. SS_{14} is effective in reducing, both *in vivo* and *in vitro*, basal GH release, as well as physiologically and pharmacologically stimulated GH secretion. However, SS actions may be somewhat species-specific. For example, SS has been shown to have no effect on basal GH release in cultures of chicken (8) and bovine (9) pituitary cells. In contrast, SS is effective in reducing basal GH release in rat (10), sheep (11) and goldfish (12) pituitary cultures. As such, studies examining SS regulation of somatotrope function in a variety of different secretory systems may reveal novel insights into SS biology and/or somatotrope regulation.

In goldfish (*Carassius auratus*), at least three distinct SS genes, each encoding for a different peptide, are expressed in the hypothalamus (13). In addition, *in vivo* GH release is known to be regulated by a variety of stimulatory and inhibitory factors, and the signal transduction cascades of several of these factors have been extensively studied *in vitro* (reviewed in (14)). The result is an excellent system in which to study SS regulation of GH release, the intracellular mechanisms mediating this regulation, and the interactions of these mechanisms with the second messenger systems of other regulatory factors.

Although the role of SS as a hypothalamic regulator of GH secretion has been well established, SS peptides are also known to be physiological regulators of cellular

functions in pancreatic islets, the gastrointestinal tract and the immune system. In addition, the SS system has also been implicated in several pathophysiological disease states, such as Alzheimer's disease, Huntington's disease and epilepsy (reviewed in (15-18)). Obviously, the biological importance of SS peptides is not limited to that of GH inhibition. However, it is impossible to address such diversity in a single review. As such, the focus of this chapter will be on the SS peptide family, and its regulation of GH secretion in mammals and teleost fish, in particular goldfish.

1.2 SS Peptides in Mammals

Like other peptide hormones, SS peptides are derived from larger prohormones that are enzymatically cleaved to yield mature hormones. In mammals, there are two biologically active forms of SS, SS_{14} and mammalian (m)SS_{28} (Fig. 1.1). Both peptides are derived from the same gene product, preprosomatostatin-I (PPSS-I). PPSS-I is rapidly cleaved into the prohormone prosomatostatin (PSS-I), which is subsequently processed to SS_{14} and mSS_{28} through endoproteolytic processing (Fig. 1.1). Cleavage at a dibasic Arg-Lys site, likely by the pro-protein convertases PC1 and PC2, yields SS_{14} and an 8 kDa peptide while cleavage at a monobasic Arg, likely by the trans Golgi peptidase furin (19), yields mSS_{28} and a 7 kDa peptide. In addition, there is a monobasic Lys processing site in the N-terminal region of the prohormone which generates the decapeptide antrin, also referred to as prosomatostatin$_{(1\text{-}10)}$. In all, there are six known cleavage products of mammalian PSS-I (Fig. 1.1); however, SS_{14} and mSS_{28} are the only biologically active products. Antrin and the remaining 3 peptides are devoid of any known activity (reviewed in (2, 19, 20)).

SS_{14} and mSS_{28} are cyclic in nature as a result of a disulfide bond between Cys^3 and Cys^{14} (1). Structure-function studies have revealed that Phe^6-Phe^7-Trp^8-Lys^9-Thr^{10}-Phe^{11} plus the disulfide bridge are necessary for SS_{14} inhibition of GH release (21); however, Phe^7 and Thr^{10} can undergo minor substitutions, such as $Phe^7 \rightarrow Tyr^7$ and $Thr^{10} \rightarrow Gly^{10}$ (reviewed in (22)).

Recently, a related neuropeptide, cortistatin (CST), was cloned from human, mouse and rat sources (23, 24). In humans and rats, preprocortistatin processing putatively yields short and long form cleavage products analogous to SS_{14} and mSS_{28}. These products consist of human (h)CST_{17} and its homologue rat (r)CST_{14}, as well as $hCST_{29}$ and $rCST_{29}$. There is little structural similarity between the N-terminal 15/14 amino acids of $hCST_{29}$ and mSS_{28}. However, there is a great deal of structural similarity in the C-terminal regions of these peptides. For example, rCST shares 11 of its 14 amino acid residues with SS_{14}, including the two cysteine residues that render SS_{14} cyclic. As in the case of SS_{14} and mSS_{28}, the predicted secondary structures of the CST peptides are cyclic (reviewed in (25)).

1.2.1 Anatomical Distribution of SS_{14}, mSS_{28} and CST in Mammals

Both SS_{14} and mSS_{28} have been isolated from the hypothalamus and, as such, are theoretically capable of regulating pituitary function in a neuroendocrine manner (1, 26). Although the co-existence of SS_{14} and mSS_{28} is not restricted to the hypothalamus, the relative content of SS_{14} and mSS_{28} varies according to the tissue being considered (27). In the cerebral cortex and retina, SS_{14} exceeds mSS_{28} by a ratio of approximately 4:1, while in the retina, peripheral nerves, pancreas and stomach, SS_{14} is virtually the only form found (28, 29). In contrast, mucosal cells within the gut synthesize mainly mSS_{28}. Both SS_{14} and mSS_{28} are detectable within the blood (27), with circulating SS peptides being rapidly inactivated by the liver and kidneys. In humans, the plasma half-life of SS_{14} is 2-3 min, while mSS_{28} is slightly more resistant to inactivation (2).

In comparison, CST was named for its predominantly cortical expression pattern. Using *in situ* hybridization, CST messenger ribonucleic acid (mRNA) expression has been shown to be essentially restricted to the cerebral cortex and hippocampus (24, 30). However, expressed sequence tags for CST complementary (c) deoxyribonucleic acid (DNA) obtained from some peripheral tissues including fetal heart, fetal lung, prostate and certain tumors have been discovered in the expressed sequence tag database (GenBank) (25).

1.2.2 Regulation of SS Gene Expression and Secretion in Mammals

1.2.2.1 SS Gene Structure

The rat SS gene consists of exons of 238 and 367 base pairs (bp) separated by an intron of 621 bp. The 5' upstream region contains a number of regulatory elements, including, among other things, a TATA box, a 3',5'-cyclic adenosine 5'-monophosphate (cAMP) response element (CRE), and two silencer elements (reviewed in (20, 31)).

1.2.2.2 Regulation of Hypothalamic SS Gene Expression and Secretion

Hypothalamic PSS-I mRNA levels and the secretion of the mature peptides are differentially controlled by a variety of regulatory factors. Briefly, GH, GH-releasing hormone (GHRH) and dopamine (DA) all act on hypothalamic somatostatinergic neurons to increase PSS-I mRNA (32-35) and SS peptide secretion (33, 34, 36-39), whereas, SS autoregulates itself by diminishing hypothalamic PSS-I mRNA (40) and SS peptide secretion (41). Short-term glucocorticoid treatment (42), as well as testosterone (43), estrogen (44), insulin-like growth factor-I (32) and numerous cytokines have also been shown to elevate PSS-I mRNA levels (reviewed in (31)). Opposing these stimulatory factors are SS-release inhibitors, which include, γ-aminobutyric acid (45, 46) and leptin (47). Leptin (47) and long-term exposure to glucocorticoids (42) both negatively regulate PSS-I mRNA expression.

Of the stimulatory factors known to increase PSS-I mRNA, only the molecular mechanisms underlying regulation by glucocorticoid, which activates gene transcription through the cAMP/CRE pathway, has been elucidated (48). The molecular mechanisms underlying stimulation of SS gene transcription by other factors, such as cytokines, remain to be clarified. Similarly, the mechanism underlying inhibition of SS gene transcription are also unknown.

1.3 SS Peptides in Goldfish

In goldfish, PSS-I is thought to be cleaved at a dibasic Arg-Lys site yielding an SS_{14} whose predicted amino acid sequence is identical to that found in mammals (13) (Fig. 1.2). Unlike mammalian PSS-I, goldfish PSS-I does not contain a monobasic Arg cleavage site capable of yielding a 28 amino acid SS. Although there is a cleavage site capable of producing a 26 amino acid SS, such a product has not been identified (13).

Unlike mammals, teleost fish, such as goldfish, possess a second PSS, PSS-II. PSS-II cDNA, or their gene products, which have been identified in numerous other teleost species, including anglerfish, rainbow trout, coho salmon and several others (reviewed in (4)), all contain [Tyr^7, Gly^{10}]SS_{14} in the C-terminus of the mature peptide (reviewed in (49)). Although goldfish PSS-II contains cleavage sites capable of generating both SS_{14} and SS_{28} peptides, studies in anglerfish have shown that SS_{14} and SS_{28} are separate, independent products of PSS-I and PSS-II, respectively (50-53). As such, goldfish PSS-II is believed to yield a 28 amino acid peptide. This peptide, which contains [Glu^1, Tyr^7, Gly^{10}]SS_{14} at the C-terminus (13) (Fig. 1.2), is referred to as goldfish brain (gb)SS_{28}, as the cDNA encoding PSS-II was first identified in goldfish brain. However, a second 28 amino acid SS, which differs from the predicted sequence of $gbSS_{28}$ by 5 amino acids has been isolated from goldfish intestine, (gi)SS_{28} (54) (Fig. 1.2). Both $gbSS_{28}$ and $giSS_{28}$ contain $Phe^7 \rightarrow Tyr^7$ and $Thr^{10} \rightarrow Gly^{10}$ substitutions within the active region of the C-terminal SS_{14} (Fig. 1.2), and the existence of a C-terminal [Tyr^7, Gly^{10}]SS_{14} in both $gbSS_{28}$ and $giSS_{28}$ suggests that they are both products of PSS-II genes. However, whether these two forms of SS_{28} are products of the same gene or represent two different genes is unknown.

Having multiple PSS-II peptides is not unique to goldfish. Two PSS-II peptides, PSS-II1 and PSS-II2, capable of yielding a 28 and a 25 amino acid SS respectively, have been identified in rainbow trout (55). The existence of these two genes is believed to be the result of a Salmonidae tetraploidization event (55, 56). Whether tetraploidization is also responsible for the existence of $gbSS_{28}$ and $giSS_{28}$ is unknown. The PSS-II gene has not been identified in tetrapods (49), and it has been suggested that the PSS-II gene arose out of a duplication event within the teleost lineage prior to tetraploidization (4, 55, 56).

Interestingly, a third SS-encoding cDNA sequence has been identified in goldfish (13). This is the first documented occurrence of three SS genes within in a single vertebrate species (57). This third cDNA sequence encodes for a PSS-III containing [Pro^2]SS$_{14}$ at the C-terminus (Fig. 1.2). PSS-III contains a potential cleavage site for a 14 amino acid peptide, but not for a 28 amino acid peptide. There are, however, Arg monobasic cleavage sites capable of yielding a 24 and a 29 amino acid peptide. However, a [Pro^2]SS$_{14}$ peptide has also been identified in Russian sturgeon (58), thus, the occurrence of a [Pro^2]SS$_{14}$ peptide, rather than a longer 24 and/or 29 amino acid product, is a likely possibility in goldfish. Phylogenetic analysis suggests that PSS-III in goldfish is related to the [Pro^2, Met^{13}]SS$_{14}$ precursor in frog and the CST precursor in mammals (57, 59, 60).

1.3.1 Anatomical Distribution of SS$_{14}$, [Pro^2]SS$_{14}$ and gbSS$_{28}$ in Goldfish

mRNA for PSS-I, -II and -III have all been identified in goldfish brain. Unfortunately, a cDNA sequence encoding for giSS$_{28}$ has not yet been identified; consequently, whether the giSS$_{28}$ gene is expressed within the goldfish brain is unknown. Within the brain, the mRNA for SS$_{14}$, [Pro^2]SS$_{14}$ and gbSS$_{28}$ display differential, yet overlapping, patterns of expression. In particular, only [Pro^2]SS$_{14}$ mRNA is present within the olfactory bulb and tract, while both SS$_{14}$ and [Pro^2]SS$_{14}$ mRNA are detectable in the posterior brain. In contrast, mRNA for SS$_{14}$, [Pro^2]SS$_{14}$ and gbSS$_{28}$ can all be detected within the telencephalon, hypothalamus and optic tectum-thalamus regions (13).

Within the brain, hypothalamic lesioning studies have established that the preoptic area is the origin of some of the immunoreactive (ir)SS fibres that innervate the pituitary and control GH secretion (61). Which SS isoform(s) is/are contained within these projections is not known, as the antibody used in this study recognized both SS$_{14}$ and mSS$_{28}$, and its ability to recognize [Pro^2]SS$_{14}$ and gbSS$_{28}$ was not ascertained (61). Interestingly, in addition to its role as a hypothalamic neuroendocrine regulator of pituitary function, SS$_{14}$ may also be regulating pituitary function in a paracrine/autocrine

manner. This possibility is supported by the detection of PSS-I mRNA within freshly excised pituitary fragments (13).

Throughout the body, PSS-I, -II and -III mRNA are differentially expressed. Only PSS-I mRNA is present in the kidney; whereas, in the liver, PSS-II and -III mRNA, but not PSS-I mRNA, are detectable. Both PSS-I and -II mRNA are detectable within the gastrointestinal tract (62), as is the $giSS_{28}$ peptide (54).

1.3.2 Regulation of SS Gene Expression in Goldfish Forebrain

The regulatory regions of the goldfish PSS-I, -II and -III genes have not been ascertained. As such, the molecular mechanisms underlying their regulation cannot be discussed. However, sex- and season-dependent variations in the expression of these genes within the forebrain (which is comprised of the telencephalon, as well as the preoptic region and hypothalamus), as well as some of the factors that are likely responsible for these changes, have been identified.

In females, PSS-I mRNA is most abundant in December and April, and lowest in July; whereas in males, PSS-I mRNA levels are similar in April, July and December (13). This suggests that seasonal changes in PSS-I mRNA levels are only occurring in females. Seasonal changes in PSS-II mRNA also occur, however, the pattern of expression is the same for both males and females. In contrast to PSS-I, PSS-II mRNA is highest in July, and lowest in April and December (13). PSS-III mRNA levels also vary seasonally in both males and females. For males, PSS-III mRNA levels are high in July and December, and low in April. For females, PSS-III mRNA levels are high in July, and low in April and December (13). How these changes in PSS mRNA levels relate to possible seasonal variations in GH release is unclear. However, brain and pituitary irSS levels have been reported to fluctuate inversely with serum GH levels, which are highest in June and lowest in November (63). Interestingly, this pattern of change in brain irSS content is consistent with the observed seasonal variation in PSS-I mRNA levels in female, but not male, goldfish (13, 63). Seasonal changes in PSS-II and -III mRNA levels do not

correspond with seasonal variations in either GH or irSS in both male and female fish (13, 63).

Seasonal changes in PSS mRNA levels suggests that steroid hormones may be influencing PSS expression. In support of this, estradiol has been shown to significantly increase PSS-I and PSS-III, but not PSS-II, mRNA in the forebrains of both male and female goldfish (64).

Another regulator of SS gene expression in goldfish is the catecholamine DA (65). Both type 1 (D1) and type 2 (D2) DA receptors are involved in the regulation of all three SS genes in the goldfish brain. In male and female goldfish in the early stages of sexual recrudescence, D1 activation decreases PSS-I and -II mRNA. Interestingly, D1 regulation of PSS-III appears to be time-dependent. D1 receptor activation initially increases and then subsequently decreases PSS-III mRNA. In comparison, D2 actions on PSS-I expression are inhibitory in male and female fish in early gonadal recrudescence and stimulatory in late gonadal recrudescence. In male and female goldfish in the late stages of sexual recrudescence PSS-I and -II mRNA are increased by D2 mechanisms, whereas PSS-III mRNA is initially inhibited and then stimulated by D2 receptor activation. The fact that DA regulation is dependent upon the reproductive stage of the fish suggests an interaction between the sex steroids and dopaminergic pathways in the regulation of the PSS genes.

1.4 SS Receptors in Mammals

1.4.1 Receptor Pharmacology

Early work with SS analogues led to the recognition of two pharmacological subgroups of SS receptors (sst's). Group 1 receptors bound the octapeptide agonist SMS 201-995 (Octreotide) with high affinity, while group 2 receptors did not (66). Subsequently, in 1992, five different receptor subtypes (sst_{1-5}), all belonging to the seven transmembrane domain guanosine 5'-trisphosphate-binding protein (G-protein)-coupled surperfamily, were cloned (67-70). The pharmacologically defined group 1 receptors consist of sst_2, sst_3, sst_5, while the group 2 receptors consist of sst_1 and sst_4.

Both SS_{14} and mSS_{28} bind to all five sst subtypes with nanomolar affinity (71-74), as do $hCST_{17}$ and $rCST_{29}$ (75, 76) (Table 1.1). However, $hsst_5$ has been shown to exhibit a 10-15 fold selectivity for mSS_{28} compared to SS_{14} (75, 77). Recently, The Merck Research Group developed a non-peptide agonist for each of the five hsst's (78) (Table 1.1). The availability of these high-affinity, subtype-selective agonists was a major advancement that has enabled the physiological functions of the different sst's to be explored in non-transfected systems expressing more than one receptor subtype (79, 80) (see Section 1.4.3).

1.4.2 Neuroanatomical Distribution of Sst Subtypes

The neuroanatomical distribution of the sst subtypes have been well characterized in rodent and human tissues using Northern Blots, reverse transcriptase (RT)-polymerase chain reaction (PCR) and *in situ* hybridization. These studies have revealed an intricate, overlapping pattern of sst expression that is both subtype and tissue specific. For example, in rat, mRNA for sst_{1-5} have all been localized to the cerebral cortex, hippocampus, amygdala, olfactory bulb and preoptic area (81). However, relative to the other subtypes, sst_2 is expressed predominately within the cerebral cortex (82). Sst_5 mRNA is expressed throughout the rat brain (83), while sst_3 is preferentially located in the cerebellum (84). Compared to the other four receptors, sst_4 is the least well expressed subtype (83). Interestingly, the anatomical distribution of sst mRNA also appears to be species specific. For example, in rat, sst_5 mRNA is expressed throughout the brain (83), yet in the human brain, the expression of sst_5 mRNA is minimal (85, 86).

1.4.3 Sst Subtype-Specific Functions

The existence of multiple receptor subtypes that possess distinct, yet overlapping, patterns of expression has lead to the hypothesis that the different receptor subtypes are responsible for regulating different physiological and/or cellular functions. For example, in rat pituitary, mRNA for sst_1, sst_3 and sst_4 are present at very low levels, while sst_2 and sst_5 mRNA are highly expressed (reviewed in (87)). As a result, it has been proposed that

sst_2 and sst_5 are the primary regulators of somatotrope function. The use of subtype-specific agonists has since confirmed that sst_2 and sst_5, but not sst_1, sst_3 and sst_4, are coupled to the inhibition of GH release (78, 79).

1.5 Sst's in Goldfish

As with mammals, goldfish (gf)sst's are members of the seven transmembrane domain G-protein-coupled receptor family. To date, 8 gfsst's ($gfsst_{1A, 1B, 2, 3A, 3B, 5A, 5B, 5C}$) have been cloned from brain tissues (88-92). Pharmacological characterization of $gfsst_{5A}$ has shown that although it binds SS_{14}, $[Pro^2]SS_{14}$ and $gbSS_{28}$ with high affinity, it displays some selectivity for the 28 amino acid SS's (89). In COS cells expressing recombinant $gfsst_2$, cAMP levels are reduced by SS_{14} and $[Pro^2]SS_{14}$, but not by $gbSS_{28}$ (91). This suggests that SS action depends not only on what receptor subtypes are expressed, but also on the SS isoform present. Consistent with mammalian studies, $gfsst_2$ and $gfsst_5$ mRNA are more abundant than other gfsst subtype mRNA in the pituitary (87, 89, 91).

Somatostatin binding sites have also been characterized in goldfish brain using radiolabelled ^{125}I-$[Tyr^{11}]SS_{14}$ (93). Analysis revealed specific binding that was saturable, reversible, and time- and pH-dependent. Radiolabelled SS_{14} displacement by mSS_{28} and salmon (s)SS_{25}, which may be a homologue of $gbSS_{28}$ (94), was similar to that of unlabelled SS_{14} (93). Although $[Pro^2]SS_{14}$ and $gbSS_{28}$ were not directly examined in this study, it was hypothesized that they too would displace radiolabelled SS_{14} in a similar fashion (93).

Anatomically, SS_{14} binding sites seem to be associated with 3 different systems in goldfish brain: (1) the preoptic-hypothalamic area, which is involved in the regulation of pituitary hormone secretion, (2) the optic tectum, which is involved in visual processing, and (3) the facial and vagal lobes, which are important in the regulation of feeding (93).

1.6 Ca^{2+} Signalling

Elevations in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), often referred to as Ca^{2+} signals, are an essential component of the transduction mechanisms mediating extracellular ligand-stimulated exocytosis. Not surprisingly, the molecular mechanisms underlying Ca^{2+} signal generation are dynamic and complex. The intracellular signalling cascades involved in regulating somatotrope GH release, including Ca^{2+} signal involvement, in both mammals and goldfish are discussed later in this chapter (Sections 1.7 and 1.8 respectively). The underlying molecular machinery responsible for Ca^{2+} signal generation are introduced below.

Ca^{2+} is compartmentalized within cells. Cytosolic Ca^{2+}, which is predominately bound to soluble Ca^{2+}-binding proteins, represents 10-20% of the total cellular Ca^{2+} (95). The remainder of the Ca^{2+} is contained within intracellular organelles, such as the endoplasmic reticulum, Golgi network, mitochondria, endosomes, lysosomes and secretory vesicles. Typically, in unstimulated secretory cells, the $[Ca^{2+}]$ in the cytosol is in the hundreds of nanomolar range, while the $[Ca^{2+}]$ within the intracellular organelles is in the millimolar range (95). A third, and practically unlimited source of Ca^{2+} for the cell, is the extracellular medium, which generally has a $[Ca^{2+}]$ of approximately 1 mM (96).

Cells elevate $[Ca^{2+}]_i$ through two, non-mutually exclusive, mechanisms. First, cells can allow extracellular Ca^{2+} to enter the cell. This may occur through voltage-sensitive Ca^{2+} channels (VSCC), voltage-independent ion channels, or other ion exchange systems. Second, the Ca^{2+} contained within intracellular stores can pass through intracellular Ca^{2+} channels located on these stores and enter the cytosol (reviewed in (97-100)). There are two major classes of intracellular Ca^{2+} channels, inositol (1,4,5)-trisphosphate (IP_3)-sensitive receptor (IP_3R) channels and ryanodine (Ry)-receptor (RyR) channels (101). Another essential component of the intracellular Ca^{2+} signalling machinery are the Ca^{2+} pumps , which are responsible for removing Ca^{2+} from the cytosol.

1.6.1 IP_3R Channels

Activation of plasma membrane receptors capable of triggering phospholipase C (PLC), such as certain seven-transmembrane-domain receptors and tyrosine kinase receptors, can result in the hydrolysis of phosphatidylinositol-4,5-bisphosphonate to IP_3 and diacylglycerol (DAG) (102, 103). DAG remains in the plasma membrane to act on protein kinase C (PKC), while IP_3 is free to diffuse into the cytosol and release Ca^{2+} from intracellular stores expressing IP_3R channels. In the presence of stimulatory concentrations of IP_3, low concentrations of cytosolic Ca^{2+} promote IP_3R channel activity while high concentrations of Ca^{2+} inhibit it (104). In addition to IP_3, several other factors are known to modulate the activity of IP_3R channels, including luminal Ca^{2+}, protein kinase A (PKA), PKC, Ca^{2+}/calmodulin (CaM)-dependent protein kinase (K) II and pH. As such, IP_3R channels not only initiate Ca^{2+} signals, but are also actively involved in the integration of different intracellular signals.

1.6.2 RyR Channels

Initially isolated by their ability to bind the plant alkaloid ryanodine (105, 106), the RyR channels constitute a second class of intracellular Ca^{2+}-release channel. There are three members of the RyR family, type I, II and III (reviewed in (107)). Cyclic adenosine diphosphate (ADP) ribose, which is synthesized by ADP-ribosyl cyclase in response to the activation of certain membrane bound receptors, stimulates RyR type II to release Ca^{2+} (reviewed in (107)). Endogenous ligands for type I and III RyR channels are currently unknown, however, an increase in $[Ca^{2+}]_i$ can in itself activate all three RyR channel types, thereby inducing further Ca^{2+} release. This processes is referred to as Ca^{2+}-induced Ca^{2+} release (CICR) (101, 108). At high concentrations, caffeine sensitizes the RyR to resting $[Ca^{2+}]_i$, enabling CICR to occur at resting $[Ca^{2+}]_i$ levels (100).

1.6.3 Ca^{2+} Pumps

The clearance of Ca^{2+} from the cytosol occurs through two well-studied pathways, the Ca^{2+}-adenosine 5'-triphosphatases (ATPases) and the electrogenic Ca^{2+} uniporters.

The Ca^{2+}-ATPases consists of the sarcoplasmic reticulum/endoplasmic reticulum Ca^{2+}-ATPases (SERCA's) and the plasma membrane Ca^{2+}-ATPases (PMCA's). Electrogenic Ca^{2+} uniporters, which operate in mitochondria, utilise the mitochondrial membrane potential to draw in Ca^{2+}, while SERCA's and PMCA's actively remove Ca^{2+} from the cytosol in an ATP-dependent manner (reviewed in (96)). There are several specific SERCA inhibitors, including thapsigargin (Tg) and 2,5-di(*tert*-butyl)-1,4-benzohydroquinone (BHQ). Tg has a high affinity for SERCA's and irreversibly blocks them in their Ca^{2+}-free state. Following long term treatment with SERCA inhibitors, agonists are unable to induce Ca^{2+} release from SERCA-expressing stores due to a complete discharge of their luminal Ca^{2+} (reviewed in (96)).

1.6.4 Intracellular Ca^{2+} Buffers

A rise in $[Ca^{2+}]_i$ can occur locally, in close proximity to one or more Ca^{2+} channels, or globally, when the Ca^{2+} signal spreads throughout the cytoplasm. However, the diffusion of Ca^{2+} within the cytosol is limited by the presence of Ca^{2+} buffers. The cytosol contains slowly diffusible Ca^{2+}-binding proteins capable of rapidly buffering elevations in cytosolic Ca^{2+} (reviewed in (100)). In fact, the ratio of free to bound Ca^{2+} in the cytosol is approximately 1:100 (109). As a result, the spread of a Ca^{2+} signal is a balance between the entry of Ca^{2+} into the cytosol, and its binding to and release from soluble Ca^{2+}-buffering proteins.

1.6.5 Ca^{2+}-Controlled Enzymes

One of the most important Ca^{2+}-binding proteins in non-muscle cells is CaM, which has been linked to the control of over 20 different intracellular enzymes (96). The Ca^{2+}-CaM complex can bind directly to proteins to modulate their activity, or the activated CaM can exert its regulatory effects through the activation of a host of CaM-dependent protein kinases and phosphatases (96, 110). The Ca^{2+}-CaM-dependent kinase family consists of myosin light chain kinase, phosphorylase kinase and the CaM kinases I, II, III and IV (reviewed in (96)). CaM KII is the most multifunctional member of the

family, having been implicated in the regulation of numerous different cellular processes (111).

However, even in the absence of CaM, Ca^{2+} is able to regulate the activity of certain intracellular enzymes. Most notably, several isozymes of PKC are known to have their activity modulated by changes in $[Ca^{2+}]_i$ (112, 113). PKC is essential in the control of gene expression, modulation of ion channels and exocytosis. Interestingly, these cellular processes are also controlled by Ca^{2+} independent of Ca^{2+} regulation of PKC activity (96). In addition to PKC, Ca^{2+} also regulates PLC and phospholipase A_2 (PLA_2) activity, both of which can also be regulated by PKC (96, 114). The result is an intracellular signalling network that allows for numerous regulatory interactions between the molecules involved.

1.6.6 Ca^{2+} Signals and Exocytosis

Increases in $[Ca^{2+}]_i$, resulting either from the application of natural ligands or pharmacologic compounds, can trigger hormone release in endocrine cells (96). Unfortunately, the process(es) through which Ca^{2+} signals are translated into increases in hormone release is not well understood. The complex interaction of the numerous cytoplasmic and membrane-bound proteins responsible for the fusion of secretory granules with the plasma membrane is referred to as the SNARE hypothesis (reviewed in (115, 116)). It has been proposed that one of these proteins, synaptotagmin I, functions as the Ca^{2+} sensor responsible for initiating membrane fusion in the presence of elevated $[Ca^{2+}]_i$ (117). However, how this final fusion step is related to agonist-evoked Ca^{2+} signals is unclear. Although it is likely that localized Ca^{2+} signals provide the Ca^{2+} necessary to trigger exocytosis, Ca^{2+} is a dynamic second messenger that is able to control the activity of several of the enzymes involved in stimulating secretion. As such, it seems likely that the function of Ca^{2+} signals is not merely to provide the Ca^{2+} necessary to trigger exocytosis.

1.7 Regulation of GH Release in Mammals

In mammals, the most well-studied regulators of somatotrope GH release are the stimulatory factor GHRH and the inhibitory factor SS. The release of GH from the pituitary is pulsatile, which is thought to result from the oscillating release of SS and GHRH into the hypothalamo-hypophysial portal system. This is known as the dual regulator hypothesis. However, due in large part to gonadal steroids, the pulsatility of GH release is sexually dimorphic. In rats, females have rapid, irregular GH pulses that are lower in amplitude than those seen in males (118). In male rats, GH pulses are more regular, occurring every 3.3 h; between pulses basal GH levels are undetectable (119). The generation of these rhythms is the result of a complex interplay of pulsatile GHRH release, interactions between SS- and GHRH-containing neurons, sex steroids and the negative feedback of GH on its own release through GH receptors on SS neurons in the brain (reviewed in (118, 120)).

Recently, the dual regulator hypothesis has been challenged. In particular, additional compounds, such as ghrelin and pituitary adenylate-cyclase activating polypeptide (PACAP) are emerging in the literature as important regulators of the GH axis. The mechanisms underlying GHRH and SS regulation of GH release are discussed below, for information on the mechanisms mediating PACAP and ghrelin regulation of somatotrope function in mammals, the reader is referred to several excellent reviews (121-124).

1.7.1 GHRH

Two forms of GHRH have been identified in human hypothalamus, a 44 amino acid form, $GHRH_{44}$, and a 40 amino acid form which differs from $GHRH_{44}$ by the absence of the four C-terminal amino acids (reviewed in (125)). Both forms are encoded by a single gene. In mice and other non-human species, short forms of GHRH analogous to human $GHRH_{40}$ are absent (reviewed in (125)).

GHRH stimulation of GH release involves a classical adenylate cyclase (AC)/cAMP/PKA signal transduction cascade (126). The GHRH receptor is positively coupled to AC through a G_s-protein (127). The increased level of cAMP activates PKA,

which subsequently phosphorylates VSCC's, thereby increasing their probability of opening (128). Accompanying the increased VSCC activity is a dramatic increase in $[Ca^{2+}]_i$ (129). In the absence of extracellular Ca^{2+}, GHRH is unable to stimulate GH release, suggesting that Ca^{2+} influx and the corresponding increase in $[Ca^{2+}]_i$ are essential for GHRH stimulation of GH release (129, 130).

However, a limited number of studies have raised the possibility that additional signalling mechanisms, in particular IP_3 and intracellular Ca^{2+} stores, are also involved in GHRH-mediated GH release (121, 131). Whether this signalling heterogeneity represents signalling differences within somatotrope subpopulations or different GHRH receptors is still under investigation.

1.7.2 SS

In mammals, SS_{14} and mSS_{28} act through the same family of receptors to inhibit GH secretion. Given that mSS_{28} is an N-terminal extension of SS_{14}, the majority of mammalian studies have considered these two peptides to be functionally equivalent. Consequently, any differences in SS_{14} and mSS_{28} signalling that may exist have received very little attention. As such, SS_{14} and mSS_{28} will, for this section, be collectively referred to as SS unless otherwise stated.

In vivo administration of SS in humans blocks GH secretory responses to exercise, hypoglycemia and arginine (132-134). Similarly, i*n vitro*, SS has a profound inhibitory effect on GH release in response to all physiological and pharmacological stimuli. The pronounced ability of SS to regulate GH release has been widely attributed to two key mechanisms. Firstly, SS couples negatively to AC to inhibit cAMP production (135-137). Secondly, SS limits Ca^{2+} influx either directly through actions on Ca^{2+} channels (138-141) or indirectly by activating hyperpolarizing K^+ channels (142-144). Reducing Ca^{2+} influx is believed to be the principal mechanism responsible for the reduction in $[Ca^{2+}]_i$ brought on by SS exposure (130, 141, 145-147). SS-induced membrane hyperpolarization and reductions in $[Ca^{2+}]_i$ occur independently of changes in intracellular cAMP levels (147).

However, SS is also able to inhibit GH secretion stimulated pharmacologically by cAMP and Ca^{2+} (reviewed in (20)). Furthermore, the ability of SS_{14} to suppress Ca^{2+}-dependent exocytosis by acting distal to elevations in intracellular Ca^{2+} has been documented in several other secretion systems, including pancreatic α-cells (148), β-cells (149), and pituitary GC (80) and GH_4C_1 (150) cells. These findings suggest an additional more distal site(s) of action for SS. The molecular mechanisms underlying this distal regulatory mechanisms are unclear; however, it is believed to be mediated by a pertussis toxin-sensitive G-protein (151). At least two different mechanisms have been proposed. The first is based on the fact that transient increases in GH secretion, colloquially referred to as GH rebounds, occur following removal of SS inhibition both *in vitro* and *in vivo* (130, 152, 153). Furthermore, these rebounds can be amplified by application of a secretagogue during SS treatment (130). Thus it is thought that SS blocks membrane fusion, leading to an accumulation of readily releasable secretory granules beneath the plasma membrane (154). Following the removal of SS inhibition, the accumulated granules fuse with the plasma membrane, resulting in the GH rebound.

The second explanation for SS inhibition of cAMP- and Ca^{2+}-stimulated GH release involves the depriming of secretory granules through the activation of the serine/threonine phosphatase calcineurin (148, 149). In neuroendocrine cells, secretory granules can be functionally divided into a reserve pool and a release-competent pool. Results from pancreatic β-cells (155), pituitary melanotrophs (156) and adrenal chromaffin cells (157) suggest that ATP hydrolysis is required for secretory granules to become release competent. This process is referred to as priming. Granule depriming through calcineurin activation would reduce the number of release-competent granules and thereby decrease hormone secretion in response to agonist treatment. Calcineurin activity is also thought to underlie galanin-, as well as adrenaline-evoked reductions in insulin secretion (149).

1.8 Regulation of GH Release in Goldfish

As with all teleosts, goldfish lack a functional median eminence; instead, neuroendocrine regulation of pituitary function is via hypothalamic nerve terminals innervating the pituitary and terminating in close proximity to the pituitary cells (158). In addition, unlike the situation in mammals and the grass carp (159), *in vivo* GH release does not appear to occur in a regular, reproducible daily rhythm in goldfish (63).

1.8.1 Factors Stimulating GH Release in Goldfish

GH release is stimulated by a number of hypothalamic factors, including GHRH, DA, gonadotropin-releasing hormone (GnRH), PACAP, ghrelin (S Unniappan, personal communication) neuropeptide Y, thyrotropin-releasing hormone, cholecystokinin, bombesin and activin (reviewed in (160)). Of these, the transmembrane and intracellular signalling cascades of GnRH, DA and PACAP have been extensively studied. GnRH and DA have been identified in neuronal fibers and terminals within the pars distalis and have been shown, through both *in vitro* and *in vivo* studies, to play a physiologically relevant role in the regulation of GH release (reviewed in (161)). Recently, the presence of irPACAP in the goldfish pituitary, as well as the ability of PACAP to stimulate GH release *in vivo*, as well as *in vitro*, have been confirmed (162).

1.8.1.1 GnRH

The decapeptide GnRH is a primary regulator of reproduction (163); however, in goldfish, GnRH also participates in the control of GH secretion (reviewed in (164)). As with most vertebrates, two forms of GnRH have been identified in the goldfish brain. In goldfish, these are chicken (c)GnRH-II and salmon (s)GnRH. Both act through the same receptor population (165-168) to stimulate PKC and increase, both acutely and over extended periods of time, GH release (161). Both cGnRH-II- and sGnRH-stimulated GH release are also dependent upon increases in $[Ca^{2+}]_i$, which is due, at least in part, to the mobilization of Ca^{2+} from intracellular pool(s). Pharmacological characterization of intracellular Ca^{2+} pools responsive to the GnRH's revealed that they are sensitive to the

intracellular Ca^{2+} channel blocker TMB-8 and the intracellular Ca^{2+} mobilizer caffeine, but are insensitive to the SERCA inhibitor Tg (169).

In recent years it has become apparent that there are differences between the sGnRH and cGnRH-II signalling cascades, especially with respect to their reliance on intracellular Ca^{2+} stores. For example, Ry-sensitive Ca^{2+} stores are only involved in cGnRH-II signalling, and then only in sexually regressed fish (170). Whereas sGnRH-stimulated GH release is sensitive to the IP_3 antagonist, xestospongin C, cGnRH-II-stimulated GH release is not (170). In addition, mitocondrial buffering of agonist-induced Ca^{2+} signals seems to occur for sGnRH- but not cGnRH-II stimulated GH release (171).

Ca^{2+} signalling is often mediated through the multifunctional CaM KII. Both acute and prolonged GnRH-stimulated GH secretion are dependent upon CaM KII; this involvement is likely distal to PKC activation (172). Interestingly however, the ability of the PKC activator tetradecanoyl phorbol acetate (TPA) to stimulate GH release in the presence of TMB-8 (169) suggests that PKC can act distal to intracellular Ca^{2+} release to stimulate GH release. Alternatively, it is also possible that PKC-stimulated GH release is, at least in part, independent of Ca^{2+} released from intracellular stores. GnRH/PKC-evoked GH responses also involve extracellular Na^+ and an amiloride-sensitive Na^+-H^+ exchanger (173).

Although nitric oxide synthase (NOS) activation and nitric oxide (NO) production are not likely involved in the regulation of basal GH release from goldfish pituitary cells, NO is believed to mediate GnRH-stimulated GH release (174-176). Brain NOS- and inducible-NOS immunoreactivity have been demonstrated in pituitary cells (175). In particular irNOS has been localized to both somatotropes and gonadotropes (176). Consequently, whether the NO involved in mediating GnRH-induced GH release is originating from within somatotropes, and/or nearby gonadotropes is unknown (176). Irrespective of its origin, NO appears to be acting through a soluble guanylate cyclase/3',5'-cyclic guanosine 5'-monophosphate (cGMP) signalling mechanism to induce GH release (175, 176). The exact order of events and interaction between GnRH receptor binding, NO formation, guanylate cyclase activation and the eventual secretion of GH from somatotropes is complex, uncertain and discussed elsewhere (176). One

possible scenario is that sGnRH and cGnRH-II activation of NOS is mediated by mobilization of Ca^{2+} from pharmacologically distinct intracellular stores (176), although this has not yet been proven.

1.8.1.2 DA

Another well-studied stimulator of GH release in goldfish is DA. Acute and prolonged DA stimulation of GH release is mediated by D1 receptors coupled to an AC /cAMP/PKA signal transduction cascade (177). D1 signalling is thought to be highly dependent on extracellular Ca^{2+} entry through VSCC (177), which is likely required for the maintenance of intracellular Ca^{2+} stores (178). This hypothesis is consistent with experiments showing that DA increases Ca^{2+} currents in somatotropes (14). Intracellular Ca^{2+} stores sensitive to TMB-8, Tg and BHQ, but insensitive to Ry, are known to participate in DA-stimulated GH release (178). In a separate study, TMB-8 blocked forskolin-stimulated GH release, suggesting that intracellular Ca^{2+} release channel involvement is downstream of cAMP mobilization (169). The mobilization of arachidonic acid (AA) by PLA_2 and its metabolism by lipoxygenase are also known to be involved in mediating the GH response to DA at some point distal to cAMP generation, as is CaM KII (172).

Results with inhibitors of NOS and guanylate cyclase enzymes indicate that NO is also involved in the regulation of D1-stimulated GH secretion in a cGMP-dependent manner (174, 176). However, this appears to be a time-dependent event, as acutely stimulated GH release is not affected by the broad spectrum NOS inhibitor AGH, suggesting that NOS/NO/cGMP may only play a significant role in mediating long-term DA stimulation (176). Compared with its importance in GnRH action, evidence suggests that NO plays a somewhat less critical role in D1-stimulated GH release.

1.8.1.3 PACAP

Like DA, acute and prolonged PACAP-stimulated GH release is mediated by an AC /cAMP/PKA system that is dependent on extracellular Ca^{2+} entry through VSCC's

(178-180). This is consistent with the known coupling of the goldfish PACAP type 1 receptor (PAC_1) to AC in mammalian cell expression systems (162, 181). Similar to DA, PACAP-stimulated GH release is dependent upon CaM KII (178). DA and PACAP share a similar dependence on intracellular Ca^{2+} stores. Both are sensitive to TMB-8, BHQ and Tg but not Ry. In addition, PACAP appears to also rely on a TMB-insensitive Ca^{2+} store that D1 signalling does not employ (178). In preliminary experiments, NOS inhibitors had no effect on PACAP-stimulated GH release in static culture. Surprisingly, soluble guanylate cyclase inhibitors did prevent PACAP-stimulated GH responses (176). This would suggest that PACAP may be signalling though cGMP independent of NOS activation (176); however, more investigations are needed before any conclusions can be drawn.

1.8.2 Factors Inhibiting GH Release in Goldfish

In goldfish, SS, norepinephrine (NE) and serotonin (5-HT) are all known to inhibit GH release from pituitary somatotropes (reviewed in (160)).

1.8.2.1 5-HT

5-HT has been shown to reduce GH secretion from pituitary fragments (182), as well as, dispersed pituitary cells (183). In addition, 5-HT inhibits, but does not abolish DA-, SKF-38393 (a D1 agonist)- and sGnRH-stimulated GH release from goldfish pituitary fragments, as well as SKF-38393-stimulated GH release from dispersed goldfish pituitary cells (183).

1.8.2.2 NE

In vitro studies examining NE inhibition of basal and stimulated GH release establish that NE decreases GH secretion by way of α_2-adrenoreceptor activation (184). NE reduces basal GH secretion, and treatment with the α_2-agonist clonidine decreases both released and cellular basal cAMP levels (185). However, treatment with NE had no

effect on basal $[Ca^{2+}]_i$ and NE is incapable of lowering basal GH secretion in the presence of the VSCC agonist Bay K 8644 (185). Interestingly, both $[Ca^{2+}]_i$ rebounds and GH rebounds are seen following removal of NE, and the magnitude of the GH rebound increases if secretagogues are applied during NE treatment. NE is also capable of inhibiting GnRH- and DA-stimulated GH release, as well as GH secretion resulting from pharmacological activation of their respective signalling cascades (185).

1.8.2.3 SS

As discussed earlier (Section 1.3.2), changes in the brain SS system have been linked to the control of GH release. In addition, *in vitro* studies employing pituitary fragments or cultures of mixed dispersed pituitary cells have shown that SS_{14} reduces basal GH secretion and inhibits GH responses to GnRH, SKF-38393 and pharmacological activators of their respective signalling cascades (12, 186-188). *In vivo*, injections of SS_{14} have been linked to transient decreases in GH blood levels (189). $[Pro^2]SS_{14}$ has also been shown to dose-dependently inhibit GH secretion from perifused pituitary fragments (13). Taken together, these results suggest that, in goldfish, SS peptides play a physiologically relevant role in the regulation of GH release.

Surprisingly, a related PSS-II peptide, sSS_{25}, did not alter pituitary fragment GH release, despite containing SS_{14} within the C-terminus (188) (Fig. 1.2). Interestingly, sSS_{25} has $Phe^7 \rightarrow Tyr^7$ and $Thr^{10} \rightarrow Gly^{10}$ substitutions within the active region of SS_{14} at the same positions as $gbSS_{28}$ (Fig. 1.2). It is curious that although sSS_{25} is capable of displacing radiolabelled SS_{14} from goldfish brain (93), it does not appear to be biologically active (188). Another teleost SS, catfish (c)SS_{22} (190) has also been shown to be ineffective in lowering basal GH release from goldfish pituitary fragments (186). In cSS_{22}, 7 of the 14 amino acids at the C-terminus differ from those of SS_{14}, including $Phe^6 \rightarrow Tyr^6$, $Thr^{10} \rightarrow Ser^{10}$ and $Phe^{11} \rightarrow Arg^{11}$ substitutions in the active region of the C-terminal SS_{14} (Fig. 1.2). In addition, Thr^5 is glycosylated (190). Both of these features may explain why cSS_{22} has no effect on GH secretion in goldfish (186).

1.9 Research Objectives

One of the major questions in current SS biology research is whether, and how, different SS peptides contribute to the selective regulation of cell function in tissues where more than one isoform is present. In this thesis, I have used the goldfish pituitary as an experimental model in which to begin addressing this question. The expression of PSS-I, -II and -III mRNA within the goldfish hypothalamus and pituitary was examined to evaluate the possibility that more than one SS isoform may be regulating pituitary cell function. The ability of the corresponding mature peptides, SS_{14}, $gbSS_{28}$ and $[Pro^2]SS_{14}$, to regulate basal GH secretion, as well as GH release stimulated by a number of known hypothalamic regulators or pharmacological activators of their respective intracellular signalling cascades was then explored. Results from these experiments are presented in Chapter 2. The remainder of my thesis research focused on how one of these SS peptides, SS_{14}, affects elevations in $[Ca^{2+}]_i$, and how these effects relate to the ability of SS_{14} to inhibit GH release. Chapter 3 examines the ability of SS_{14} to regulate basal, as well as GnRH-stimulated GH release by modulating $[Ca^{2+}]_i$. Chapter 4 examines the ability of SS_{14} to regulate SKF-38393- and PACAP-stimulated GH release via actions on $[Ca^{2+}]_i$. Finally, in Chapter 5, the major findings of this study are summarized, and their limitations, implications and future directions are addressed.

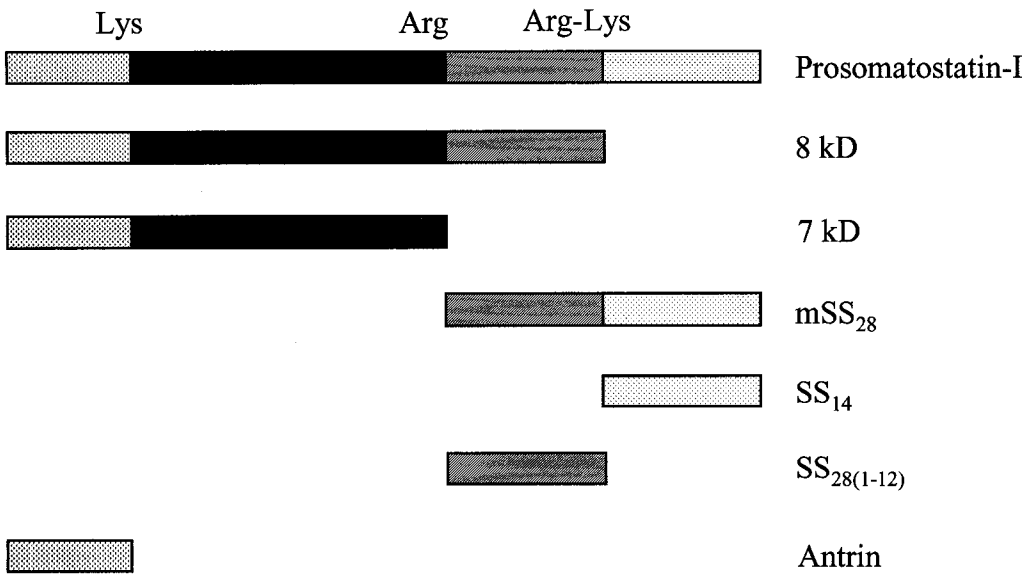

Fig. 1.1. Schematic Representation of Mammalian PSS-I and its Cleavage Products.

Enzymatic processing occurs at either paired (Arg-Lys) or single (Arg or Lys) basic amino acid residues. Only SS_{14} and mSS_{28} are known to be biologically active. Adapted from (19).

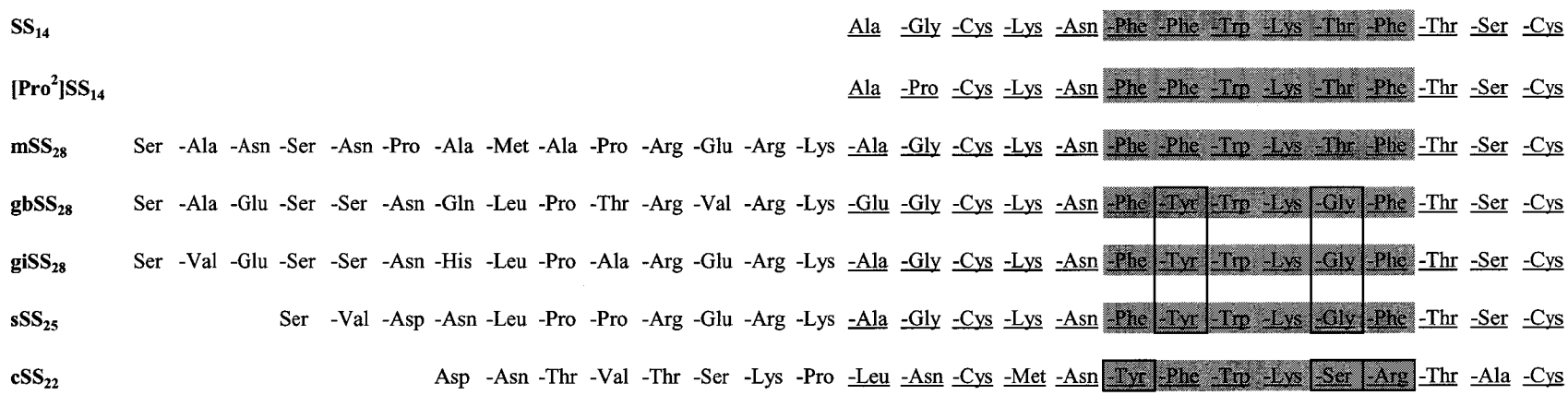

Fig. 1.2. The Amino Acid Sequences of Seven Different SS Isoforms.

SS_{14} and its C-terminal variants as contained within N-terminal extended SS isoforms are underlined. The putative bioactive regions of each of the peptides, amino acids 6 through 11 of SS_{14}, are shaded. Amino acid substitutions within the bioactive regions are boxed. See text for abbreviations.

Table 1.1. Binding Selectivity of Different SS's, SS-Related Peptides and Non-Peptide Agonists for Cloned hsst's [1].

	IC_{50} (nM)					
	sst_1	sst_2	sst_3	sst_4	sst_5	References
SS-like peptides						
SS_{14}	0.1-2.26	0.2-1.3	0.3-1.6	0.3-1.8	0.2-0.9	(71-74)
mSS_{28}	0.1-2.2	0.2-4.1	0.3-6.1	0.3-7.9	0.05-0.4	(71-74)
$hCST_{17}$	7	0.6	0.6	0.5	0.4	(76)
$rCST_{29}$	2.8	7.1	0.2	3	13.7	(75)
Non-peptide agonists						
L-797,591	1.4	1875	2240	170	3600	(78)
L-779,976	2760	0.05	729	310	4260	(78)
L-796,778	1255	>10000	24	8650	1200	(78)
L-803,087	199	4720	1280	0.7	3880	(78)
L-817,818	3.3	52	64	82	0.4	(78)

[1] adapted from (20)

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Chapter 2 – Endogenous Hypothalamic SS's Differentially Regulate GH Secretion from Goldfish Pituitary Somatotropes *In Vitro* [1]

2.1 Introduction

The SS neuropeptide family is comprised of multiple genes and gene products. In mammals, there are two biologically active forms of SS, SS_{14} and its N-terminal extension mSS_{28}; both of which are derived from the same precursor molecule, PSS-I (reviewed in (1) and Section 1.2). cDNA's for PSS-I have also been cloned from numerous non-mammalian species, including chicken, frog and several teleost fish. Teleosts in which PSS-I has been cloned include anglerfish, rainbow trout, sturgeon and goldfish ((2); reviewed in (3)). Similar to mammalian PSS-I, goldfish PSS-I is capable of yielding SS_{14} (reviewed in Section 1.3). In fact, the SS_{14} derived from goldfish PSS-I is identical to mammalian SS_{14} in amino acid sequence (4). However, unlike mammalian PSS-I, goldfish PSS-I does not contain a monobasic Arg cleavage site capable of yielding SS_{28}. Although there is a cleavage site capable of yielding a 26 amino acid SS (4), a SS_{26} has not been isolated.

In addition to PSS-I, goldfish possess two additional PSS's, PSS-II and PSS-III (reviewed in Section 1.3). $gbSS_{28}$, which is contained within PSS-II, differs from mSS_{28} in two ways. First, in addition to differing by eight amino acids in the N-terminus, it contains [Glu^1, Tyr^7, Gly^{10}]SS_{14} at its C-terminus (4). Second, it is a separate gene product, rather than an alternate cleavage product (4). Although goldfish PSS-II contains cleavage sites capable of generating both 14 and 28 amino acid peptides, evidence obtained in other teleosts suggest that goldfish PSS-II only yields $gbSS_{28}$ (5-8).

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The third goldfish PSS, PSS-III, contains a potential cleavage site for a 14 amino acid peptide, but not for a 28 amino acid peptide (4). Although PSS-III also contains monobasic Arg cleavage sites capable of yielding 24 and 29 amino acid peptides, results from Russian sturgeon (9) suggest that [Pro^2]SS_{14} is the most likely product of this message in goldfish (reviewed in Section 1.3). PSS-III is phylogenetically related to the [Pro^2, Met^{13}]SS_{14} precursor in frog and CST in mammals (4, 10).

In mammals, SS_{14} and SS_{28} are differentially expressed throughout the central nervous system, peripheral nervous system and most of the major organs of the body (11-13), while CST is expressed primarily in the cerebral cortex and hippocampus ((14, 15); reviewed in Section 1.2.1). Similarly, in several non-mammalian vertebrates, including frog (16), coho salmon (17), rainbow trout (17, 18), sturgeon (2) and goldfish (4), differential distribution of PSS-I, -II and -III have been reported.

The SS's act through a family of G-protein coupled receptors. In mammals, five sst subtypes (sst_{1-5}) have been identified (reviewed in Section 1.4). Each subtype is capable of interacting with a distinct set of intracellular signalling systems (reviewed in (19)). All five sst's bind SS_{14} and mSS_{28} with high affinity; however, sst_5 exhibits some selectivity for mSS_{28}. Although all five receptor subtypes are expressed in the pituitary, sst_2 and sst_5 are believed to be the primary regulators of somatotrope function (20). In goldfish, 8 sst's ($gfsst_{1A, 1B, 2, 3A, 3B, 5A, 5B, 5C}$) have been cloned from brain tissues ((21-24); reviewed in Section 1.5). Similar to the situation in mammals, pharmacological characterization of $gfsst_{5A}$ has shown that although it binds SS_{14}, [Pro^2]SS_{14} and $gbSS_{28}$ with high affinity, it displays some selectivity for 28 amino acid SS's (22). Consistent with mammalian studies, $gfsst_2$ and $gfsst_5$ mRNA are predominantly expressed in the pituitary as compared to other brain regions (22, 24).

The differential expression of sst subtypes is clearly one of the means by which functional specificity is achieved in the SS/sst system. However, whether (and how) the different SS peptides contribute to the selective regulation of cell function in tissues where more than one isoform are present is poorly understood. To begin exploring this possibility, the existence of PSS mRNA in goldfish hypothalamus and pituitary was

evaluated. Subsequently, the ability of the mature peptides, SS_{14}, [Pro^2]SS_{14} and $gbSS_{28}$, to regulate basal GH secretion and cAMP production was examined. In addition, the ability of these three SS peptides to inhibit GH release stimulated by several different goldfish neuroendocrine regulators, as well as GH secretion resulting from the pharmacological activation of their respective intracellular signalling cascades were evaluated. In particular, GnRH, which stimulates GH release in a PKC-dependent manner, and DA and PACAP, which act through AC/cAMP/PKA-sensitive mechanisms, were employed (reviewed in (25) and Section 1.8.1). The concept of SS isoform functional selectivity was further tested by comparing the apparent intracellular mechanisms mediating mSS_{28} and $gbSS_{28}$ inhibition of GH release.

2.2 Materials and Methods

2.2.1 Animals and Cell Preparation

All animal maintenance and experimental protocols utilized in this study were approved by the University of Alberta, Biological Sciences Animal Care Committee in accordance with national guidelines. Common goldfish (*Carassius auratus*; 8-13 cm in length) were purchased from Aquatic Imports (Calgary, AB) and maintained in flow-through aquaria (1800 l) at 16-20 C as previously described (26). As needed, goldfish were anaesthetized in 0.05% tricaine methanesulphonate and euthanised by cervical transection. Pituitaries were subsequently excised and their cells dispersed using a previously described trypsin/DNase dispersion protocol (26).

Pituitaries from male and female goldfish at different stages of the reproductive cycle were used in this study. The magnitude of SS_{14} inhibition of basal GH release has previously been reported to vary throughout the seasonal reproductive cycle (27). However, when all available data, collected over the last 5 years, concerning SS_{14} regulation of basal GH release were pooled and analyzed according to the time of year, these changes were not significant (Section 5.6, Fig. 5.2). In the present study, SS_{14}, [Pro^2]SS_{14} and $gbSS_{28}$ inhibition of basal GH release was always significant, regardless

of gonadal state. Nevertheless, where possible, the effects of the different SS peptides, either alone or against a specific secretagogue, were compared simultaneously to control for possible seasonal variation. To facilitate future comparisons, the approximate gonadal stage of the goldfish employed for each set of experiments, as determined by the time of year, are reported in the figure legends.

2.2.2 Reagents and Test Substances

All media contained Medium-199 (Invitrogen, Burlington, ON or Sigma-Aldrich, St. Louis, MO) with 0.1 g/l L-glutamine, 26 mM $NaHCO_3$, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid (HEPES), 100 mg/l streptomycin and 100 000 U/l penicillin (pH adjusted to 7.2). Dispersion medium contained Hank's salts and 0.3% bovine serum albumin (BSA, fraction V, Calbiochem, San Diego, CA). Plating medium (for overnight incubation) contained Earle's salts and 1% horse serum (Invitrogen). Testing medium was the same as dispersion medium except that BSA was reduced to 0.1%. In instances where cells were depolarized with 30 mM KCl, equimolar substitution of KCl for NaCl was employed to maintain osmolarity.

Distilled, deionised water was used to prepare stock solutions of [Pro^2]SS_{14}, $gbSS_{28}$ (synthesized by Dr. J. Rivier), SS_{14}, mSS_{28}, mammalian $PACAP_{38}$ (PACAP), sGnRH ([Trp^7, Leu^8]GnRH), cGnRH-II ([His^5, Trp^7, Tyr^8]GnRH; Peninsula Laboratories, Belmont, CA) and CsCl. 8Br-cAMP, 7β-acetoxy-8,13-epoxy-1α,6β,9α-trihydroxylabd-14-en-11-one (forskolin), (±)-1-phenyl-2,3,4,5-tetrahydro-(1*H*)-3-benzazepine-7,8-diol hydrochloride (SKF-38393), TPA (Research Biochemicals Incorporated, Natick, MA), dioctanoyl glycerol (DiC8) and A23187 (Calbiochem) were dissolved in dimethyl sulphoxide (DMSO). Ionomycin (Calbiochem) and AA (Sigma-Aldrich) were dissolved in ethanol. Sodium nitroprusside (SNP; Calbiochem) was dissolved in testing medium immediately prior to use. Concentrated stock solutions were stored at either room temperature or -20 C. Final concentrations were achieved by dilution in testing medium. Final concentrations of DMSO and ethanol never exceeded

0.1% and had no effect on basal GH release (28), $[Ca^{2+}]_i$ (29), or ionic currents in identified goldfish somatotropes (30).

Trizol reagent, Taq DNA polymerase and SuperScript II RNase H$^-$ Reverse Transcriptase were purchased from Invitrogen. Hybond nylon membrane, Rediprime II random prime labelling system and [α-^{32}P] deoxy-CTP (dCTP) were purchased from Amersham Biosciences (Buckinghamshire, England) while the QIAquick Nucleotide Removal Kit was obtained from Qiagen (Mississauga, ON).

2.2.3 RT-PCR and Southern Blot Analysis

Total RNA was extracted from freshly excised hypothalamus and pituitary, as well as dispersed pituitary cells that had been cultured overnight, using Trizol RNA isolation reagent (Invitrogen). Total RNA was reverse transcribed into cDNA using SuperScript II RNase H$^-$ Reverse Transcriptase (Invitrogen). PCR amplifications were carried out using primers specific for PSS-I, -II and -III mRNA (4). The primer sets were:

SS1-F2 (5'GCGTATCCAGTGCGCACTGGC3') and
SS1-R2 (5'GTGAAAGTTTTCCAGAAGAA3') for PSS-I mRNA,
SS2-F1 (5'CGAATCACAGCTACAAAGAGTC3') and
SS2-R1 (5'CAAGCGAGGGCCTGAGCAGG3') for PSS-II mRNA,
SS3-F1 (5'GGAGCTACAAGACTTCAAC3') and
SS3-R1 (5'CTGTGTCAGAGTAAGTCCACG3') for PSS-III mRNA.

PCR conditions were denaturation for 1 min at 95 C, annealing for 1 min at either 51 C for SS_{14} or 54 C for SS_{28} and $[Pro^2]SS_{14}$, and extension at 73 C for 1 min for a total of 30 cycles, with a final extension of 5 min at 73 C (4). The reactions were then electrophoresed on 1% agarose gels, transferred to Hybond nylon membranes (Amersham Biosciences) by capillary transfer and fixed by baking at 80 C for 2 h. The membranes were prehybridized at 65 C for 1 h in a hybridization solution containing 0.5 M $NaHPO_4$ (pH 7.2), 7% sodium dodecyl sulfate (SDS), 1 mM ethylenediamine tetra-acetate (EDTA; pH 8.0) and 1% BSA. The membranes were then transferred into fresh

hybridization solution to which [α-^{32}P]dCTP-labelled probe was added. Probes for SS_{14}, [Pro^2]SS_{14} and $gbSS_{28}$ were labelled using the Rediprime II random prime labelling system (Amersham Biosciences) and purified using the QIAquick Nucleotide Removal Kit (Qiagen) according to the manufacturers instructions. Hybridization was carried out overnight at 65 C. The membranes were subsequently washed twice with wash solution containing 0.04 M $NaHPO_4$ (pH 7.2), 1% SDS, 1 mM EDTA (pH 8.0) at 65 C and exposed to a Phosphorscreen (Molecular Dynamics, Sunnyvale, CA) for 1 h. The screen was scanned using a PhosphorImager 445 SI (Molecular Dynamics) and analyzed using the IMAGEQUANT software (Molecular Dynamics). As a negative control, PCR's were performed in the absence of cDNA to examine cross-contamination of samples. As an internal control of the RT step, PCR amplification was carried out for 35 cycles of 94 C for 1 min, 50 C for 1 min and 73 C for 1 min with primers designed on the basis of β-actin partial cDNA sequence in goldfish (31) (unpublished sequence, GenBank accession number AF079831).

2.2.4 Static Incubation Experiments Assessing GH Release

Following dispersion, cells were plated at a density of 0.25 x 10^6/well in 24-well plates (Falcon Primaria, Becton Dickinson Labware, Franklin Lakes, NJ) and cultured overnight at 28 C, 5% CO_2 and saturated humidity (26). The next day, following a rinse in testing media, cells were cultured in the presence of natural ligands and/or pharmacological agents for 2 h (26). Experiments were performed in either triplicate or quadruplicate on each plate, and each experiment was repeated a minimum of three times, using different cell preparations each time. The testing media was subsequently removed and stored at –26 C until GH content was measured using a previously validated radioimmunoassay (RIA) (32). Hormone release was normalized as a percentage of the mean basal control value and compared using analysis of variance (ANOVA) followed by least significant difference (LSD) multiple comparisons. Differences were considered significant when probability (P)<0.05. All secretagogues employed in this study caused

significant elevations in GH release relative to basal control values. Hormone release results are presented as mean ± standard error of the mean (SEM). Regression lines and the median inhibitory concentrations (IC_{50}'s) were calculated using SigmaPlot v 7.0.

2.2.5 Static Incubation Experiments Assessing cAMP Levels

Freshly dispersed pituitary cells were plated and cultured overnight using the same procedure as described above. The next day, cells were washed with clear testing media (testing media without phenol red) and subsequently cultured for 2 h in clear testing media supplemented with varying concentrations of one of the three different SS isoforms. Experiments were performed in triplicate on each incubation plate, and each experiment was repeated a minimum of three times, using different cell preparations each time. Following drug treatment, 800 µl of clear testing media was collected to assess cAMP release, while cellular cAMP was extracted by lysing the cells with 1 ml distilled, deionised water and subsequent 30-sec sonication. All samples, released and cellular, were placed in a boiling water bath for 10 min to denature phosphodiesterases. Samples were then acetylated and assayed for cAMP content using a cAMP enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA). To facilitate pooling of data from replicate experiments, cAMP levels were normalized as a percent of the mean basal control value and compared using ANOVA followed by LSD multiple comparisons. Differences were considered significant when $P<0.05$. Results are presented as mean ± SEM. Regression lines were calculated using SigmaPlot v 7.0.

2.3 Results

2.3.1 Expression of PSS-I, PSS-II and PSS-III mRNA in the Hypothalamus and Pituitary

To assess whether pituitary cells may be exposed to SS_{14}, $[Pro^2]SS_{14}$ and $gbSS_{28}$, the expression of their prohormone mRNA in the hypothalamus and pituitary was examined. Since the hypothalamic neurons that directly innervate the teleost pars distalis

(33) are removed by trypsin/DNase dispersion (26), the inclusion of both pituitary fragments and primary pituitary cell cultures enabled me to examine PSS mRNA expression in pituitary preparations that still contained hypothalamic nerve terminals, as well as preparations devoid of such terminals. mRNA for all three PSS's were detected in the hypothalamus following Southern blot analysis of RT-PCR products (Fig. 2.1). Interestingly, although PSS-I and PSS-II mRNA were present in dispersed pituitary cells, no PSS mRNA was detected in pituitary fragments (Fig. 2.1).

2.3.2 SS_{14}, [Pro^2]SS_{14} and $gbSS_{28}$ on Basal GH Release

Previously, SS_{14} has been shown to dose-dependently reduce GH release from both goldfish pituitary fragments and dispersed pituitary cell preparations (27, 34); similarly, [Pro^2]SS_{14} has been shown to reduce basal GH release from pituitary fragments (4). However, results obtained from pituitary fragment preparations may be complicated by SS acting on hypothalamic neuronal terminals present within the anterior pituitary (33). Here the data establish that all three SS isoforms can dose-dependently diminish basal GH release by acting at the level of the pituitary cell (Fig. 2.2A). SS_{14} and [Pro^2]SS_{14} produced similar dose-response curves that were markedly different from that of $gbSS_{28}$. IC_{50} estimates establish that $gbSS_{28}$ is more potent than either SS_{14} or [Pro^2]SS_{14} in reducing basal GH release (Table 2.1). Maximal inhibition, as calculated by regression analysis, was 71.90 ± 5.06% for SS_{14}, 72.73 ± 0.46% for [Pro^2]SS_{14} and 53.24 ± 6.18% for $gbSS_{28}$.

2.3.3 SS_{14}, [Pro^2]SS_{14} and $gbSS_{28}$ Actions on cAMP and cAMP/PKA-Dependent GH Secretion

In goldfish, as in other vertebrate systems, cAMP/PKA-dependent signalling cascades have been implicated in the regulation of basal GH secretion (35-37). Here the ability of SS_{14}, [Pro^2]SS_{14} and $gbSS_{28}$ to modulate both released and intracellular basal cAMP levels in naïve pituitary cells was examined. All three isoforms significantly

reduced intracellular cAMP levels. However, while 1 μM SS_{14} and 1 μM [Pro^2]SS_{14} were equal in their ability to reduce intracellular cAMP levels, 1 μM $gbSS_{28}$ was significantly less effective (Fig. 2.2B). In contrast, none of the three SS isoforms tested significantly altered the levels of released cAMP (Fig. 2.2C). Significant reductions in basal GH release (Fig. 2.2A) did not directly correlate with significant decreases in either intra- or extracellular cAMP levels (Fig. 2.2B and C).

I subsequently examined the ability of maximally effective concentrations of [Pro^2]SS_{14} (100 nM; Fig. 2.2A) and $gbSS_{28}$ (10 nM; Fig. 2.2A) to affect cAMP/PKA-dependent GH secretion, and compared these findings to those previously observed with a maximally effective concentration of SS_{14} (1 μM; Table 2.1) (27, 38). Activation of PACAP and DA receptors have been shown to stimulate GH secretion from goldfish pituitary cells through cAMP/PKA-dependent mechanisms (reviewed in (25)). Accordingly, treatments with maximal stimulatory concentrations of the DA D1-agonist SKF-38393 (1 μM) (28) and PACAP (10 nM) (39) significantly elevated GH release in this study (Fig. 2.3A). Similarly, direct activation of the cAMP/PKA cascade with the AC activator forskolin (1 μM) and the cell permeant cAMP analogue 8Br-cAMP (1 mM) resulted in significant increases in GH secretion (Fig. 2.3A). As had been demonstrated for SS_{14} (27), [Pro^2]SS_{14} abolished the ability of PACAP, SKF-38393, forskolin and 8Br-cAMP to induce GH release (i.e., GH responses to treatment with SS plus secretagogue were not significantly different from responses to SS alone; Fig. 2.3A). In contrast, $gbSS_{28}$ only partially reduced the GH responses to SKF-38393 and PACAP, and did not alter GH responses to forskolin or 8Br-cAMP (Fig. 2.3B). These results establish that, $gbSS_{28}$, unlike SS_{14} and [Pro^2]SS_{14}, does not act distal to cAMP formation to suppress GH responses. To further test this hypothesis, I examined the ability of all 3 SS's to inhibit AA-stimulated GH release. Previous work from this lab has shown that AA mediates DA-stimulated GH secretion subsequent to cAMP formation (40). While both SS_{14} (1 μM) and [Pro^2]SS_{14} inhibited GH responses to 50 μM AA (Fig. 2.4A and B), $gbSS_{28}$ had no effect (Fig. 2.4C).

2.3.4 SS_{14}, [Pro^2]SS_{14} and $gbSS_{28}$ on PKC-Dependent GH Secretion

In goldfish, PKC-dependent transduction mechanisms act independently of the cAMP/PKA pathway to stimulate GH secretion. Two endogenous GnRH's, sGnRH and cGnRH-II, have been shown to evoke GH release through activation of PKC, while SS_{14} is known to inhibit GH responses to PKC activation (reviewed in (25)) (Table 2.1). In order to compare the ability of different SS's to affect PKC-dependent GH release, the effects of [Pro^2]SS_{14} and $gbSS_{28}$ on GH responses to maximal stimulatory concentrations of sGnRH (100 nM) (26), cGnRH-II (100 nM) (26) and two PKC activators, TPA (100 nM) (41) and DiC8 (100 μM) (41) were examined. In the presence of 100 nM [Pro^2]SS_{14} or 10 nM $gbSS_{28}$, the GH-releasing ability of both cGnRH-II and sGnRH was completely abolished, while the responsiveness to DiC8 was significantly reduced (Fig. 2.5A). These observations are similar to those previously observed with SS_{14} (27) (Table 2.1). GH responses to TPA were also greatly reduced by [Pro^2]SS_{14} (Fig. 2.5A), as was reported for SS_{14} (Table 2.1). In contrast, $gbSS_{28}$ was completely ineffective against TPA-stimulated GH release (Fig. 2.5B). These observations reveal a surprising difference in the ability of these three goldfish SS's to affect TPA-sensitive, but not DiC8-induced GH secretion.

2.3.5 SS_{14}, [Pro^2]SS_{14} and $gbSS_{28}$ on NO-Mediated GH Release

NO signalling has been implicated in both the GnRH and DA signalling pathways regulating GH secretion in goldfish ((42, 43); reviewed in Section 1.8.1). Consequently, I examined the effectiveness of the goldfish SS's against NO-stimulated GH release, using maximally effective concentrations of SNP (100 μM) (42), an NO donor. In this study, SNP significantly increased GH release, and this stimulation was abolished by both SS_{14} and [Pro^2]SS_{14} (Fig. 2.4A and B). However, $gbSS_{28}$ had no effect on SNP-stimulated GH secretion (Fig. 2.4C).

2.3.6 [Pro^2]SS$_{14}$ and gbSS$_{28}$ Actions on Ca^{2+}-Stimulated GH Secretion

Ca^{2+} mobilization, from both intracellular and extracellular sources, is a component of both GnRH and DA stimulation of GH release (37, 44). Previously, this lab has demonstrated that SS_{14} is capable of inhibiting Ca^{2+}-ionophore-stimulated GH release (27) (Table 2.1). Here the abilities of three goldfish SS's to affect GH responses to elevations of $[Ca^{2+}]_i$ were compared. This was achieved through the use of the Ca^{2+} ionophores A23187 (10 μM) and ionomycin (10 μM), both of which increased $[Ca^{2+}]_i$ in goldfish pituitary cells in previous experiments (45), and significantly increased GH secretion in this study (Fig. 2.6). Co-incubation with [Pro^2]SS$_{14}$ significantly inhibited both A23187- and ionomycin-stimulated GH release (Fig. 2.6A). In contrast, gbSS$_{28}$ significantly inhibited ionomycin-evoked GH release but did not affect A23817-stimulated GH release (Fig. 2.6B). Treatment with a depolarizing concentration of KCl (30 mM) has been shown to increase $[Ca^{2+}]_i$ (46). Here, this treatment significantly increased GH secretion (Fig. 2.7). All three goldfish SS's completely abolished 30 mM KCl-stimulated GH release (Fig. 2.7). These data show that apart from one exception (i.e., gbSS$_{28}$ on A23187) all three SS's are able to alter GH responses to elevated $[Ca^{2+}]_i$.

2.3.7 K^+ Channel Involvement in Mediating SS Actions On Basal GH Release

Results from ovine somatotropes have demonstrated the importance of inwardly rectifying K^+ channels (K_{ir}) in regulating GH secretion (47). Furthermore, in rat somatotropes, SS_{14} has been shown to activate K_{ir} as a means of reducing basal GH secretion (48). Since K_{ir} channels display a particularly high affinity for monovalent and divalent cations (49), I examined K_{ir} involvement in SS_{14}, [Pro^2]SS$_{14}$ and gbSS$_{28}$ inhibition of basal GH secretion using 5 mM extracellular CsCl. Application of CsCl had no effect on basal GH release and did not affect the ability of either SS_{14} or [Pro^2]SS$_{14}$ to significantly inhibit GH release (Fig. 2.8). However, gbSS$_{28}$ was unable to inhibit GH secretion in the presence of CsCl (Fig. 2.8), suggesting that gbSS$_{28}$, but not SS_{14} and [Pro^2]SS$_{14}$, may rely on K_{ir} channels to inhibit GH release in this system.

2.3.8 Mammalian SS_{28} Differs from $gbSS_{28}$ in its Ability to Inhibit GH Release

The ability of mSS_{28} to inhibit GH secretion in goldfish has been previously shown (34). However, whether the mechanisms employed by mSS_{28} to modulate GH release differ from those activated by $gbSS_{28}$ has not been considered. Using mSS_{28} at the same concentration as $gbSS_{28}$ (10 nM) and the secretagogues at the same concentrations as above, I examined the ability of mSS_{28} to act upon both the PACAP/DA and GnRH signalling cascades. mSS_{28} abolished the GH responses to PACAP, SKF-38393, sGnRH, cGnRH-II and SNP, and reduced those induced by forskolin, 8Br-cAMP, DiC8, TPA and AA (Fig. 2.9). This profile of inhibition differed noticeably from that of $gbSS_{28}$ (Table 2.1), particularly in terms of effects on forskolin-, 8Br-cAMP-, SNP-, AA- and TPA-induced secretion. These results demonstrate that despite identical concentrations, $gbSS_{28}$ and mSS_{28} differ in the mechanisms underlying their inhibition of somatotrope GH release.

2.4 Discussion

In this chapter, evidence suggesting that, in goldfish, pituitary cells may be exposed to multiple SS isoforms is presented. Furthermore, I demonstrate that three different hypothalamic SS's, SS_{14}, $[Pro^2]SS_{14}$ and $gbSS_{28}$, differentially regulate GH secretion. Lastly, I establish that the goldfish somatotrope is capable of differentiating between endogenous $gbSS_{28}$ and mSS_{28} when tested at the same concentration.

2.4.1 Exposure of Pituitary Cells to Multiple SS Isoforms

As had been previously shown in goldfish of unspecified sexual states (4), the existence of three different PSS mRNAs in hypothalami obtained from sexually regressed goldfish was demonstrated in the present study. Therefore, it is conceivable that the mature peptides, SS_{14}, $gbSS_{28}$ and $[Pro^2]SS_{14}$, are participating in the neuroendocrine

regulation of goldfish pituitary function. In addition, mRNA for PSS-I and PSS-II was also identified within preparations of dispersed pituitary cells. This suggests that SS_{14} and $gbSS_{28}$ may also be produced locally at the level of the pituitary. Synthesis of hypothalamic neuropeptides within the pituitary has been well documented (reviewed in (50)). Immunoreactivity and/or mRNA for vasoactive intestinal polypeptide (51, 52), GnRH (53, 54), thyrotropin-releasing hormone (53, 55) and SS (56, 57) have all been found within mammalian anterior pituitary tissues. In addition $[Pro^2, Met^{13}]SS_{14}$ synthesis has been localized to melanotropes within the intermediate lobe of the frog pituitary (58), and $[Pro^2]SS_{14}$ has been purified from the pituitary of the Russian sturgeon (9). Although PSS mRNA has not been localized to a specific pituitary lobe or cell type, the occurrence of PSS mRNA within dispersed cells suggests that local, pituitary level peptide production may be occurring. In combination with the observed expression of PSS-I, -II and -III mRNA in the hypothalamus, it is plausible that the different SS peptides participate in the neuroendocrine, as well as paracrine and/or autocrine, regulation of goldfish pituitary physiology.

However, if hypophyseal SS synthesis were occurring, PSS mRNA should have also been detected within pituitary fragments. Although it is conceivable that the presence of other tissues within the pituitary fragments diluted the level of PSS mRNA transcripts, at least two other explanations present themselves as more likely alternatives. PSS-I, -II and -III mRNA levels in the forebrain have been shown to vary seasonally and to differ between males and females ((4); reviewed in Section 1.3.2). Furthermore, studies have demonstrated that, in the forebrains of both male and female fish, PSS-I and PSS-III expression is increased by estradiol (59). In the present study, pituitary fragment cDNA was made from pooled tissues collected from sexually regressed males and females (July) while dispersed pituitary cell cDNA was made from pooled tissues collected from sexually recrudescing males and females (November). As such, it is plausible that the absence of PSS mRNA transcripts within the pituitary fragments was the result of low steroid levels. Interestingly, in a previous study, PSS-I mRNA, but not PSS-II or -III mRNA, was detected within goldfish pituitary fragments by Northern Blot

analysis (4). Unfortunately, the gonadal stage of the fish used was not reported. Nevertheless, the hypothesis that SS peptides are being synthesized within pituitary cells and undergoing seasonal, sex steroid-dependent regulation, is consistent with this previous report.

It is also conceivable that the reason PSS mRNA was detected in cultured pituitary cells, and not freshly excised pituitaries fragments, was because the PSS-I and PSS-II genes were transcribed only after the pituitary cells were deprived of hypothalamic influences. It should be noted that PSS-I and -II mRNA within cultured pituitary cells could not be visualized by ethidium bromide staining, even after 30 cycles of PCR. This indicates that the PSS mRNA levels within the pituitary cells were quite low. Such a finding is consistent with the possibility that PSS gene transcription commenced during overnight culture. However, PSS-I mRNA has been previously detected within freshly excised pituitary fragments (4). Thus, it seems unlikely that PSS transcription following removal of hypothalamic innervation is solely responsible for the disparity between the results obtained from dispersed cells and pituitary fragments in the current study. Regardless of whether steroid and/or removal of hypothalamic influences modulate pituitary PSS mRNA expression, the data presented here are strongly suggestive of *in vivo* pituitary level peptide production. Future studies on SS release by, and/or immunocyctochemical localization of SS in dispersed pituitary cells would be an interesting test of this hypothesis.

2.4.2 SS_{14}, [Pro^2]SS_{14} and $gbSS_{28}$ Differentially Affect GH Secretion

In this study, I demonstrate that three different hypothalamic SS's differ in their ability to alter GH release (Table 2.1). Of the SS's, $gbSS_{28}$ was a more potent inhibitor of basal GH secretion than either SS_{14} or [Pro^2]SS_{14}. In addition, results with CsCl suggest that K_{ir} may channels participate in mediating $gbSS_{28}$, but not SS_{14} and [Pro^2]SS_{14}, inhibition of basal GH secretion. $gbSS_{28}$ also differs from SS_{14} and [Pro^2]SS_{14} in its ability to alter stimulated GH secretion. For example, the differential ability of these three

goldfish SS's to inhibit forskolin-, 8Br-cAMP- and AA-induced GH secretion suggests that the 14 amino acid SS's are able to act subsequent to cAMP formation to inhibit GH release while $gbSS_{28}$ is not (Fig. 2.10). Furthermore, $gbSS_{28}$ differed from SS_{14} and $[Pro^2]SS_{14}$ in that it was not as effective at inhibiting GH release resulting from the activation of PKC or liberation of NO. The ability of $gbSS_{28}$ to affect Ca^{2+} ionophore-induced, as well as D1- and PACAP-stimulated GH release also differed from that of SS_{14} and $[Pro^2]SS_{14}$. Overall, SS_{14} and $[Pro^2]SS_{14}$ are very similar in terms of their spectrum of activity; however, their activity differs markedly from that of $gbSS_{28}$. Although we cannot yet conclude from these findings that the SS's are differentially regulating GH secretion or any other cellular functions in a physiologically relevant manner, differences in intracellular signalling, such as these, would be requisite.

Where possible, the effects of the different SS isoforms were compared simultaneously. However, some of the SS_{14} data presented in Table 2.1 was collected from other sources (27, 38). Although SS_{14} regulation of basal GH release in perifusion does not vary significantly over the course of the year (Fig. 5.2), there appear to be seasonal differences in sGnRH and cGnRH-II signalling cascades (Section 1.8.1.1) (63). As such, relying on previously published data obtained from fish at different sexual stages may introduce uncontrolled seasonal and experimental errors which may limit our ability to directly compare SS actions. However, the ability of SS_{14} to inhibit SKF-38393- and forskolin-stimulated GH release in static culture has been demonstrated throughout the year (WK Yunker, unpublished). Furthermore, the data presented in Figures 2.2, 2.3, 2.6, and 2.7, were compiled from experiments using pituitary cells obtained from fish at more than one single gonadal stage. As such, the differences revealed in Table 2.1 likely represent robust differences in SS action that persist throughout the year.

It is also apparent that the goldfish GH secretion system not only differentiates between the three endogenous hypothalamic SS's, but also mSS_{28}. Unlike $gbSS_{28}$, mSS_{28} was able to inhibit forskolin-, 8Br-cAMP-, AA-, SNP- and TPA-induced GH secretion, as well as abolish GnRH-evoked responses, when used at the same dose. These

characteristics of mSS_{28} action resemble those of SS_{14} and $[Pro^2]SS_{14}$. This is not surprising given that the C-terminus of mSS_{28} is identical to that of SS_{14}.

Consistent with a previous study in frogs demonstrating the ability of SS_{14} and $[Pro^2, Met^{13}]SS_{14}$ to regulate basal cAMP formation (60), all three goldfish SS peptides suppressed basal cellular cAMP production. However, the data also demonstrate that whereas $gbSS_{28}$ was the most potent inhibitor of GH release, it was the least effective at lowering cellular cAMP levels. This provides further evidence to support the idea that SS_{14}, $[Pro^2]SS_{14}$ and $gbSS_{28}$ differentially couple to intracellular effector systems. Although it is possible that the sensitivity of the cAMP assay prevented a proper examination of the relationships between declining cAMP levels and a reduction in basal GH release, the data suggest that these two events are not tightly coupled. This is consistent with results in rat showing that SS_{14} lowers basal GH release without altering intracellular cAMP levels and blocks GHRH-stimulated GH release while only partially attenuating cAMP production (61).

The cellular mechanisms responsible for the differences in SS function presented here are not known. However, it seems likely that the different sst subtypes are involved. In mammals, each sst subtype couples to a distinct set of intracellular signalling pathways, and although all five receptor subtypes bind SS_{14} and mSS_{28} with high affinity, sst_5 does exhibit selectivity for mSS_{28} (reviewed in (19)). Similarly, characterization of $gfsst_{5A}$ revealed that although it binds all three endogenous goldfish brain SS ligands, it displays selectivity for the 28 amino acid SS's (22). In addition, $gfsst_2$ can be differentially activated by these three goldfish SS's. In COS-7 cells expressing $gfsst_2$, SS_{14} and $[Pro^2]SS_{14}$, but not $gbSS_{28}$, are able to inhibit forskolin-stimulated cAMP formation (24). Interestingly, $gfsst_2$ and $gfsst_5$ mRNA are predominantly expressed in the pituitary as compared to other brain regions (22, 24) and mammalian studies have shown that sst_2 and sst_5 are the primary regulators of somatotrope function (20). Thus, it is conceivable that $gbSS_{28}$ is acting mainly through $gfsst_5$ while SS_{14} and $[Pro^2]SS_{14}$ are acting more through $gfsst_2$. This hypothesis is currently being evaluated using non-peptidyl sst-selective agonists.

However, this hypothesis cannot explain the differences in mSS_{28} and $gbSS_{28}$ activity observed in the present study. Since $gfsst_5$ binds with, and can be activated by, both mSS_{28} and $gbSS_{28}$ with similar affinity (62), differences in the ability of these two 28 amino acid SS's to affect GH secretion may be mediated through sst_2. This remains a speculation at present since nothing is known regarding the ability of mSS_{28} to bind to and activate $gfsst_2$. Nevertheless, the presence of the SS_{14} sequence in the C-terminus of mSS_{28} is consistent with such a hypothesis.

2.4.3 Summary

Regardless of the mechanisms responsible for the differences in SS action presented, these differences have some very interesting physiological implications. In goldfish, GH secretion is regulated by a variety of neuropeptides and hypothalamic factors, some of which, stimulate GH release through different intracellular mechanisms. For example, sGnRH and cGnRH-II signalling, is mediated by intracellular Ca^{2+} stores, extracellular Ca^{2+} entry and PKC (63). In contrast, DA and PACAP stimulate GH secretion through AC/cAMP/PKA-sensitive mechanisms (reviewed in (25)). Furthermore, our lab has also shown that, in pituitary cells, the Ca^{2+} stores regulating hormone mRNA levels, as well as hormone secretion, storage and production are different (64, 65). The result is a system wherein ligands employ distinct signalling cascades to affect not only GH release, but also the steps involved in hormone synthesis. Given the differences in SS_{14}, $[Pro^2]SS_{14}$ and $gbSS_{28}$ action presented here (Table 2.1), it seems likely that, *in vivo*, they are responsible for regulating different aspects of cell function.

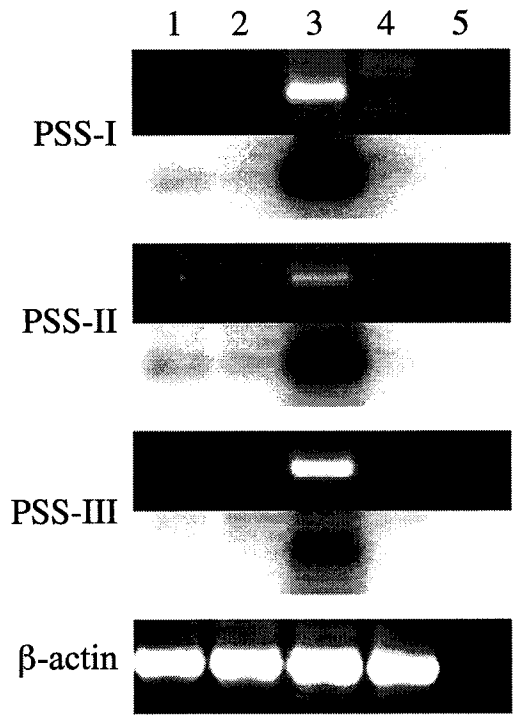

Fig. 2.1. RT-PCR and Southern Blot Analysis for PSS-I, -II and -III mRNA in the Goldfish Hypothalamus and Pituitary.

Southern blot analysis (black background panels) of RT-PCR (white background panels) for PSS-I, -II and -III mRNA in the goldfish hypothalamus and pituitary was performed. The different lanes represent the following: 1 and 2, dispersed pituitary cells; 3, hypothalamus; 4, pituitary fragments; 5, negative control. Pituitary fragment cDNA was made from pooled tissues collected from sexually regressed males and females (July) while dispersed pituitary cell cDNA was made from pooled tissues collected from sexually recrudescing males and females (November). PCR for goldfish β-actin was used as an internal control for the RT step.

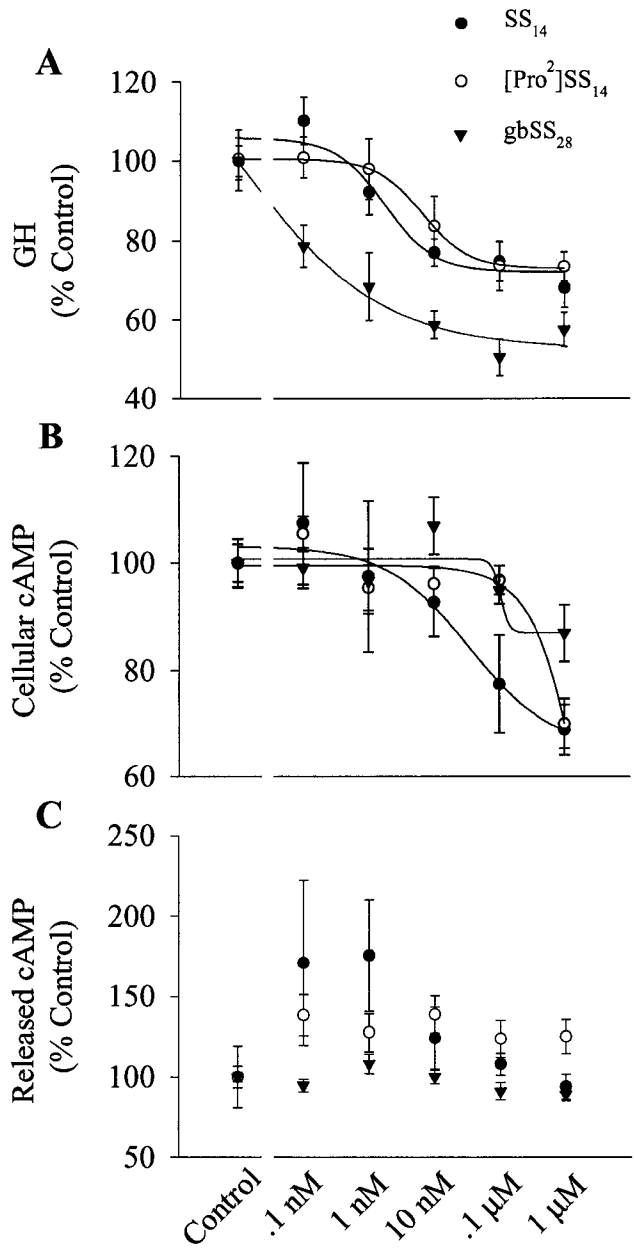

Fig. 2.2. Dose-Dependent Actions of SS_{14}, $[Pro^2]SS_{14}$ and $gbSS_{28}$.

The dose-dependent effects of three endogenous SS isoforms on (A) basal GH secretion, (B) intracellular cAMP levels and (C) released cAMP levels were examined. Dispersed pituitary cells from goldfish in the late stages of gonadal maturity (May) were used for (A) while cells obtained from goldfish undergoing gonadal recrudescence (December and January) were used for (B) and (C). Average basal GH was 969.06 ± 65.76 ng/ml (n=36), average basal cellular cAMP was 0.15 ± 0.01 pmol/ml (n=27) and average basal released cAMP was 6.58 ± 0.77 pmol/ml (n=27).

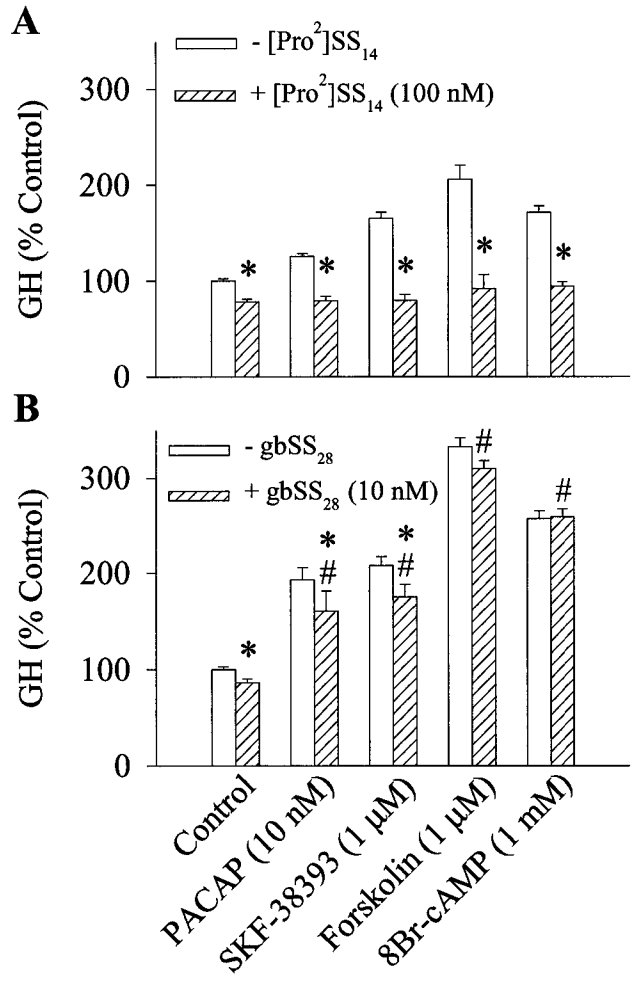

Fig. 2.3. Effects of [Pro^2]SS$_{14}$ and gbSS$_{28}$ on PACAP-, SKF-38393- and 8Br-cAMP-Stimulated GH Release.

The effects of (A) [Pro^2]SS$_{14}$ and (B) gbSS$_{28}$ on PACAP-, SKF-38393-, forskolin- and 8Br-cAMP-stimulated GH secretion were examined. Dispersed pituitary cells obtained from goldfish at times of late gonadal recrudescence (March) and regression (June) were used for (A). Cells used in (B) were obtained from goldfish with regressed gonads and from fish undergoing gonadal recrudescence (September through February). Average basal GH levels were 2165 ± 187.60 ng/ml (n=36) in (A) and 1723 ± 167.3 ng/ml (n=41) in (B). An asterisks (*) represents a significant reduction in GH release compared to the non-SS exposed column of the pair. A number sign (#) represents a significant difference compared to the SS-treated control.

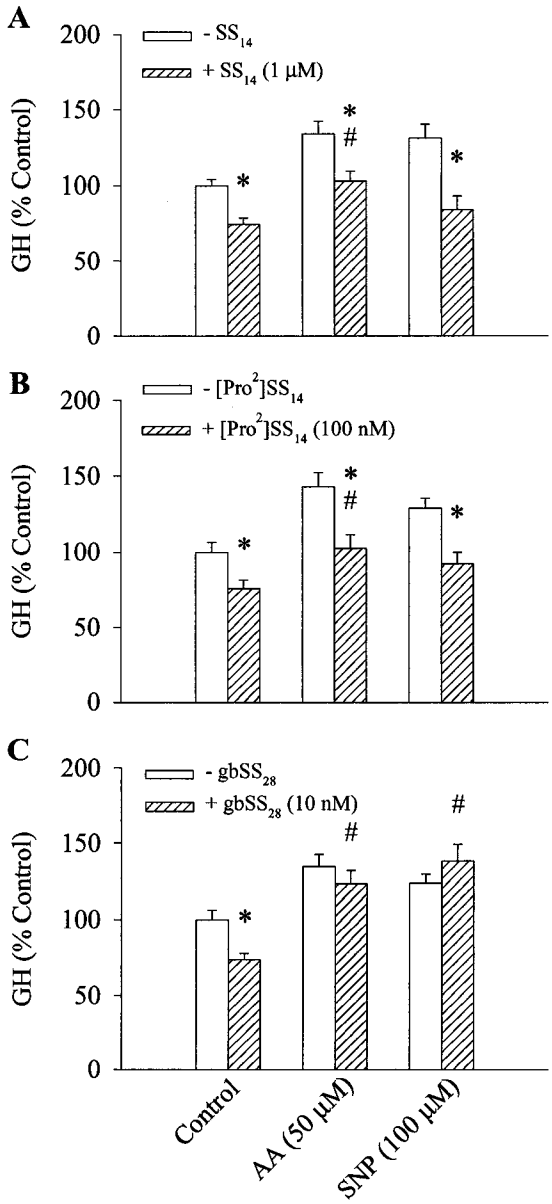

Fig. 2.4. Effects of SS_{14}, $[Pro^2]SS_{14}$ and $gbSS_{28}$ on AA- and SNP-Stimulated GH Release.

The effects of (A) SS_{14}, (B) $[Pro^2]SS_{14}$ and (C) $gbSS_{28}$ on AA- and SNP-stimulated GH secretion were examined. Dispersed pituitary cells obtained from goldfish at times of early gonadal recrudescence (October) were used for all experiments. Average basal GH levels were 1362 ± 147.2 ng/ml (n=24) in (A), 1519 ± 192.7 ng/ml (n=20) in (B) and 1442 ± 278.4 ng/ml (n=20) in (C). An asterisks (*) represents a significant reduction in GH release compared to the non-SS exposed column of the pair. A number sign (#) represents a significant difference compared to the SS-treated control.

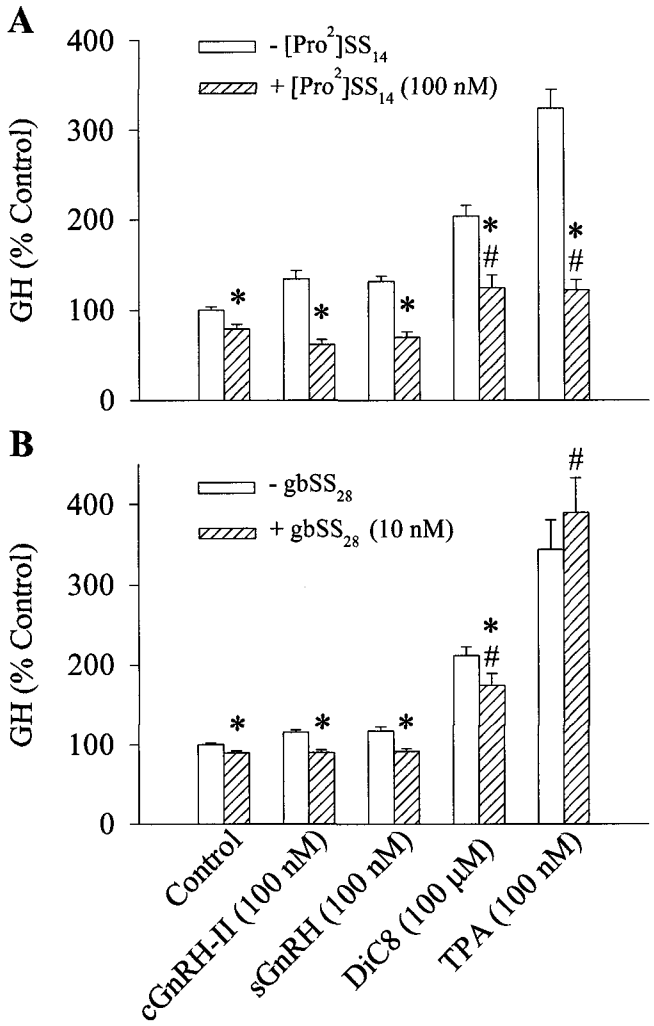

Fig. 2.5. Effects of [Pro^2]SS$_{14}$ and gbSS$_{28}$ on GnRH- and PKC-Stimulated GH Release.

The effects of (A) [Pro^2]SS$_{14}$ and (B) gbSS$_{28}$ on GnRH- and PKC-stimulated GH secretion were examined. Dispersed pituitary cells obtained from goldfish at times of gonadal recrudescence (January and March) were used for all experiments. Average basal GH levels were 2004 ± 266.3 ng/ml (n=22) in (A) and 2056 ± 175.1 ng/ml (n=38) in (B). An asterisks (*) represents a significant reduction in GH release compared to the non-SS exposed column of the pair. A number sign (#) represents a significant difference compared to the SS-treated control.

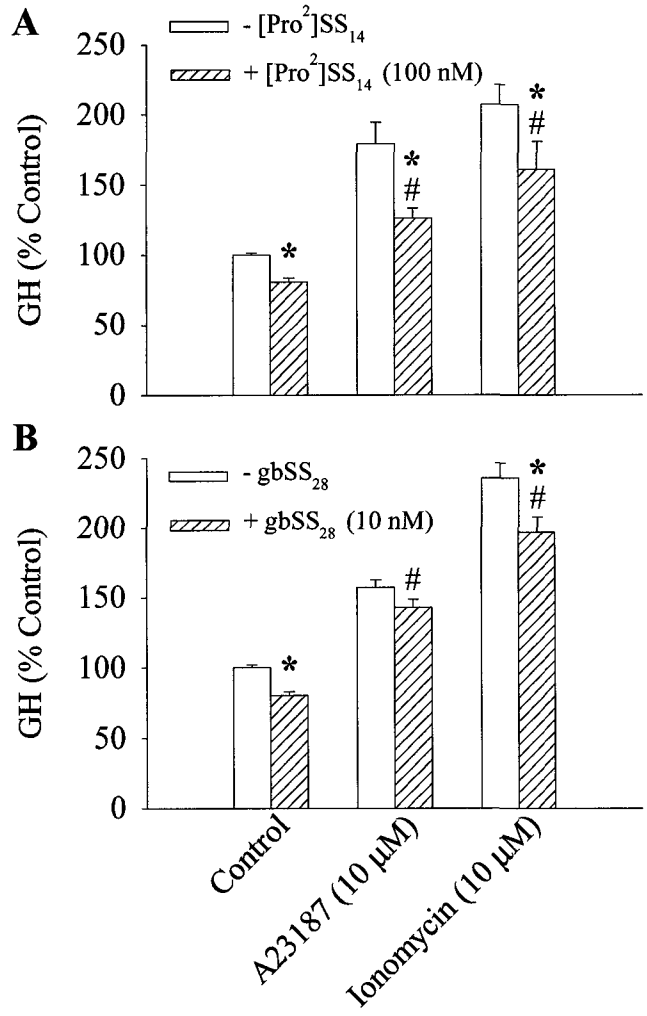

Fig. 2.6. Effects of [Pro^2]SS$_{14}$ and gbSS$_{28}$ on Ca^{2+} Ionophore-Stimulated GH Release.

The effects of (A) [Pro^2]SS$_{14}$ and (B) gbSS$_{28}$ on A23187- and ionomycin-stimulated GH secretion were examined. Dispersed pituitary cells obtained from sexually regressed goldfish (June) were used for (A), while cells obtained from goldfish undergoing sexual recrudescence (January, February) were used for (B). Average basal GH levels were 1726 ± 96.1 ng/ml (n=16) in (A) and 2345 ± 114.2 ng/ml (n=32) in (B). An asterisks (*) represents a significant reduction in GH release compared to the non-SS exposed column of the pair. A number sign (#) represents a significant difference compared to the SS-treated control.

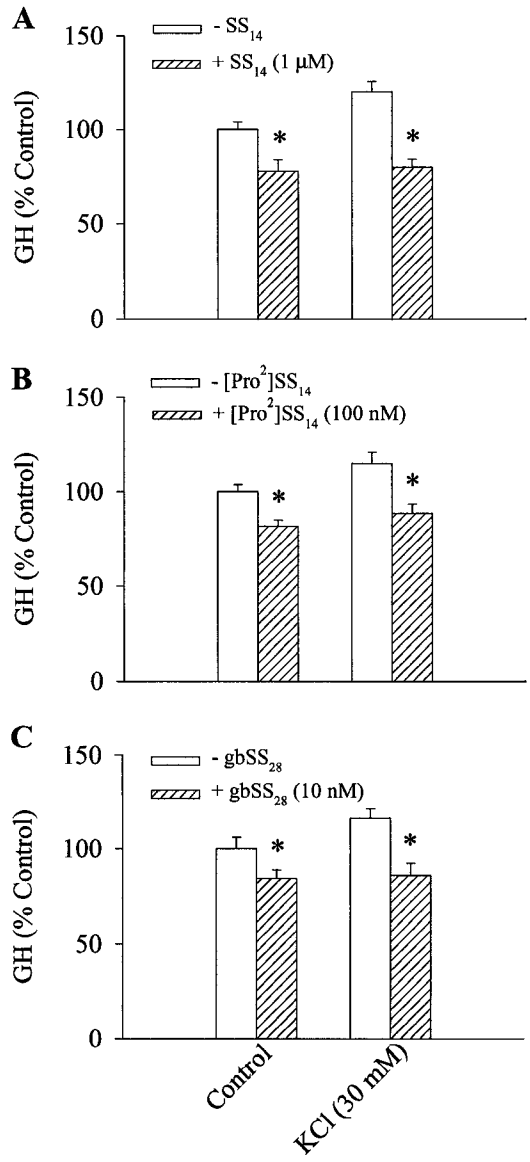

Fig. 2.7. Effects of SS_{14}, $[Pro^2]SS_{14}$ and $gbSS_{28}$ on 30 mM KCl -Stimulated GH Release.

The effects of (A) SS_{14}, (B) $[Pro^2]SS_{14}$ and (C) on $gbSS_{28}$ on 30 mM KCl-stimulated GH secretion were examined. Dispersed pituitary cells obtained from either sexually regressed fish or fish at the early stages of gonadal recrudescence (July, September and October) were used for all experiments. Average basal GH levels were 613 ± 81.4 ng/ml (n=24) in (A), 772.0 ± 105.7 ng/ml (n=24) in (B) and 676.8 ± 119.7 ng/ml (n=16) in (C). An asterisks (*) represents a significant reduction in GH release compared to the non-SS exposed column of the pair.

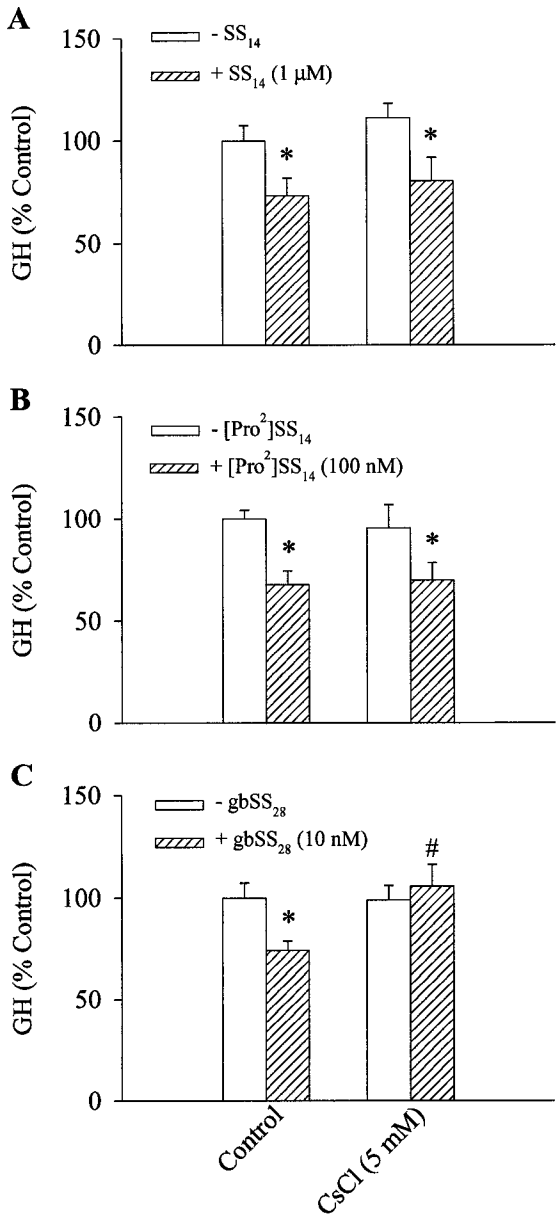

Fig. 2.8. Effects of 5 mM Extracellular CsCl on SS_{14}, $[Pro^2]SS_{14}$ and $gbSS_{28}$ Inhibition of Basal GH Secretion.

The effects of 5 mM extracellular CsCl on (A) SS_{14}, (B) $[Pro^2]SS_{14}$ and (C) $gbSS_{28}$ inhibition of basal GH secretion were examined. Dispersed pituitary cells obtained from sexually regressed goldfish (July, August and September) were used for all experiments. Average basal GH levels were 336 ± 37.1 ng/ml (n=16) in (A), 354 ± 33.3 ng/ml (n=16) in (B) and 417 ± 50.6 ng/ml (n=20) in (C). An asterisks (*) represents a significant reduction in GH release compared to the non-SS exposed column of the pair. A number sign (#) represents a significant difference compared to the SS-treated control.

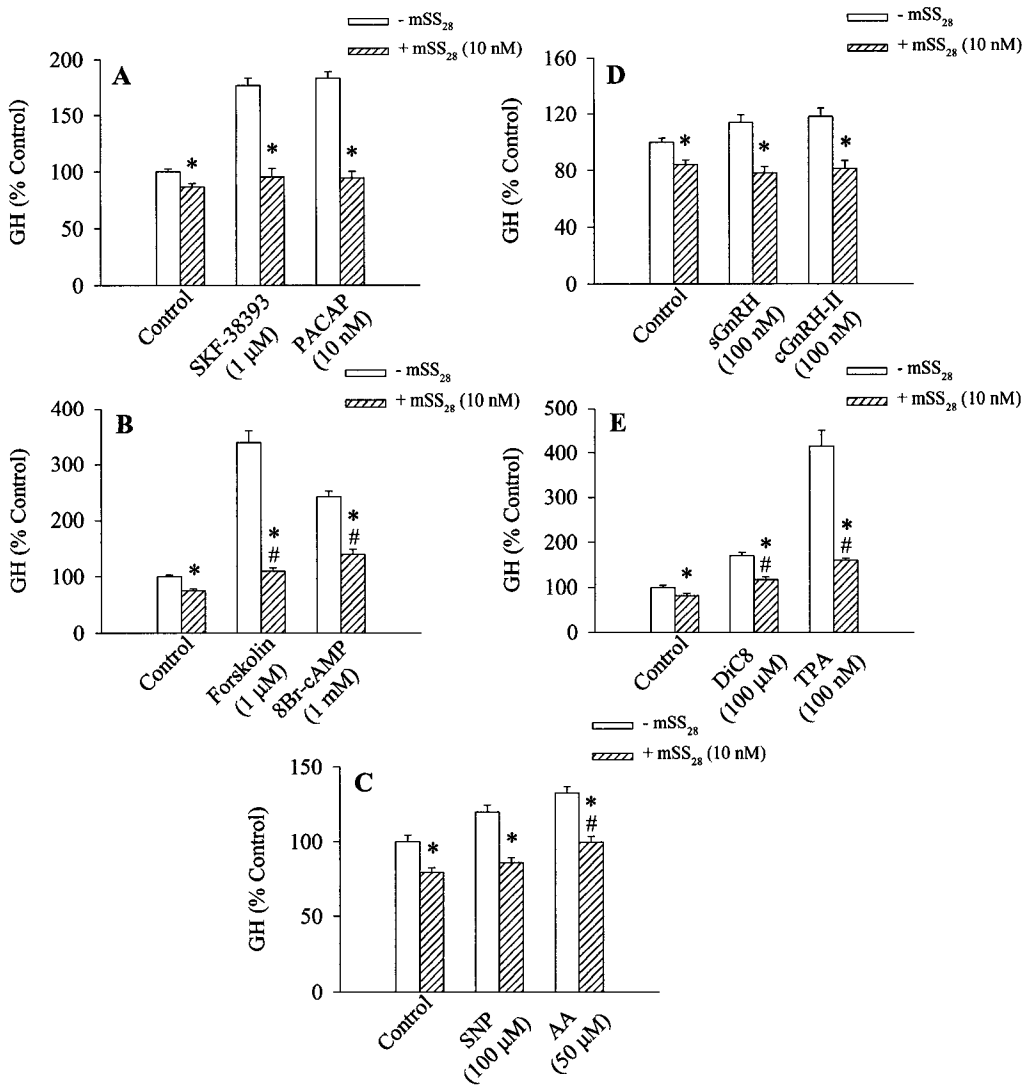

Fig. 2.9. Effects of mSS_{28} on Stimulated GH Release.

The effects of mSS_{28} on (A) SKF-38393- and PACAP-stimulated GH secretion, (B) forskolin- and 8Br-cAMP-stimulated GH secretion, (C) SNP- and AA-stimulated GH secretion, (D) sGnRH- and cGnRH-II-stimulated GH secretion and (E) DiC8- and TPA-stimulated GH secretion were examined. Dispersed pituitary cells obtained from goldfish undergoing sexual recrudescence (December) were used for all experiments. Average basal GH levels were 1051 ± 103.7 ng/ml (n=20) in (A), 1140 ± 51.0 ng/ml (n=20) in (B), 1034 ± 99.1 ng/ml (n=20) in (C), 777 ± 59.2 ng/ml (n=20) in (D) and 969 ± 59.7 ng/ml (n=12) in (E). An asterisks (*) represents a significant reduction in GH release compared to the non-SS exposed column of the pair. A number sign (#) represents a significant difference compared to the SS-treated control.

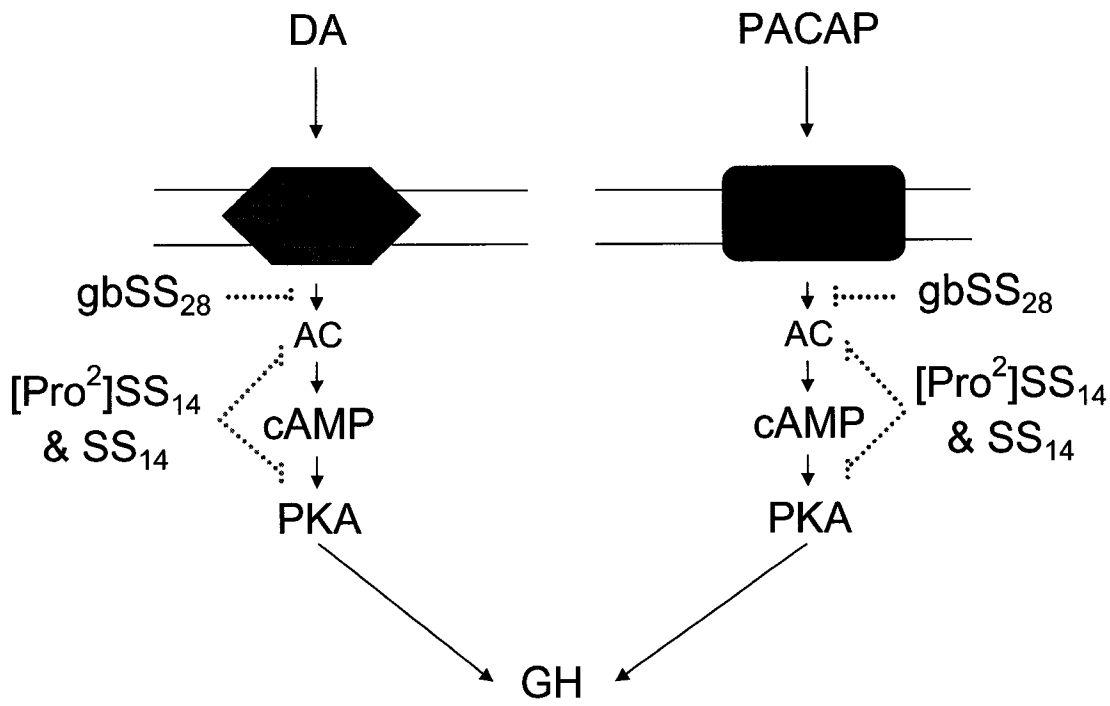

Fig. 2.10. A Hypothetical Model of the Regulation of DA- and PACAP-Stimulated GH Release by Endogenous SS Peptides in Goldfish.

SS_{14} and $[Pro^2]SS_{14}$ are capable of acting at point(s) distal to cAMP formation to regulate GH release, while $gbSS_{28}$ regulation is the result of a mechanism(s) acting prior to cAMP formation.

Table 2.1. Summary of the Effects of Four Different SS Isoforms on Basal and Stimulated GH Secretion from Primary Cultures of Dispersed Goldfish Pituitary Cells.

Treatment	*Effects on GH release in static incubation*			
	SS_{14}	**[Pro^2]SS_{14}**	**$gbSS_{28}$**	**mSS_{28}**[3]
Basal GH Secretion				
SS alone	↓	↓	↓	↓
IC_{50}	1.73 nM	6.69 nM	0.16 nM	
Maximally effective concentration	1 μM	100 nM	10 nM	
SS plus 5 mM CsCl	↓	↓	↔	
Stimulated GH Secretion				
GnRH Cascade				
sGnRH	X[1]	X	X	X
cGnRH-II	↓[1]	X	X	X
DiC8	↓[1]	↓	↓	↓
TPA	↓[1]	↓	↔	↓
DA/PACAP Cascade				
PACAP	X[2]	X	↓	X
SKF-38393	X[1]	X	↓	X
Forskolin	X[1]	X	↔	↓
8Br-cAMP	X[1]	X	↔	↓
AA	↓	↓	↔	↓
NO Cascade				
SNP	X	X	↔	X
Ca^{2+} Ionophores				
A23178	↓[1]	↓	↔	
Ionomycin	↓[1]	↓	↓	
Depolarization				
30 mM KCl	X	X	X	

X = abolished ↓ = reduced ↔ = not affected

[1] taken from (27), [2] taken from (38), [3] mSS_{28} contains SS_{14} within its C-terminus

2.5 References

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Chapter 3 – SS_{14} Actions on Basal and GnRH-Evoked Ca^{2+} Signals and GH secretion [2]

3.1 Introduction

Since its discovery in 1973 (1), the role of hypothalamic SS_{14} as an inhibitor of pituitary GH secretion has received considerable attention. Early studies concluded that regulation of basal and stimulated increases in cAMP levels and $[Ca^{2+}]_i$ were the principal mechanisms underlying SS_{14} inhibition of basal and stimulated GH release ((2, 3); reviewed in Section 1.7.2). Accordingly, SS_{14} has been shown to couple negatively to AC to inhibit cAMP production (4-6), and to limit Ca^{2+} influx, either directly through actions on Ca^{2+} channels (7-10) or indirectly by activating hyperpolarizing K^+ channels (11-13). Reducing Ca^{2+} influx is believed to be the principal mechanism responsible for the reduction in $[Ca^{2+}]_i$ brought on by SS_{14} exposure (2, 10, 14-16).

SS_{14} is known to exert its effects through a family of five heptahelical, G-protein-coupled sst's (reviewed in Section 1.4). All five receptors are capable of coupling, through G-proteins, to the inhibition of AC, activation of phosphotyrosine phosphatase and modulation of mitogen-activated protein kinase. However, various sst subtypes have also been shown to differentially couple to a number of other effector systems, including K^+ channels and VSCC's (reviewed in (17)).

Unfortunately, most of our knowledge regarding these intracellular mechanisms are the result of studies examining SS_{14} actions on GHRH-stimulated GH secretion, which is dependent upon an AC/cAMP/PKA, extracellular Ca^{2+} entry signalling system (reviewed in (18, 19) and Section 1.7.1). The intracellular mechanisms mediating SS_{14} inhibition of other, non-AC-activating GH secretagogues have received considerably less

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attention. For example, GH-releasing peptide (GHRP) stimulation of GH release is associated with activation of PLC- and PKC-dependent mechanisms (reviewed in (18, 20)). Although SS_{14} is known to inhibit GHRP-stimulated GH release (21), the intracellular mechanisms mediating this inhibition are not as well understood.

The goal of this chapter is to ascertain if, similar to what has been proposed for SS_{14} regulation of GHRH-stimulated GH release, SS_{14} inhibits PKC-dependent GH secretion by inhibiting stimulated increases in $[Ca^{2+}]_i$. To do so, the goldfish somatotrope was used. In goldfish, GH secretion is effectively and consistently stimulated by two endogenous forms of GnRH, cGnRH-II and sGnRH (reviewed in (22, 23) and Section 1.8.1.1). Both GnRHs participate in the physiological stimulation of GH release, and the GH-releasing actions of both peptides are attenuated by endogenous SS_{14} (24). The intracellular signalling systems responsible for sGnRH- and cGnRH-II-stimulated GH release have been extensively studied and are reviewed in detail elsewhere (22, 23, 25). Briefly, both sGnRH- and cGnRH-II-stimulation of GH release are dependent upon Ca^{2+} release from an intracellular pool(s) (26), extracellular Ca^{2+} influx and PKC.

Using single, identified somatotropes loaded with the Ca^{2+}-sensitive dye Fura-2, I characterized $[Ca^{2+}]_i$ under basal, as well as GnRH- and DiC8-stimulated conditions, and subsequently examined the influence of SS_{14} on these Ca^{2+} signals. These findings were compared to GH release data obtained from similar experiments conducted on populations of mixed pituitary cells in column perifusion. The results demonstrate that neither reductions in $[Ca^{2+}]_i$ or an abolition of stimulated Ca^{2+} signals are required for SS_{14} inhibition of either basal or PKC-dependent GH responses. The data also suggest the presence of novel SS_{14} actions proximal, as well as distal, to Ca^{2+} signal generation.

3.2 Materials and Methods

3.2.1 Animals and Cell Preparation

Animal maintenance, as well as pituitary cell dispersion and culture were performed as described in Section 2.2.1. Freshly dispersed cells were then plated on

either poly-L-lysine-coated coverslips (0.25 x 10^6 cells/ml), for intracellular Ca^{2+} imaging (27), or preswollen Cytodex-I beads (28) (Sigma-Aldrich, 1.5 x 10^6 cells/column) for column perifusion and cultured overnight prior to experimentation.

Pituitaries from male and female goldfish at different stages of the gonadal reproductive cycle were used in this study. Although the magnitude of GH responses to different neuroendocrine regulators varies throughout the seasonal reproductive cycle (reviewed in (29, 30)), the ability of SS_{14} to inhibit stimulated GH secretion is independent of gonadal state. Nevertheless, to facilitate future comparisons, the approximate gonadal stage of the fish employed for each set of experiments, as determined by the time of year, are reported in the figure legends.

3.2.2 Reagents and Test Substances

5-(2-(2-(bis(2- ((acetyloxy)methoxy)-2-oxoethyl)amino)- 5-methylphenoxy)ethoxy)-2-benzofuranyl)- , (acetyloxy)methyl ester (Fura-2 AM; Molecular Probes) was made up daily in a 20% (w/v) pluronic F-127/DMSO solution (Molecular Probes) (31). Final concentrations were achieved by dilution in testing medium. Ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA; Sigma-Aldrich) was dissolved directly into testing medium. All other media and reagents were the same as described in Section 2.2.2.

3.2.3 Cell Identification

Somatotropes were identified using unique morphological characteristics visible under Nomarski Differential Interference Contrast (DIC) microscopy as previously described (32). Briefly, somatotropes possess a large ovoid nucleus containing a centrally placed nucleolus, numerous granules within the cytoplasm and small cytoplasmic extensions. The ability of this technique to correctly identify somatotropes in primary culture has been shown to be 94% accurate (32). However, I also verified my ability to

reliably employ this technique to identify GH cells by immunostaining with GH anti-serum. My identification of GH cells was >95% accurate (W. K. Yunker, unpublished).

3.2.4 Measurements of $[Ca^{2+}]_i$ in Single, Identified Somatotropes

Following overnight incubation, cells were loaded with Fura-2 by 35-40 minute incubation at 28 C, 5% CO_2 and saturated humidity in testing medium containing 10 μM Fura-2 AM. Cells were subsequently washed twice, first with testing medium and then with clear testing medium. Following loading incubation, all procedures were conducted at room temperature. Using an open bath imaging chamber and peristaltic pump, the cells were perifused with clear testing medium at a rate of 1 ml/min. The Fura-2 was excited with a Hg-Xe arc lamp (Hamamatsu, Japan) at 340 nm and 380 nm wavelengths using a computer-controlled filter wheel (Empix Imaging, Mississauga, ON). Emission fluorescence (510 nm) was recorded through a 100x oil-immersion objective (1.3 N.A. Fluor) on a Zeiss Axiovert 135 inverted microscope (Carl Zeiss, Canada, Don Mills, ON) using a Paultek Imaging ICCD camera (Grass Valley, CA) and National Institutes of Health Image software (version 1.61, available on the Internet by anonymous file transfer protocol from zippy.nimh.nih.gov) running Ca^{2+} Ratiometrics (version 3, a macro written by Dr. Calvin JH Wong). Pairs of images were collected every 15 sec. Exposure time and camera gain were used to optimize the signal-to-noise ratio, while neutral density filters (Omega Optical, Brattleboro, VT) were employed to reduce photobleaching. The ratio of emission intensity at 510 nm from alternate 340 nm and 380 nm excitation was converted to $[Ca^{2+}]_i$ estimates (33) off-line using constants determined specifically for these experimental conditions through the use of an *in vitro* Ca^{2+} calibration kit (Molecular Probes) (31).

Ca^{2+} tracings from individual cells are presented as nanomolar estimations of $[Ca^{2+}]_i$. All tracings were normalized as a percentage of average $[Ca^{2+}]_i$ from time 0 to 2 min (referred to as basal $[Ca^{2+}]_i$), expressed as % pretreatment, analyzed as detailed below and pooled. A 2-min baseline period was chosen because the application of GnRH

was 2 min in duration. Each Ca^{2+} profile was analyzed in three ways. The average Ca^{2+} response was calculated as the average $[Ca^{2+}]_i$ during the drug application period. The maximum amplitude of the Ca^{2+} response was the maximum $[Ca^{2+}]_i$ recorded during either the drug application period or the five minutes following drug application. The time to maximum amplitude was calculated as the amount of time between the start of drug application and maximum amplitude. These parameters were compared using Mann-Whitney U-test, while the proportions of cells displaying different types of Ca^{2+} responses were compared using Fisher's Exact Test. Differences were considered significant when $P<0.05$.

3.2.5 Column Perifusion Studies

Cells cultured on Cytodex beads were placed in temperature controlled (18 C) columns and perifused with testing medium as previously described (28). Experiments began with the collection of six 5-min fractions of perifusate, after which fractions were collected every 30 sec to enhance temporal and kinetic resolution during and following drug application. Perifusates were stored at –26 C until GH content was measured by a previously validated RIA (34). GH release for each column was normalized as a percentage of the average values obtained for the first 6 fractions (% pretreatment). Net hormone responses were quantified by determining the change in GH levels (i.e., area under the curve) for the duration of drug application plus 5 min following drug removal and expressed as a percentage of pretreatment values (35). This was done by subtracting the GH values at each time point during drug application and the subsequent 5-min period from the pre-pulse mean, which was defined as the average of the three time points prior to drug application, and summing the results together for a net GH response (% pretreatment). Statistical analysis was performed using Student's *t*-test. Differences were considered significant when $P<0.05$. Hormone release results are presented as mean ± SEM.

3.3 Results

3.3.1 $[Ca^{2+}]_i$ in Unstimulated Somatotropes and Responsiveness to K^+

In mixed populations of dispersed goldfish pituitary cells, ~20% of cells possess GH immunoreactivity, and may be readily identified using established morphological criteria (32). Of all the morphologically identified somatotropes examined in this chapter, ~88% (144 of 163 cells) were quiescent, exhibiting stable basal intracellular Ca^{2+} values (Fig. 3.1A). The average $[Ca^{2+}]_i$ from time 0 to 2 min for these cells was 151 ± 11 nM (mean ± SEM) with a coefficient of variation (CV; calculated as the standard deviation divided by the average) of 11.2 ± 0.7 %. The remaining 19 cells were spontaneously active, demonstrating highly variable fluctuations in $[Ca^{2+}]_i$ (Fig. 3.1B and C). Average $[Ca^{2+}]_i$ from time 0 to 2 min for these cells was 216 ± 25 nM with a CV of 28.0 ± 3.8 %. Overall, quiescent cells remained as such, while spontaneously active cells remained active. Cells exhibiting transitions from a quiescent to a spontaneously active state, or vice versa, were not observed. All data presented herein were obtained from quiescent somatotropes.

As a control to establish that reductions in $[Ca^{2+}]_i$ are possible and detectable within single, identified goldfish somatotropes, I attempted to lower $[Ca^{2+}]_i$ by removing extracellular Ca^{2+} during perifusion. Changeover from Ca^{2+}-containing to nominally Ca^{2+}-free testing media containing 4 mM EGTA resulted in a decrease in basal $[Ca^{2+}]_i$ (Fig. 3.2A). On average, for the 5 min following removal of extracellular Ca^{2+}, $[Ca^{2+}]_i$ decreased to 75.9 ± 5.1 % of pretreatment levels (n=8). These data suggest a role for extracellular Ca^{2+} in the maintenance of basal $[Ca^{2+}]_i$ levels and establish that reductions in $[Ca^{2+}]_i$ are possible and detectable in this system. Furthermore, to approximate the relative range of increases in $[Ca^{2+}]_i$ to be expected in goldfish somatotropes, cells were depolarized with 30 mM K^+. Treatment with 30 mM K^+ induced rapid, reversible increases in $[Ca^{2+}]_i$ (Fig. 3.2B) to an average maximal amplitude of 600 ± 92 nM. Average $[Ca^{2+}]_i$ during 2 min K^+ exposure increased to 545.1 ± 150.7% pretreatment levels (n=15).

3.3.2 Effects of SS_{14} on Basal $[Ca^{2+}]_i$ and GH Release

Long-term exposure to a maximally effective dose of SS_{14} (1 μM) (34) has previously been shown to reduce basal and inhibit stimulated GH release in static incubation (24) and perifused pituitary fragments (34). In the present study, the effects of acute exposure to 1 μM SS_{14} on GH release and $[Ca^{2+}]_i$ were investigated. In column perifusion, 5-min application of SS_{14} significantly reduced basal GH release (Fig. 3.3A). Upon removal of SS_{14} a GH rebound, such as has been reported for mammalian pituitary cells (16), was not observed. It should be noted that in this, as well as all subsequent experiments, SS_{14} was employed at a concentration of 1 μM, a dose which is ~100 to 1000 times greater than what is commonly used in mammalian research. Goldfish lack a typical hypothalamo-hypophyseal portal system, instead, hypothalamic neurons directly innervate the anterior pituitary, releasing neuroendocrine factors in the direct vicinity of the pituitary cells (reviewed in (22, 25)). It is therefore reasonable to expect that the concentration of neuroendocrine factors surrounding goldfish pituitary cells would be considerably higher than that of their mammalian counterpart, and that as such, the maximally effective concentration of the regulatory factors would be higher than those in mammalian model systems.

Next, I tested the hypothesis that SS_{14}-regulated changes in GH secretion are accompanied by parallel reductions in $[Ca^{2+}]_i$, as has been proposed for mammalian somatotropes (16). When single identified somatotropes loaded with Fura-2 were exposed to 1 μM SS_{14} for 5 min, no changes in $[Ca^{2+}]_i$ were observed (n=9; Fig. 3.3B). Average Ca^{2+} prior to and during SS_{14} application was 97.2 ± 2.3 and 102.4 ± 3.3 % pretreatment respectively. Similarly, 8-min application of 1 μM SS_{14} also had no effect (n=5, data not shown) on $[Ca^{2+}]_i$. These results establish that although SS_{14} is capable of significantly lowering basal GH release, it has no detectable effect on resting $[Ca^{2+}]_i$ in goldfish somatotropes.

3.3.3 SS_{14} Inhibits cGnRH-II-Stimulated GH Release Despite Elevations in $[Ca^{2+}]_i$

In cell column perifusion experiments, 2-min application of a maximally effective dose of cGnRH-II (0.1 μM) (28) stimulated GH release (Fig. 3.4). However, in the presence of SS_{14}, the GH response was completely abolished (Fig. 3.4).

In mammals, GHRH-evoked Ca^{2+} signals are completely abolished by SS_{14}; furthermore, $[Ca^{2+}]_i$ is decreased in a manner similar to what is seen during SS_{14}-induced reductions of basal $[Ca^{2+}]_i$ (16). I tested whether this phenomenon similarly occurs with cGnRH-II stimulation of goldfish somatotropes. When data from all cells exposed to cGnRH-II (0.1 μM) were pooled together, $[Ca^{2+}]_i$ increased to an average of 140.1 ± 9.0 % pretreatment (n=24) during the 2-min application of cGnRH-II. Of these 24 cells, 12 responded with increases in $[Ca^{2+}]_i$ of >20% pretreatment (Fig. 3.5A; average $[Ca^{2+}]_i$=166.5 ± 16.3% pretreatment, Table 3.1; i.e., robust Ca^{2+} responses that are at least approximately double the normal %CV observed in quiescent somatotropes). For the remaining 12 cells, the average increase in $[Ca^{2+}]_i$ in response to cGnRH-II was 4.7% pretreatment. To examine the ability of SS_{14} to modulate cGnRH-II-evoked Ca^{2+} signals, a 2-min pulse of cGnRH-II was applied during a 5-min exposure to SS_{14}. When data from all cells exposed to cGnRH-II during SS_{14} treatment were pooled together, $[Ca^{2+}]_i$ increased to an average of only 119.9 ± 5.2% pretreatment during GnRH application (n=19). Of the 19 cells examined, 5 responded with a >20% pretreatment increase in $[Ca^{2+}]_i$ during cGnRH-II application (Fig. 3.5B; Table 3.1). In the remaining 14 cells, the average increase in $[Ca^{2+}]_i$ in response to cGnRH-II in the presence of SS_{14} was 5.7% pretreatment.

Comparisons between the 5 cells responding with >20% pretreatment increases in $[Ca^{2+}]_i$ during cGnRH-II application in the presence of SS_{14} and the 12 cells responding with >20% pretreatment increases in $[Ca^{2+}]_i$ upon exposure to cGnRH-II alone revealed that, in cells displaying robust increases in $[Ca^{2+}]_i$, SS_{14} had no effect on either the maximal amplitude of the Ca^{2+} response or average Ca^{2+} response during GnRH application (Table 3.1). However, the time to maximal amplitude in the 5 cells demonstrating robust Ca^{2+} signals in the presence of SS_{14} was significantly delayed

compared to what was observed when the somatotropes were exposed to cGnRH-II alone (Fig. 3.5C; Table 3.1). A similar delay in the time to maximal amplitude in response to cGnRH-II in the presence of SS_{14} was also seen when data from all 19 cells were pooled (data not shown). These results establish that SS_{14} is modulating cGnRH-II-evoked Ca^{2+} responses in two ways. First, SS_{14} is reducing the number of cells responding to cGnRH-II with robust Ca^{2+} signals. Second, in cells displaying robust Ca^{2+} responses, SS_{14} is altering the temporal characteristics of the Ca^{2+} signal.

3.3.4 SS_{14} Inhibits sGnRH-Stimulated GH Release Despite Elevations in $[Ca^{2+}]_i$

The ability of SS_{14} to interfere with the actions of the other endogenous GnRH found in goldfish, sGnRH, to stimulate GH release and Ca^{2+} signalling was also examined. In perifusion studies, 2-min application of a maximally effective dose of sGnRH (0.1 μM) (28) increased GH release (Fig. 3.6). As was the case with cGnRH-II, the ability of sGnRH to stimulate GH release was completely abolished in the presence of SS_{14} (Fig. 3.6).

In individual somatotropes loaded with Fura-2, 2-min application of sGnRH increased $[Ca^{2+}]_i$ to an average of 151.3 ± 23.4% pretreatment (n=22) during the application period. Of these 22 cells, 8 responded to sGnRH treatment with an increase in $[Ca^{2+}]_i$ of >20% (Fig. 3.7A; Table 3.1). As was the case for cGnRH-II, the peak of the Ca^{2+} response was typically observed during the sGnRH application period. In the remaining 14 cells, the average increase in $[Ca^{2+}]_i$ in response to sGnRH was 4.0% pretreatment. In the presence of SS_{14}, $[Ca^{2+}]_i$ increased to 117.9 ± 4.4% pretreatment (n=20) during sGnRH application. Of these 20 cells, only 2 responded with a >20% pretreatment increase in $[Ca^{2+}]_i$ (Fig. 3.7B; Table 3.1). In the remaining 18 cells, the average increase in $[Ca^{2+}]_i$ in response to sGnRH while in the presence of SS_{14} was 4.4% pretreatment.

Comparison of the sGnRH-elicited Ca^{2+} responses in cells exhibiting responses of >20% pretreatment, in either the presence or absence of SS_{14}, revealed that the maximal

amplitude of the Ca^{2+} response, as well as the time to peak amplitude were significantly altered by SS_{14} (Fig. 3.7C; Table 3.1). Changes in all of these response characteristics were also observed when all somatotropes treated with sGnRH during SS_{14} exposure were included in the analysis (data not shown).

These results demonstrate that SS_{14} significantly reduced the number of cells producing robust Ca^{2+} signals in response to sGnRH, but did not completely abolish the ability of somatotropes to produce such Ca^{2+} signals. Furthermore, as was found with cGnRH-II-stimulated Ca^{2+} signals, the profile of the sGnRH-stimulated Ca^{2+} signal are also a target of SS_{14} action. However, SS_{14} appears to modulate sGnRH- and cGnRH-II-stimulated Ca^{2+} signals differently.

3.3.5 SS_{14} Inhibits DiC8-stimulated GH Release Despite Elevations in $[Ca^{2+}]_i$

Both sGnRH and cGnRH-II are believed to stimulate GH release through a PKC-sensitive signalling mechanism (reviewed in (23)). The synthetic diacylglycerol, DiC8, was used to bypass the GnRH receptor and directly activate PKC, thereby enabling me to investigate whether Ca^{2+} signals can be generated distal to PKC activation and whether SS_{14} is able to modulate events subsequent to PKC activation.

In column perifusion studies, 5-min applications of 10 μM DiC8 (a concentration which has been previously shown to consistently induce a GH response that is ~85% of the maximal DiC8 response (36)) stimulated GH release (Fig. 3.8). However, when DiC8 was administered during an 8-min SS_{14} treatment, the ability of DiC8 to stimulate GH release was completely abolished (Fig. 3.8). Interestingly, exposure to DiC8 appeared to potentiate SS_{14} inhibition of GH release in a rapid, reversible manner. Upon application of DiC8, GH release was further suppressed, while upon removal of DiC8 this additional suppression was alleviated (Fig. 3.8).

In single-cell Ca^{2+}-imaging experiments, 5-min application of DiC8 (10 μM) induced dramatic increases in $[Ca^{2+}]_i$. On average, $[Ca^{2+}]_i$ rose to 163.0 ± 14.9% of the pretreatment values (n=13) during DiC8 application. Of these 13 cells, 10 responded to

DiC8 application with a >20% increase in $[Ca^{2+}]_i$ (Fig. 3.9A; Table 3.1) In the remaining 3 cells, the average increase in $[Ca^{2+}]_i$ in response to DiC8 was 8.9% pretreatment. In the presence of SS_{14}, DiC8 was still capable of inducing robust increases in $[Ca^{2+}]_i$, generating responses that on average were 238.5 ± 53.8% of pretreatment levels (n=14). Of these 14 cells, 7 responded with >20% increases in $[Ca^{2+}]_i$ (Fig. 3.9B; Table 3.1), while the remaining 7 cells responded with an average increase in $[Ca^{2+}]_i$ of 0.2% pretreatment.

In cells displaying >20% pretreatment increases in $[Ca^{2+}]_i$, the average $[Ca^{2+}]_i$ during DiC8 application, as well as the maximal amplitude of the Ca^{2+} response were significantly higher in SS_{14}-treated cells relative to their non-SS_{14} exposed controls (Fig. 3.9C; Table 3.1). In addition, the time to maximal amplitude was significantly reduced in the presence of SS_{14} (Table 3.1). Similar SS_{14}-induced changes were also seen when results from all somatotropes treated with DiC8 were pooled (data not shown).

These findings demonstrate that although PKC activation is capable of evoking robust Ca^{2+} signals that are not inhibited by treatment with SS_{14}, the GH response to PKC activation is susceptible to SS_{14} inhibition. Furthermore, these data establish that PKC activation during exposure to SS_{14} has the paradoxical effect of further inhibiting GH release while generating a larger Ca^{2+} signal.

3.4 Discussion

The ability of activated sst's to couple negatively to AC and Ca^{2+} influx has been extensively studied (reviewed in (17)). However, the intracellular mechanisms through which SS_{14} inhibits cAMP-independent neuroendocrine regulators of GH release is less well understood. In this study, the extensively studied goldfish somatotrope was employed to ask whether inhibition of Ca^{2+} signals was being used as a mechanism for SS_{14}-induced inhibition of the PKC-dependent GH secretagogue, GnRH.

3.4.1 Ca^{2+} Signals in Goldfish

A previous study from our laboratory established that some goldfish somatotropes display spontaneous fluctuations in $[Ca^{2+}]_i$ (26). Here I report that quiescent to active somatotropes occur in a ratio of ~ 8:1. The low occurrence of spontaneously active cells not only precludes a separate statistical analysis of this subpopulation, but also suggests that these cells may not contribute substantially to the overall GH response. The idea of cell heterogeneity, especially among pituitary cells has been well documented, (37-41); unfortunately, this phenomena has not yet been investigated in this system. It is interesting to note that basal $[Ca^{2+}]_i$ in quiescent cells was considerably lower than that observed in spontaneously active cells.

Although the relative contribution of Ca^{2+} entry through VSCC activity to the maintenance of resting $[Ca^{2+}]_i$ in goldfish somatotropes has not yet been evaluated, spontaneous Ca^{2+} activity was observed in EGTA-containing, Ca^{2+}-free media (n=3, data not shown). This observation supports the idea that release from intracellular stores, and not extracellular Ca^{2+} influx is responsible for the spontaneous activity observed in goldfish somatotropes (26). Nevertheless, VSCC are present and play a role in the regulation of GH secretion in goldfish (42). Consistent with the known presence of L-type VSCC in goldfish somatotropes (42), treatment with depolarizing concentrations of K^+ elevated GH secretion (26), and in the present study, evoked increases in $[Ca^{2+}]_i$ that were ~1.5 times greater in magnitude than those elicited by neuroendocrine receptor agonists. These observations, together with the fact that basal GH secretion in goldfish is sensitive to reductions in extracellular Ca^{2+} and additions of VSCC blockers (43, 44), indicate that lowering $[Ca^{2+}]_i$ is possible and detectable in our system, and that such reductions result in diminished GH release. It is surprising therefore, that, unlike SS_{14} actions in mammalian pituitary cells (3), SS_{14} does not alter basal $[Ca^{2+}]_i$ as a means of reducing basal GH release (see below).

3.4.2 SS_{14} Action

Previous studies have shown that in mammalian model systems, SS_{14} negatively couples to $[Ca^{2+}]_i$ to inhibit basal, as well as non-cAMP-dependent stimulated GH release (2, 3, 14, 16, 45, 46). In contrast, our results establish that in goldfish somatotropes, it is possible for SS_{14} to modulate basal GH secretion without inducing parallel reductions in $[Ca^{2+}]_i$. Similarly, although SS_{14} reduces the proportion of somatotropes responding to GnRH with robust Ca^{2+} signals, it does not abolish GnRH- or DiC8-stimulated Ca^{2+} signals, and yet is still effective in completely inhibiting the associated GH response. These findings establish that neither reduction of $[Ca^{2+}]_i$ nor abolition of Ca^{2+} signal generation are plausible explanations of the secretion-inhibiting effects of SS_{14} in this system.

These conclusions assume that the amount of GH released by somatotropes selected for $[Ca^{2+}]_i$ determination should be detectable by our RIA. The morphological cell identification is dependent upon the orientation of the somatotropes on the culture plate. As a result, it is extremely difficult to ascertain the exact percentage of all somatotrope cells that could meet our morphological criteria given the appropriate orientation. However, using histologically fixed cells immunostained for GH reactivity, a minimum of 1/3 of all GH staining cells meet our morphological criteria (WK Yunker, JD Johnson, unpublished). In addition, the RIA consistently has a minimal effective detection level of better than 2.5 ng/ml, while basal GH levels in column perifusion are typically between 30 to 40 ng/ml. Therefore, if morphologically identified somatotropes represent 1/3 of all GH cells, the assay would be more than capable of detecting the resulting 10 to 13 ng/ml fluctuations in GH levels. This assumes relatively equal secretion rates among the GH cells. If however, secretion rates among GH cells are not equal, and the somatotropes selected for $[Ca^{2+}]_i$ measurement do not contribute a measurable amount of GH, there exists the equally intriguing possibility that a sizeable subpopulation of somatotropes which exhibit Ca^{2+} signals in response to both natural ligands and pharmacological stimulation but do not contribute to the overall release of GH.

In goldfish, the two endogenous GnRHs are capable of acting on the same somatotrope (26), possibly through the same population of GnRH receptors (47-49) to stimulate GH release. Acute stimulation of GH release by either sGnRH or cGnRH-II is dependent upon Ca^{2+} release from a caffeine-sensitive, Tg-insensitive intracellular Ca^{2+} store(s) (26) and activation of PKC (reviewed in (23)). In the present study, the GH responses to both GnRHs were abolished by SS_{14} and the corresponding Ca^{2+} signals were significantly altered. Surprisingly, SS_{14} modulated the cGnRH-II and sGnRH-stimulated Ca^{2+} signals differently (Table 3.1). This differential modulation of cGnRH-II- and sGnRH-stimulated Ca^{2+} signals suggests that, despite acting through the same receptor population, sGnRH and cGnRH-II are initiating different intracellular responses.

Although a definitive explanation of the cellular mechanisms underlying these responses can not be provided, several possibilities do exist. Although both GnRH's utilize intracellular Ca^{2+} pools sensitive to caffeine but not to Tg, sGnRH and cGnRH-II can also specifically target IP_3- and Ry-sensitive Ca^{2+} stores, respectively ((50); reviewed in Section 1.8.1.1). Thus SS_{14} may be exerting differential modulatory effects on the release of Ca^{2+} from these putative GnRH-selective stores, or the filling state of these stores, to affect GnRH-stimulated Ca^{2+} signals. It is also possible, that these observations are the result of differential expression of various sst subtypes on somatotrope cells, or of multiple somatotrope subtypes each possessing different SS_{14} signalling cascades.

Changes in the characteristics of the GnRH-induced Ca^{2+} signals (i.e., time to maximum amplitude and magnitude of maximum amplitude) suggest that the rate of increase in $[Ca^{2+}]_i$ (in addition to possible changes in the average $[Ca^{2+}]_i$) is reduced by treatment with SS_{14}. The rate of increase in $[Ca^{2+}]_i$ has been shown to be an important factor in determining the effectiveness of a Ca^{2+} signal in stimulated exocytosis in rat pituitary cells (51) and neurons (52); this would offer an alternate mechanism by which SS_{14} inhibits GnRH-evoked GH secretion in the presence of GnRH-induced Ca^{2+} signals. However, neither a simple slowing in the rate of increase in $[Ca^{2+}]_i$ nor a decrease in average size of the Ca^{2+} response appear to be adequate explanations in light of results

from experiments with DiC8, which actually generates a larger Ca^{2+} signal with a faster apparent rate of increase to maximum amplitude in the presence of SS_{14} (see below).

In the present study, DiC8 consistently generated large, robust Ca^{2+} signals that were similar in profile to results obtained from previous studies using populations of dispersed pituitary cells (53). Both the GH and Ca^{2+} profiles resulting from treatment with DiC8 differed from those resulting from either cGnRH-II or sGnRH. However, given that application of DiC8 results in a pharmacological, rather than natural activation of PKC, and that the experimental dose is probably not identical to the natural DAG levels experienced during GnRH challenge, there is no reason to expect identical response profiles. As was the case for cGnRH-II- and sGnRH-stimulated GH release, SS_{14} was effective in inhibiting PKC-stimulated GH release. However, SS_{14} inhibition of GH release appeared to be potentiated during DiC8 application. Surprisingly, in the presence of SS_{14}, the Ca^{2+} signal generated by PKC was significantly greater in both the average magnitude of increase and the maximal amplitude; more importantly, the time to maximal amplitude was significantly reduced (thereby ruling out decreasing the rate of increase of $[Ca^{2+}]_i$ as a putative mechanism for SS_{14}). Simultaneous application of muscarinic receptor agonists and SS_{14} has been shown to potentiate the increase $[Ca^{2+}]_i$ produced by muscarinic receptor activation alone (54, 55). Similarly, sst activation has been shown to increase $[Ca^{2+}]_i$ as a result of PLC activation (56, 57). However, the paradoxical effect of PKC activation potentiating SS_{14} inhibition of GH release while SS_{14} increases PKC-stimulated Ca^{2+} signals has never before been reported.

How this effect is achieved is not known, but several alternatives can be considered. First, previous studies in other model systems have illustrated the capacity of PKC to modulate sst function (58). Therefore, one can speculate that PKC activation may modulate the function of either the sst or the associated intracellular signalling cascade, thereby enabling SS_{14} to elevate $[Ca^{2+}]_i$, possibly through activation of PLC (56, 57). Second, although previous studies suggest that PKC does not depend upon Ca^{2+} mobilization from intracellular stores to stimulate GH release (26), the possibility that

SS_{14} treatment somehow enables activated PKC to mobilize intracellular Ca^{2+} or increase extracellular Ca^{2+} influx can not be excluded. Regardless of the mechanism underlying SS_{14}-potentiation of DiC8-evoked Ca^{2+} signals, how the augmented Ca^{2+} signals relate to enhanced inhibition of GH release is an interesting question for future Ca^{2+} signalling research.

In addition, Kwong and Chang (1997) have previously demonstrated that GH responses to Ca^{2+} ionophores in goldfish pituitary cells are suppressed by SS_{14} (24). Therefore, it is highly likely that SS_{14} also acts downstream of Ca^{2+} mobilization to reduce GH secretion. Is the downstream site of action responsible for attenuation of Ca^{2+} ionophore-stimulated GH release also responsible for this paradoxical effect? Sst activation has been linked to the activation of various different protein kinases and phosphatases (13, 59, 60), and it is plausible that these mediators are involved here. Preliminary data from this laboratory suggests that SS_{14} inhibition of basal GH release is not dependent upon protein kinases sensitive to staurosporine inhibition (WK Yunker, unpublished). Nevertheless, the effects of SS_{14} on DiC8-stimulated Ca^{2+} signals are in stark contrast to the effects of SS_{14} on GnRH-evoked Ca^{2+} signals. As a result, it seems likely that SS_{14} is acting at least two distinct points along the GnRH/PKC signalling cascade.

Studies conducted on mammalian pituitary cells report a very pronounced SS 'off response' (16, 61), or GH rebound. Furthermore, this rebound can be amplified if the cells are exposed to an agonist during treatment with SS (16). This rebound phenomena has been taken as evidence of a distal site of SS action in which secretory granules accumulate beneath the plasma membrane and are free to secrete upon removal of SS inhibition (reviewed in (62)). In some instances, "Ca^{2+} rebounds" have also been reported (16). Rebounds in GH and Ca^{2+} upon removal of SS_{14} were not observed in this study, irrespective of whether an agonist was administered during SS_{14} exposure. This would suggest that either SS_{14} effects are not readily reversible within the time frame examined, or that the mechanism(s) through which SS_{14} is inhibiting GH release in goldfish are not the same as the mechanism(s) mediating SS action in mammalian somatotropes.

Interestingly, studies on another inhibitor of goldfish GH release, NE, have shown that following acute exposure to NE there is a rapid, transient Ca^{2+} rebound (31). In addition, following 2-hr exposure to NE there is a transient increase in GH secretion of up to ~250% pretreatment (31, 63). These results not only demonstrate that "rebounds" in both secretion and $[Ca^{2+}]_i$ can occur in goldfish somatotropes, but they also suggest that two different inhibitors of GH release, SS_{14} and NE, may employ different mechanisms to inhibit basal and stimulated GH release.

In summary, the present study has demonstrated that abolition of Ca^{2+} signals is not a primary mechanism through which SS_{14} lowers basal, or inhibits GnRH-stimulated hormone release in goldfish somatotropes. Furthermore, evidence is presented supporting the hypothesis that SS_{14} acts on at least two distinct points along the GnRH/PKC signalling cascade.

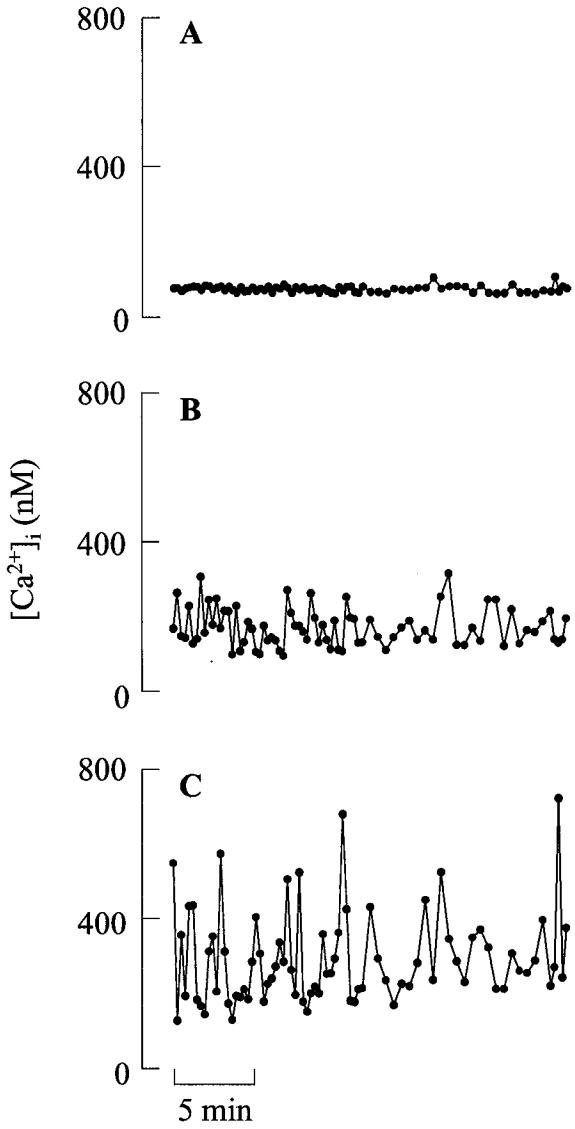

Fig. 3.1. Basal $[Ca^{2+}]_i$ in Identified Somatotropes.

Dispersed pituitary cells obtained from goldfish undergoing gonadal recrudescence (January and February) were used. (A) $[Ca^{2+}]_i$ trace of a single, identified somatotrope displaying stable basal $[Ca^{2+}]_i$ values. (B-C) Patterns of $[Ca^{2+}]_i$ in spontaneously active somatotropes. This and all subsequent $[Ca^{2+}]_i$ tracings shown are from Fura-2 loaded, morphologically identified somatotropes within populations of cultured pituitary cells.

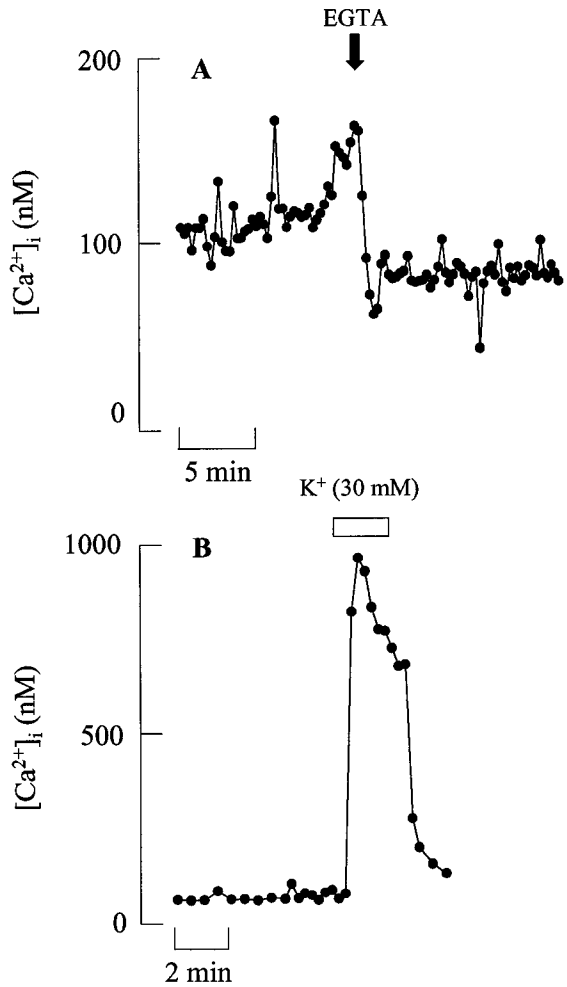

Fig. 3.2. Basal $[Ca^{2+}]_i$ is Dependent Upon Extracellular Ca^{2+}.

Dispersed pituitary cells obtained from goldfish undergoing gonadal recrudescence (January and February) were used. (A) $[Ca^{2+}]_i$ trace of a single, identified somatotrope displaying a reduction in $[Ca^{2+}]_i$ following changeover from Ca^{2+}-containing to nominally Ca^{2+}-free testing media containing 4 mM EGTA (arrow). Average $[Ca^{2+}]_i$ from time 0 to 2 min was 333 ± 68 nM (n=8). (B) K^+ applied for 2 min (open bar) as indicated, increased $[Ca^{2+}]_i$ in single, identified somatotropes. Average $[Ca^{2+}]_i$ from time 0 to 2 min was 129 ± 33 nM (n=15).

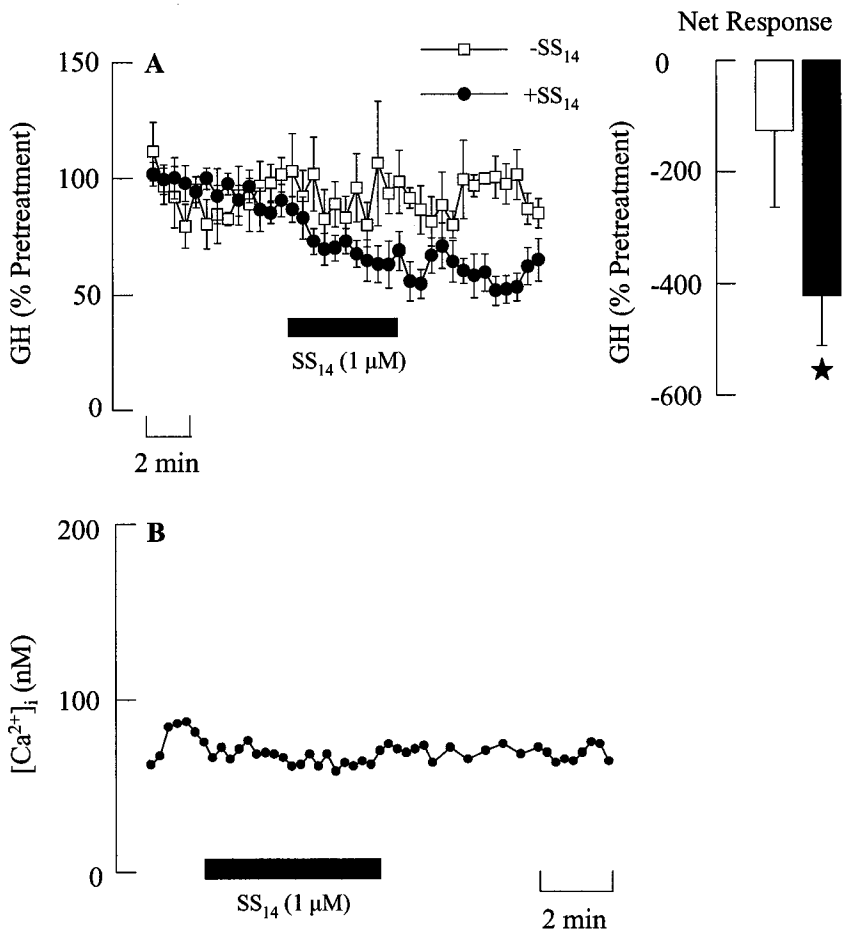

Fig. 3.3. SS_{14} Reduces Basal GH Release but not Basal $[Ca^{2+}]_i$.

Dispersed pituitary cells obtained from goldfish with regressed gonads were used (August and September). (A) Application of SS_{14} for 5 min (black bar) significantly lowered basal GH release. Vertical bars represent net GH responses in the absence (open bar) and presence (solid bar) of SS_{14}. A star represents a significant difference between the two treatments. Average basal GH levels were 45.3 ± 1.0 ng/ml (n=4) and 45.4 ± 1.2 ng/ml (n=8) in control and SS_{14}-exposed columns respectively. (B) $[Ca^{2+}]_i$ trace of a single, identified somatotrope exposed to SS_{14} for 5 min (horizontal black bar). Treatment with SS_{14} did not lower basal intracellular $[Ca^{2+}]_i$. Average $[Ca^{2+}]_i$ from time 0 to 2 min was 113 ± 20 nM (n=9).

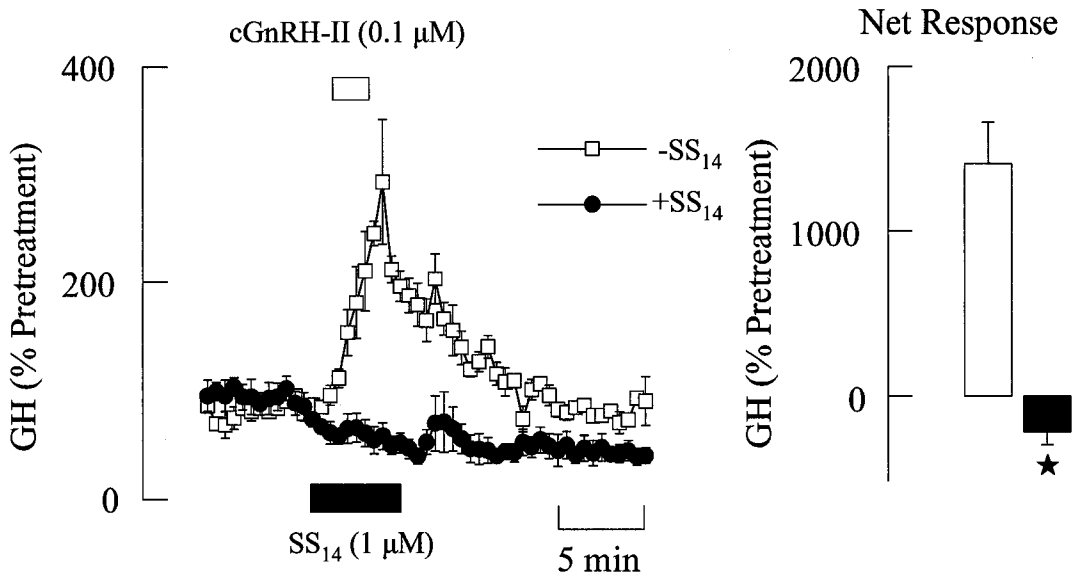

Fig. 3.4. SS_{14} Inhibits cGnRH-II-Stimulated GH Release.

Dispersed pituitary cells obtained from goldfish undergoing gonadal recrudescence and from fish at gonadal maturity (=prespawning) were used (January, March and April). cGnRH-II was applied for 2 min (open bar), while SS_{14} was applied for 5 min (black bar) as indicated. Vertical bars represent net GH responses to either cGnRH-II (open bar) or cGnRH-II plus SS_{14} (solid bar). A star indicates a significant difference between the two treatments. Average basal GH levels were 40.5 ± 2.51 (n=4) and 33.56 ± 4.6 (n=6) ng/ml in cGnRH-II- and cGnRH-II plus SS_{14}-exposed columns respectively.

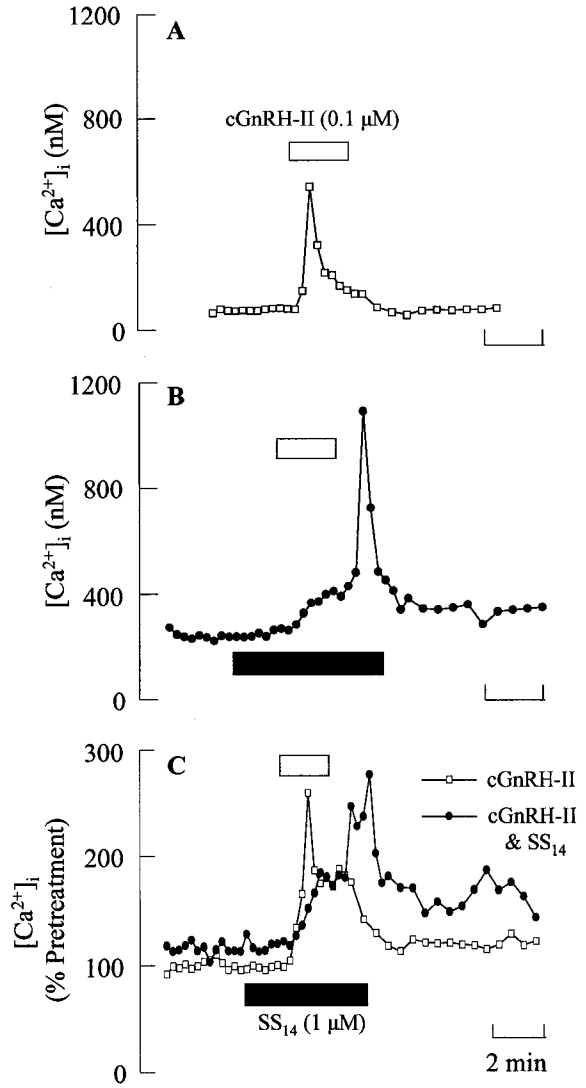

Fig. 3.5. SS_{14} Actions on cGnRH-II-Evoked Ca^{2+} Signals.

Dispersed pituitary cells obtained from goldfish either undergoing gonadal recrudescence, or goldfish at gonadal maturity (=prespawning) were used (November through May). (A) $[Ca^{2+}]_i$ trace of a single, identified somatotrope responding to a 2-min application of cGnRH-II (open bar) with an increase in $[Ca^{2+}]_i$ of >20% pretreatment. Average $[Ca^{2+}]_i$ from time 0 to 2 min for all cells treated with cGnRH-II was 235 ± 44 nM (n=24). (B) $[Ca^{2+}]_i$ trace of a single, identified somatotrope responding to cGnRH-II with an increase in $[Ca^{2+}]_i$ of >20% pretreatment during a 5-min application of SS_{14} (black bar). Average $[Ca^{2+}]_i$ from time 0 to 2 min for all cells treated with SS_{14} and cGnRH-II was 150 ± 32 nM (n=19). (C) All cells responding with >20% increases in $[Ca^{2+}]_i$ in response to cGnRH-II either alone or in the presence of SS_{14} were pooled, the resulting average Ca^{2+} profile is presented (error bars have been omitted for clarity).

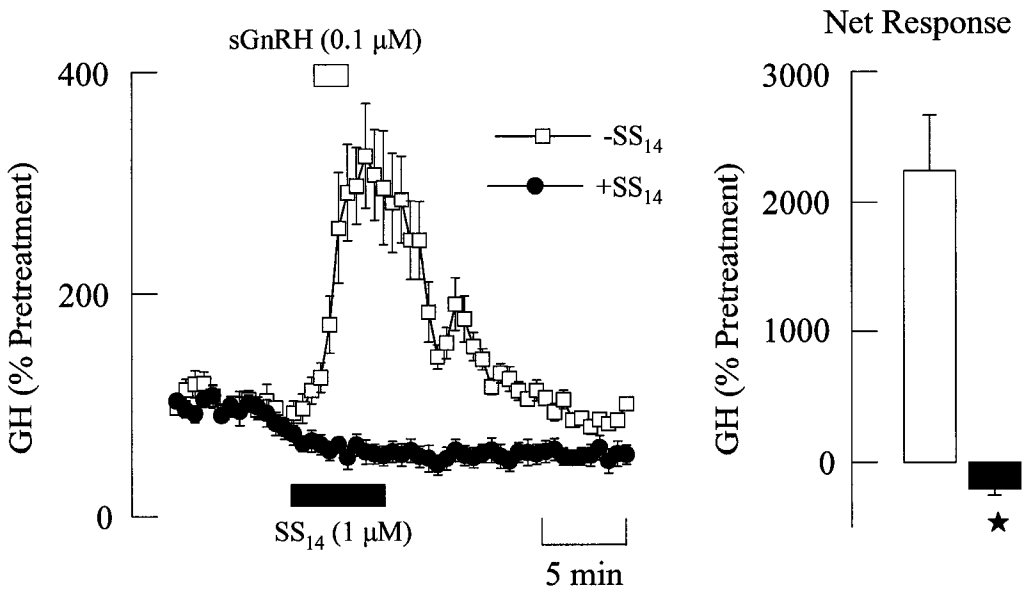

Fig. 3.6. SS_{14} Inhibits sGnRH-Stimulated GH Release.

Dispersed pituitary cells obtained from goldfish at gonadal maturity (=prespawning) were used (April). sGnRH was applied for 2 min (open bar), while SS_{14} was applied for 5 min (black bar) as indicated. Vertical bars represent net GH responses to either sGnRH (open bar) or sGnRH plus SS_{14} (solid bar). A star indicates a significant difference between the two treatments. Average basal GH levels were 40.2 ± 1.5 (n=6) and 41.5 ± 1.3 (n=6) ng/ml in sGnRH-II- and sGnRH-II plus SS_{14}-exposed columns respectively.

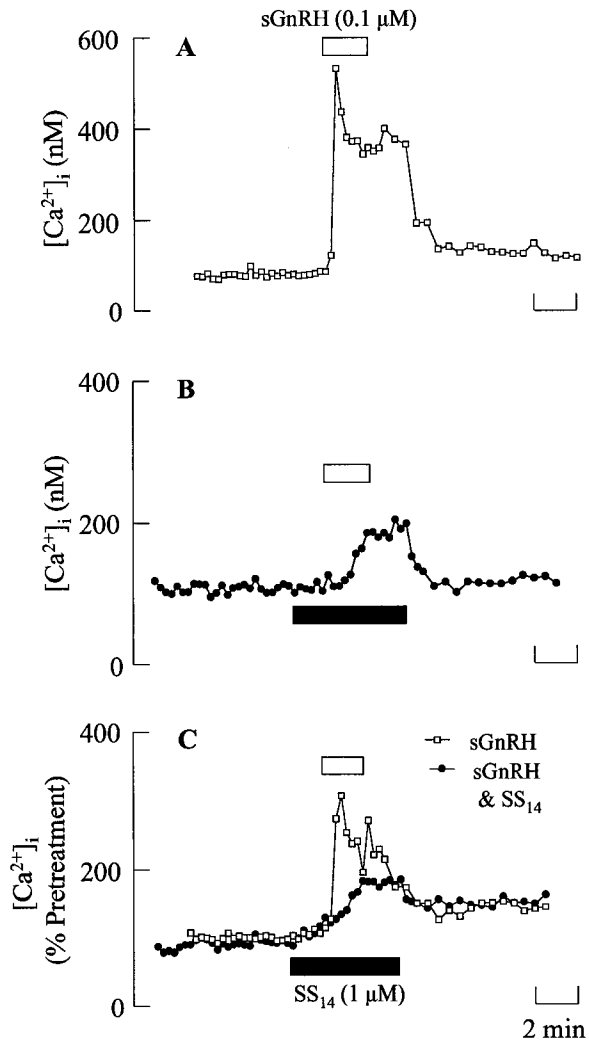

Fig. 3.7. SS_{14} Actions on sGnRH-Stimulated Ca^{2+} Signals.

Dispersed pituitary cells obtained from goldfish undergoing gonadal recrudescence as well as goldfish at gonadal maturity (=prespawning) were used (January through April). (A) $[Ca^{2+}]_i$ trace of a single, identified somatotrope responding to a 2-min application of sGnRH (open bar) with an increase in $[Ca^{2+}]_i$ of >20% pretreatment. Average $[Ca^{2+}]_i$ from time 0 to 2 min for all cells exposed to sGnRH was 138 ± 23 nM (n=22). (B) $[Ca^{2+}]_i$ trace of a single, identified somatotrope responding to sGnRH with an increase in $[Ca^{2+}]_i$ of >20% pretreatment during a 5-min application of SS_{14} (black bar). Average $[Ca^{2+}]_i$ from time 0 to 2 min for all cells exposed to SS_{14} and sGnRH was 163 ± 23 nM (n=20). (C) All cells responding with >20% increases in $[Ca^{2+}]_i$ in response to sGnRH either alone or in the presence of SS_{14} were pooled, the resulting average Ca^{2+} profile is presented (error bars have been omitted for clarity).

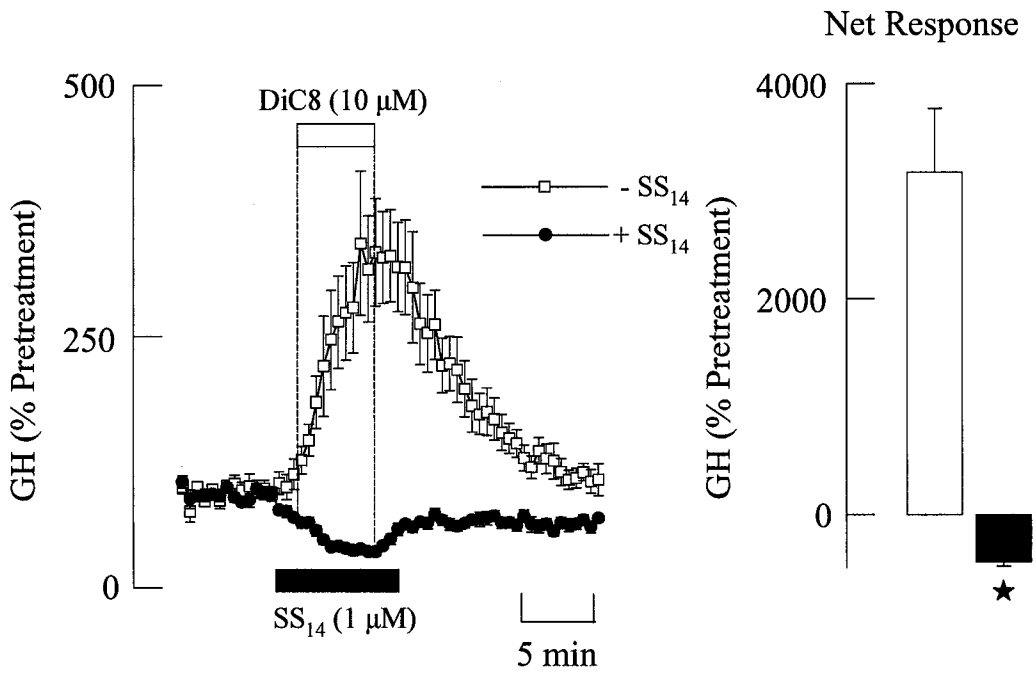

Fig. 3.8. SS_{14} Inhibits DiC8-Stimulated GH Release.

Dispersed pituitary cells obtained from goldfish with regressed gonads (June and July) were used. DiC8 was applied for 5 min (open bar), while SS_{14} was applied for 8 min (black bar) as indicated. Note that during exposure to DiC8, GH release was further decreased in columns exposed to SS_{14}. Vertical bars represent net GH responses to either DiC8 or DiC8 plus SS_{14}. A star indicates a significant difference between the two treatments. Average basal GH levels were 45.9 ± 6.6 (n=10) and 48.2 ± 3.8 (n=10) ng/ml in DiC8- and DiC8 plus SS_{14}-exposed columns respectively.

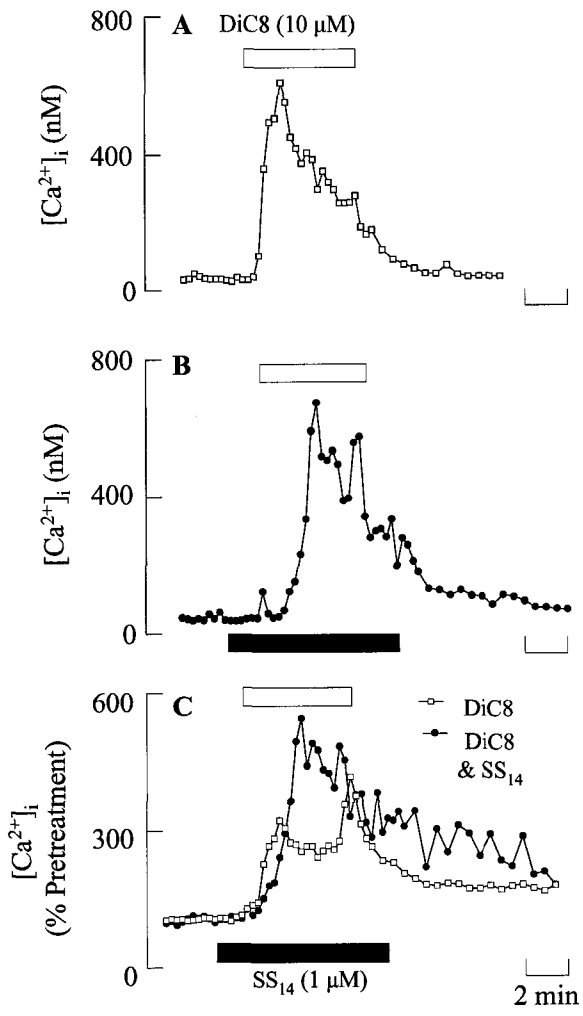

Fig. 3.9. SS_{14} Actions on DiC8-Evoked Ca^{2+} Signals.

Dispersed pituitary cells obtained from goldfish with regressed gonads (June and July) were used. (A) $[Ca^{2+}]_i$ trace of a single, identified somatotrope responding to a 5-min application of DiC8 (open bar) with an increase in $[Ca^{2+}]_i$ of >20%. Average $[Ca^{2+}]_i$ from time 0 to 2 min for all cells treated with DiC8 was 167 ± 40 nM (n=13). (B) $[Ca^{2+}]_i$ trace of a single, identified somatotrope responding to DiC8 with an increase in $[Ca^{2+}]_i$ of >20% pretreatment during an 8-min application of SS_{14}. Average $[Ca^{2+}]_i$ from time 0 to 2 min for all cells exposed to SS_{14} and DiC8 was 114 ± 23 nM (n=14). (C) All cells responding with >20% increases in $[Ca^{2+}]_i$ in response to DiC8 either alone or in the presence of SS_{14} were pooled, the resulting average Ca^{2+} profile is presented (error bars have been omitted for clarity).

Table 3.1. Effects of SS_{14} on GnRH- and DiC8-Stimulated Ca^{2+} Signals.

	% of cells[a]	Ave Ca^{2+} During (% Pretreatment)	Max Amplitude (% Pretreatment)	Time to Max Amplitude (min)
cGnRH-II	50.0 (12/24)	166.5 ± 16.3	333.6 ± 65.2	1.4 ± 0.2
cGnRH-II & SS_{14}	26.3[b] (5/19)	151.0 ± 9.7	352.8 ± 93.4	3.9 ± 0.5[b]
sGnRH	36.4 (8/22)	225.1 ± 57.2	384.9 ± 91.8	1.3 ± 0.3
sGnRH & SS_{14}	10.0[b] (2/20)	149.5 ± 17.7	196.9 ± 8.1[b]	4.4 ± 0.9[b]
DiC8	76.9 (10/13)	177.8 ± 16.7	413.7 ± 123.3	5.2 ± 0.8
DiC8 & SS_{14}	50.0 (7/14)	371.1 ± 81.6[b]	714.3 ± 176.6[b]	3.3 ± 0.8[b]

[a] % of cells responding with >20% pretreatment increases in average intracellular Ca^{2+} during secretagogue treatment. All of the Ca^{2+} parameters presented were calculated from cells that responded to a given treatment and are presented as mean ± SEM.

[b] Significantly different from non-SS_{14} treated control.

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Chapter 4 – SS_{14} Actions on SKF-38393- and PACAP-Evoked Ca^{2+} Signals and GH Secretion [3]

4.1 Introduction

Early studies examining SS_{14} inhibition of basal and stimulated GH secretion in mammals concluded that regulation of $[Ca^{2+}]_i$ was one of the principal mechanisms underlying SS_{14} inhibition of hormone release (1, 2). However, in goldfish pituitary somatotropes, GnRH- and PKC-evoked Ca^{2+} signals are present during treatment with SS_{14} (Chapter 3; (3)). The ability of SS_{14} to suppress Ca^{2+}-dependent exocytosis by acting distal to elevations in intracellular Ca^{2+} has also been documented in pancreatic α-cells (4), pancreatic β-cells (5) and pituitary GC tumour cells (6).

SS_{14} is known to exert its effects through a family of heptahelical, G-protein coupled sst's. In mammals, five subtypes, comprising six different receptors have been cloned (reviewed in (7, 8) and Section 1.4). In goldfish, eight sst's from four different subtypes have so far been cloned ((9-12); reviewed in Section 1.5). The ability of SS_{14} to inhibit exocytosis independent of Ca^{2+} signal modulation has been associated with at least two sst subtypes, sst_2 in pancreatic α-cells (13, 14) and sst_1 in pituitary GC tumour cells (6).

The goal of this study was to ascertain if, in goldfish pituitary somatotropes, SS_{14} is also capable of acting distal to elevated $[Ca^{2+}]_i$ to regulate cyclic cAMP/PKA-stimulated GH release. In goldfish, DA and PACAP respectively stimulate GH secretion through D1 and PAC_1 receptors that are coupled to an AC/cAMP/PKA signalling cascade (reviewed in (15) and Section 1.8.1). In both cases, there is also an extracellular Ca^{2+}

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[3] A version of this chapter has been submitted for publication: **Yunker WK, Chang JP**. Somatostatin-14 uncouples dopamine- and pituitary adenylate cyclase-activating polypeptide-evoked Ca^{2+} signals from growth hormone secretion. J Neuroendocrinol (Submitted April 16, 2003, MS # W2027).

entry requirement (16, 17). Although previous work from this lab has shown that SS_{14} is capable of inhibiting SKF-38393- and PACAP-stimulated GH secretion ((17, 18); Chapter 2), the importance of Ca^{2+} signals in mediating PKA-dependent GH secretion from goldfish somatotropes, and their potential modulation by SS_{14} have not been investigated.

Using single, morphologically identified somatotropes loaded with the Ca^{2+}-sensitive dye Fura-2, the effects of SS_{14} on SKF-38393-, PACAP- and cAMP-stimulated Ca^{2+} signals were examined. These findings were then compared to GH release data obtained from similar experiments conducted on populations of mixed pituitary cells in column perifusion. The results establish that although the attenuation of Ca^{2+} signals can inhibit PKA-dependent GH secretion, SS_{14} inhibits stimulated GH secretion by uncoupling Ca^{2+} signals from GH secretion. Furthermore, these results indicate that the cellular mechanisms underlying SS_{14} modulation of stimulated Ca^{2+} signals differ from those regulating GH release.

4.2 Materials and Methods

4.2.1 Animals and Cell Preparation

Animal maintenance, as well as pituitary cell dispersion and culture were performed as described in Sections 2.2.1 and 3.2.1.

4.2.2 Reagents and Test Substances

A stock solution of 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetraacetoxymethyl ester (BAPTA AM) dissolved in DMSO was made fresh daily. All other media and reagents were the same as described in Sections 2.2.2 and 3.2.2.

4.2.3 Measurements of $[Ca^{2+}]_i$ in Single, Identified Somatotropes

Experiments examining forskolin-stimulated Ca^{2+} signals (Section 4.3.4) were conducted as described in Section 3.2.4. All other Ca^{2+}-imaging experiments were conducted in the same manner, with the following exceptions. Fura-2-loaded cells were placed within a closed bath imaging chamber (RC-21B; 260 µl chamber volume; Warner Instrument Corporation, Saint-Laurent, Quebec) and perifused with clear testing medium using a gravity fed perifusion system (VC-6 Perifusion System; Warner Instrument Corporation, Hamden, CT, USA). Emission fluorescence was recorded using a Retiga EX CCD camera (Quantitative Imaging, Burnaby, BC) and Northern Eclipse imaging software (version 6.0, Empix Imaging). Pairs of images were collected every 5 sec. In instances where cells contained both Fura-2 and BAPTA, the loading incubation was performed in testing medium containing 10 µM Fura-2 AM and 50 µM BAPTA AM.

Data analysis were the same as described in Section 3.2.4. However, in the current study, the basal CV for all cells examined was 5.34 ± 0.22%. As such, a Ca^{2+} response was defined as a >10% increase in average Ca^{2+} during treatment rather than the >20% increase employed in Chapter 3.

4.2.4 Column Perifusion Studies

All column perifusion studies and related data analysis were performed as described in Section 3.2.5.

4.2.5 Static Incubation Studies

Static incubation experiments and related data analysis were performed as described in Section 2.2.4 with the following modification to allow for BAPTA-loading. Following overnight incubation, cells were rinsed with testing medium. Cells to be loaded with BAPTA were incubated for 40-min in testing media containing 50 µM

BAPTA AM while the remaining cells were incubated in testing media alone. All cells were then washed with testing media again, after which secretagogues were applied. An experiment consisted of 4 to 6 replicates per plate with each experiment being repeated at least 3 times from different cell preparations.

4.3 Results

4.3.1 SS_{14} Inhibits D1-Stimulated GH Release Despite Elevations in $[Ca^{2+}]_i$

In column perifusion, 2-min application of a maximally effective dose (1 μM) (19) of the D1 agonist SKF-38393 stimulated GH secretion (Fig. 4.1). However, if SKF-38393 was applied during a 5-min exposure to a maximally effective concentration of SS_{14} (1 μM) (20), the GH response was completely abolished (Fig. 4.1).

These findings were compared to changes in $[Ca^{2+}]_i$ by conducting similar experiments on single, morphologically identified somatotropes loaded with Fura-2. When data from all cells exposed to SKF-38393 were pooled, average intracellular Ca^{2+} during 2-min SKF-38393 application increased to 137.18 ± 9.16% pretreatment (n=22). Of these 22 cells, 19 exhibited Ca^{2+} responses (Fig. 4.2A, C). Average Ca^{2+} during SKF-38393 application for the responding cells was 141.95 ± 10.44% pretreatment (Table 4.1). The remaining 3 cells exhibited an average Ca^{2+} of 106.98 ± 1.82% pretreatment during SKF-38393 application. When SKF-38393 was applied during exposure to SS_{14}, average Ca^{2+} during SKF-38393 application for all cells examined was 129.79 ± 5.82% pretreatment (n=21). Of these 21 cells, 16 exhibited Ca^{2+} responses (Fig. 4.2B, C). Average Ca^{2+} during SKF-38393 application for the responding cells was 136.69 ± 6.87% pretreatment (Table 4.1). The remaining 5 cells exhibited an average Ca^{2+} of 107.72 ± 4.30% pretreatment during SKF-38393 application.

Comparisons of the various kinetic parameters of the Ca^{2+} responses to SKF-38393 treatment in the presence and absence of SS_{14} were performed. SS_{14} had no effect on the proportion of cells responding to SKF-38393, average Ca^{2+} during SKF-38393

treatment, or the time to maximum amplitude of the Ca^{2+} response (Table 4.1). The only kinetic parameter to be significantly altered by SS_{14} treatment was the maximum amplitude of the Ca^{2+} response. The maximum amplitude of the Ca^{2+} response to SKF-38393 in the presence of SS_{14} was significantly less than that observed in the absence of SS_{14} (Table 4.1).

4.3.2 SS_{14} Inhibits PACAP-Stimulated GH Release Despite Elevations in $[Ca^{2+}]_i$

In column perifusion, 2-min application of a maximally effective dose of PACAP (10 nM) (21) stimulated GH secretion (Fig. 4.3). However, if PACAP was applied during a 5-min pulse of SS_{14} (1 μM) the GH response was completely abolished (Fig. 4.3).

In individual somatotropes loaded with Fura-2, average Ca^{2+} increased to 165.61 ± 13.82% pretreatment (n=24) during 2-min PACAP application. Of these 24 cells, 19 exhibited Ca^{2+} responses (Fig. 4.4A, C). Average Ca^{2+} during PACAP application for the responding cells was 180.81 ± 15.70% pretreatment (Table 4.1). The remaining 3 cells exhibited an average Ca^{2+} of 107.86 ± 1.65% pretreatment during PACAP application. When PACAP was applied during a 5-min exposure to SS_{14}, average Ca^{2+} during PACAP application for all cells examined was 144.67 ± 6.86% pretreatment (n=24). Of these 24 cells, 21 exhibited Ca^{2+} responses (Fig. 4.4B, C). Average Ca^{2+} during PACAP application for the responding cells was 150.22 ± 7.04% (Table 4.1). The remaining 3 cells exhibited an average Ca^{2+} of 105.87 ± 1.17% pretreatment during PACAP application.

Comparisons of the various kinetic parameters of the Ca^{2+} responses to PACAP treatment in the presence and absence of SS_{14} were performed. SS_{14} had no effect on the proportion of cells responding to PACAP, average Ca^{2+} during PACAP application, or the time to maximum amplitude of the Ca^{2+} response (Table 4.1). The only parameter to be significantly reduced by SS_{14} treatment was the maximum amplitude of the Ca^{2+} response to PACAP (Table 4.1).

4.3.3 Ca^{2+} Signals and GH Release are Coupled in the Absence of SS_{14}

The above data suggest that SS_{14} inhibition of SKF-38393- and PACAP-stimulated GH release does not require elimination of the corresponding Ca^{2+} signals. However, it was important to verify that the observed Ca^{2+} signals were related to GH secretion. As such, I assessed the ability of the Ca^{2+} ionophore ionomycin, as well as SKF-38393 and PACAP to elevate $[Ca^{2+}]_i$ and stimulate GH secretion in cells loaded with the Ca^{2+} chelator BAPTA.

In individual somatotropes loaded with Fura-2, 2-min application of ionomycin (10 μM) generated rapid, reversible increases in intracellular Ca^{2+} (Fig. 4.5A). All cells tested (9 out of 9) responded with a >10% increase in intracellular Ca^{2+} during ionomycin application. Average Ca^{2+} during this time was 291.70 ± 36.25% pretreatment. In cells preloaded with BAPTA, only 60% of cells (6 out of 10) responded with >10% increases in intracellular Ca^{2+}. Average Ca^{2+} during ionomycin application was 137.24 ± 8.72% pretreatment for the responding cells ($P<0.05$ vs. all non-BAPTA-loaded cells treated with ionomycin) and 106.21 ± 1.51% pretreatment for the non-responding cells. When all cells examined were pooled, average Ca^{2+} increased to 124.83 ± 7.16% pretreatment during ionomycin exposure in BAPTA-preloaded cells, which was also significantly less than the average Ca^{2+} during ionomycin treatment for all non-BAPTA-loaded cells. In 2-h static incubation hormone release experiments, the GH response to ionomycin (10 μM) was significantly inhibited, but not abolished (i.e., significantly greater than BAPTA alone; Fig. 4.5B).

Pretreatment with BAPTA significantly reduced the proportion of cells responding to a 2-min application of 1 μM SKF-38393 from 86.36% (19 of 22) to 11% (1 of 9). Average Ca^{2+} during SKF-38393 treatment for all BAPTA-loaded cells examined was 105.21 ± 1.94% pretreatment, which was significantly lower than the average Ca^{2+} during SKF-38393 treatment for all non-BAPTA-loaded cells (Fig. 4.6A). In 2-h static incubation experiments, the ability of SKF-38393 to stimulate GH release was completely abolished by pretreatment with BAPTA (Fig. 4.6C).

Likewise, pretreatment with BAPTA significantly reduced the proportion of cells responding to a 2-min application of 10 nM PACAP from 79.17% (19 of 24) to 40% (4 of 10). Average Ca^{2+} during PACAP application was 127.30 ± 6.56% pretreatment for the responding cells and 106.35 ± 1.31% pretreatment for the non-responding cells. Average Ca^{2+} during PACAP exposure for all the BAPTA-loaded cells was limited to 114.73 ± 4.24% pre-treatment, which was significantly lower than the average Ca^{2+} during PACAP treatment for all the non-BAPTA-loaded cells (Fig. 4.6B). The ability of PACAP to stimulate GH release in static culture was completely abolished by pretreatment with BAPTA (Fig. 4.6C). These observations suggest that Ca^{2+} signals are a required component of the signalling cascades mediating D1 and PACAP stimulation of GH secretion.

4.3.4 SS_{14} Inhibits Forskolin-Stimulated GH Release Despite Elevations in $[Ca^{2+}]_i$

The effects of SS_{14} on Ca^{2+} signals and GH release resulting from activation of more distal aspects of the D1/PACAP signalling cascade were also examined. In column perifusion, 5-min application of the AC activator forskolin (10 μM) stimulated GH secretion (Fig. 4.7). However, if forskolin was applied during an 8-min pulse of SS_{14} (1 μM), the GH response was completely abolished (Fig. 4.7).

In individual somatotropes loaded with Fura-2, average Ca^{2+} increased to 135.86 ± 11.45% pretreatment (n=17) during 5-min forskolin application. Of these 17 cells, 11 exhibited Ca^{2+} responses (Fig. 4.8A, C). The average Ca^{2+} during forskolin application for the responding cells was 151.77 ± 15.77% pretreatment (Table 4.1). The remaining 6 cells exhibited an average Ca^{2+} of 106.69 ± 8.87% pretreatment during forskolin application. When forskolin was applied during an 8-min exposure to SS_{14}, average Ca^{2+} during forskolin application for all cells examined was 119.01 ± 5.34% pretreatment (n=12). Of these 12 cells, 9 exhibited Ca^{2+} responses (Fig. 4.8B, C). For the 9 responding cells, the average Ca^{2+} during forskolin application was 127.20 ± 3.95% pretreatment

(Table 4.1). The remaining 3 cells exhibited an average Ca^{2+} of 94.45 ± 16.37% pretreatment during forskolin application.

Comparisons of the various kinetic parameters of the Ca^{2+} response to forskolin in the presence and absence of SS_{14} were performed. SS_{14} had no effect on the proportion of cells responding to forskolin, average Ca^{2+} during forskolin application, or the maximum amplitude of the Ca^{2+} response (Table 4.1). The only parameter to be significantly altered by SS_{14} treatment was the time to maximum amplitude of the Ca^{2+} response. The time to maximum amplitude of the Ca^{2+} response to forskolin in the presence of SS_{14} was significantly longer that observed in the absence of SS_{14} (Table 4.1).

4.3.5 SS_{14} Inhibition of 8Br-cAMP-Stimulated GH Release is not Coupled to Modulation of $[Ca^{2+}]_i$

In column perifusion, 5-min application of the membrane permeant cAMP analogue, 8Br-cAMP (10 μM) stimulated GH secretion (Fig. 4.9). However, if 8Br-cAMP was applied during an 8-min pulse of SS_{14} (1 μM), the GH response was completely abolished (Fig. 4.9).

In individual somatotropes loaded with Fura-2, average Ca^{2+} increased to 122.01 ± 6.23% pretreatment (n=28) during a 5-min application of 8Br-cAMP. Of these 28 cells, 11 exhibited Ca^{2+} responses (Fig. 4.10A, C). Average Ca^{2+} during 8Br-cAMP application for the responding cells was 153.52 ± 9.64% pretreatment (Table 4.1). The remaining 17 cells exhibited an average Ca^{2+} of 101.62 ± 8.25% pretreatment during 8Br-cAMP application. When 8Br-cAMP was applied during an 8-min exposure to SS_{14}, average Ca^{2+} during 8Br-cAMP application for all cells examined was 124.06 ± 6.56% pretreatment (n=35). Of these 35 cells, 17 exhibited Ca^{2+} responses (Fig. 4.10B, C). Average Ca^{2+} during 8Br-cAMP application for the responding cells was 150.69 ± 9.07% pretreatment (Table 4.1). The remaining 18 cells exhibited an average Ca^{2+} of 98.92 ± 1.79% pretreatment during 8Br-cAMP application.

Comparisons of the various kinetic parameters of the Ca^{2+} response to 8Br-cAMP in the presence and absence of SS_{14} were performed. Treatment with SS_{14} had no significant effect on any of the kinetic parameters used to characterize the Ca^{2+} signals evoked by 8Br-cAMP (Table 4.1).

4.4 Discussion

Chapter 3 established that, in goldfish pituitary somatotropes, SS_{14} is able to regulate basal, as well as GnRH/PKC-dependent GH release by acting distal to changes in $[Ca^{2+}]_i$. In this chapter the question being asked was whether SS_{14} also regulated PKA-dependent GH release by acting independent of elevated $[Ca^{2+}]_i$.

4.4.1 Ca^{2+} Signals and GH Responses

Defining a Ca^{2+} response as an increase in average Ca^{2+} greater than twice the basal CV was the same as Chapter 3. However, the CV for basal Ca^{2+} in this study was considerably lower than that reported in Chapter 3. As such, the minimum increase in average Ca^{2+} required to consider a cell as having responded to a stimulus was 10% rather than the 20% used previously. However, when the data was examined using the more conservative definition of 20%, the results were similar (data not shown). The difference in basal CV is likely due to improvements in the imaging system. Most of the experiments presented in this chapter were conducted using a gravity-feed, closed bath imaging chamber, rather than an open bath imaging chamber perifused with a peristaltic pump. These changes resulted in a more controlled, laminar flow of media across the cells, and likely, more stable basal Ca^{2+} values.

It is conceivable that not all somatotropes express D1 or PAC_1 receptors. As such, it is not surprising that not all cells responded to SKF-38393 and PACAP. However, by defining a response as a >10% increase in average Ca^{2+}, I knowingly excluded cells that exhibited <10% increases in average Ca^{2+}. Therefore, it is likely that more cells express D1 or PAC_1 receptors than indicated by the proportion of responding cells. Interestingly,

the proportion of cells responding to SKF-38393, PACAP and forskolin were comparable. This indicates that the response criteria is consistent in its ability to detect Ca^{2+} responses from known AC activators. However, the proportion of cells responding to 8Br-cAMP was considerably less than SKF-38393, PACAP, and forskolin. Unlike the other secretagogues, 8Br-cAMP does not generate a self-amplifying signal. In addition, 8Br-cAMP is susceptible to enzymatic degradation. As such, it is not unexpected that 8Br-cAMP was less effective at generating Ca^{2+} responses.

The use of single-cell Ca^{2+} imaging in conjunction with parallel experiments examining GH secretion enables the study of stimulus-secretion coupling. However, this assumes that the Ca^{2+} signals being recording are related to GH secretion. Several lines of evidence support this assumption. When SKF-38393- and PACAP-stimulated Ca^{2+} signals were prevented by intracellular BAPTA, stimulated GH release was similarly prevented. In addition, BAPTA significantly inhibited ionomycin-stimulated GH release and attenuated the corresponding Ca^{2+} profile. However, there was a slow rise in $[Ca^{2+}]_i$ as a result of ionomycin treatment in BAPTA-loaded cells that was related to a small increase in GH release above basal levels. It is likely that this is the result of the intracellular BAPTA becoming saturated due to the massive, sustained Ca^{2+} influx induced by the ionophore. This led to delayed, yet sufficient, increases in $[Ca^{2+}]_i$ capable of evoking GH release.

Given that Ca^{2+} signals are related to GH secretion in goldfish somatotropes, what aspect of the Ca^{2+} signal determines the exocytotic response? The rate of rise in $[Ca^{2+}]_i$, with a greater rate being more effective, has been shown to be an important factor in determining the effectiveness of a Ca^{2+} signal in stimulating exocytosis in rat pituitary gonadotropes (22) and neurons (23). Similarly, this kinetic parameter is hypothesised to be an important factor in mediating GnRH stimulation of gonadotropin release from goldfish gonadotropes (24). However, the rate of rise in $[Ca^{2+}]_i$ does not appear to be a major determinant of the secretory response here. If you consider the magnitude of the GH responses to SKF-38393 and PACAP, it is clear that the response to SKF-38393 is greater. However, the apparent rate of increase in $[Ca^{2+}]_i$ is greater with PACAP than

with SKF-38393. The average Ca^{2+} during PACAP treatment, as well as the average maximum amplitude of the Ca^{2+} response evoked by PACAP, were greater than the corresponding values obtained with SKF-38393. In addition, the average time to maximum amplitude was shorter (i.e. reached more quickly) for PACAP-stimulated Ca^{2+} signals. Furthermore, treatment with forskolin resulted in a GH response that was similar in maximal amplitude to that induced by SKF-38393, but the corresponding Ca^{2+} signal had an apparent rate of rise that was less than one-half the apparent rate of rise evoked by SKF-38393. These data are not consistent with the notion of rate of rise being an important determinant in stimulating GH secretion.

4.4.2 SS_{14} Regulation of Somatotrope Function

In this chapter the data establish that, although SS_{14} modulates some aspects of the stimulated Ca^{2+} signals, Ca^{2+} signal modification is not required for SS_{14} inhibition of stimulated GH secretion. Especially interesting is the fact that none of the kinetic parameters used to characterize the Ca^{2+} signals evoked by 8Br-cAMP were affected by exposure to SS_{14}, yet GH release was completely abolished. These findings suggest that the mechanism(s) responsible for SS_{14} modulation of DA/PACAP/AC-stimulated Ca^{2+} signals differs from the mechanism(s) underlying SS_{14} inhibition of DA/PACAP/AC-stimulated GH release. Furthermore, it seems likely that the mechanism responsible for SS_{14} actions on stimulated Ca^{2+} signals is upstream of cAMP, in comparison to the more distal mechanism responsible for the uncoupling of Ca^{2+} signals from GH exocytosis. Whether SS_{14} modulates cAMP production to affect stimulated Ca^{2+} signals is not known, but is a possibility that is under investigation. Nevertheless, additional evidence that SS_{14} is able to regulate Ca^{2+} signals and exocytosis independently is available in the literature (4-6, 25). For example, in pituitary GC cells and pancreatic α-cells, SS_{14} regulation of secretion is distal (or unrelated) to Ca^{2+} signal generation and is mediated through sst_1 and sst_2 receptors, respectively. Is the regulation of Ca^{2+} signals and inhibition of GH release by SS_{14} presented in this chapter the result of differential

receptor subtype activation, or the activation of multiple signalling cascades through a single sst subtype? This question cannot be answered at this time. Although sst_2 and sst_5 are predominantly expressed in the goldfish pituitary, transcripts for sst_1, sst_2, sst_3, and sst_5 have been identified in the goldfish hypophysis and are capable of being activated by SS_{14} (9-12). Unfortunately, the use of non-peptidyl sst-selective agonists to evaluate sst subtype-specific effects in primary cultures of goldfish somatotropes has, to date, been uninformative (WK Yunker, unpublished). Identifying the mechanism responsible for SS_{14} modulation of stimulated Ca^{2+} signals, in addition to assigning specific functions to various receptor subtypes will be an interesting, and challenging part of future research.

The physiological significance of regulating Ca^{2+} signals and GH release independently is unknown. However, it is known that numerous cellular functions are Ca^{2+}-signal dependent, including the mechanisms underlying hormone synthesis (reviewed in (26)). In addition, the data suggest that the different intracellular Ca^{2+} pools regulate GH mRNA levels, cellular GH content, and GH secretion differentially in goldfish somatotropes (27). Therefore it is possible, that SS_{14} is regulating other aspects of cell function, such as mRNA transcription or translation, through its actions on Ca^{2+} signals while simultaneously inhibiting GH release through a more distal mechanism.

Although these data do not allow for an identification of the mechanism(s) through which SS_{14} inhibits GH release distal to elevated Ca^{2+}, current and past data do provide some insight. Rebounds in GH secretion following removal of SS inhibition (reviewed in (28)), have been taken as evidence for the accumulation of readily releasable secretory granules beneath the plasma membrane, possibly due to SS_{14} disruption of microfilaments (29). Once SS inhibition is removed, the accumulated granules are thought to fuse with the plasma membrane, resulting in a rapid, transient increase in GH release. Given however, that removal of SS_{14} inhibition does not result in GH rebounds in our system (this chapter and Chapter 3), this explanation seems unlikely.

On the other hand, ATP hydrolysis-dependent priming of secretory granules, which is thought to be required for secretory granules to become release-competent (30-32), may be an important target for SS_{14}. Work in pancreatic α-cells suggests that SS_{14}

may be inhibiting exocytosis by depriming secretory granules through the activation of the serine/threonine phosphatase calcineurin (4). Granule depriming would not result in a GH surge following removal of SS_{14} inhibition as readily releasable granules would not accumulate beneath the plasma membrane. Calcineurin activity has also been shown to mediate SS_{14} inhibition of exocytosis in β-cells, as well as galanin- and adrenaline-evoked reductions in insulin secretion (5). It would be interesting to examine this hypothesis in goldfish somatotropes by examining whether inhibitors of calcineurin (i.e., cyclosporin or calcineurin inhibitory peptide) can antagonize SS_{14} action.

In summary, using single-cell Ca^{2+} imaging in parallel with column perifusion studies, SS_{14} inhibition of PKA-dependent GH secretion from primary cultures of dispersed goldfish pituitary cells was investigated. The results establish that SS_{14} does not abolish stimulated Ca^{2+} signals as a means of inhibiting stimulated GH release. Furthermore, it seems likely that the cellular mechanisms underlying SS_{14} actions on Ca^{2+} signalling are upstream of cAMP and may be unrelated to those responsible for inhibiting GH release.

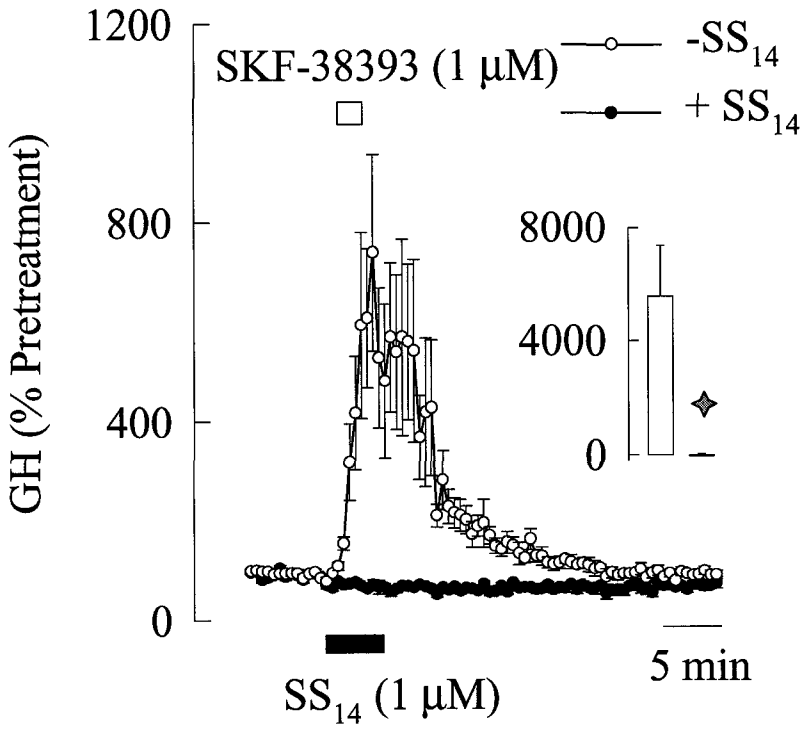

Fig. 4.1. SS_{14} Inhibits SKF-38393-Stimulated GH Release.

SKF-38393 was applied for 2 min (open bar), while SS_{14} was applied for 5 min (black bar). Dispersed pituitary cells from goldfish at times of gonadal regression and early recrudescence (July, October, and November) were used. Basal GH levels were 27.38 ± 3.60 (n=8) and 25.86 ± 4.74 (n=10) ng/ml in the SKF-38393- and SKF-38393 plus SS_{14}-treated columns respectively. Vertical bars (inset) represent net GH responses to either SKF-38393 (open bar) or SKF-38393 plus SS_{14} (solid bar). A star represents a significant difference between the two treatment groups.

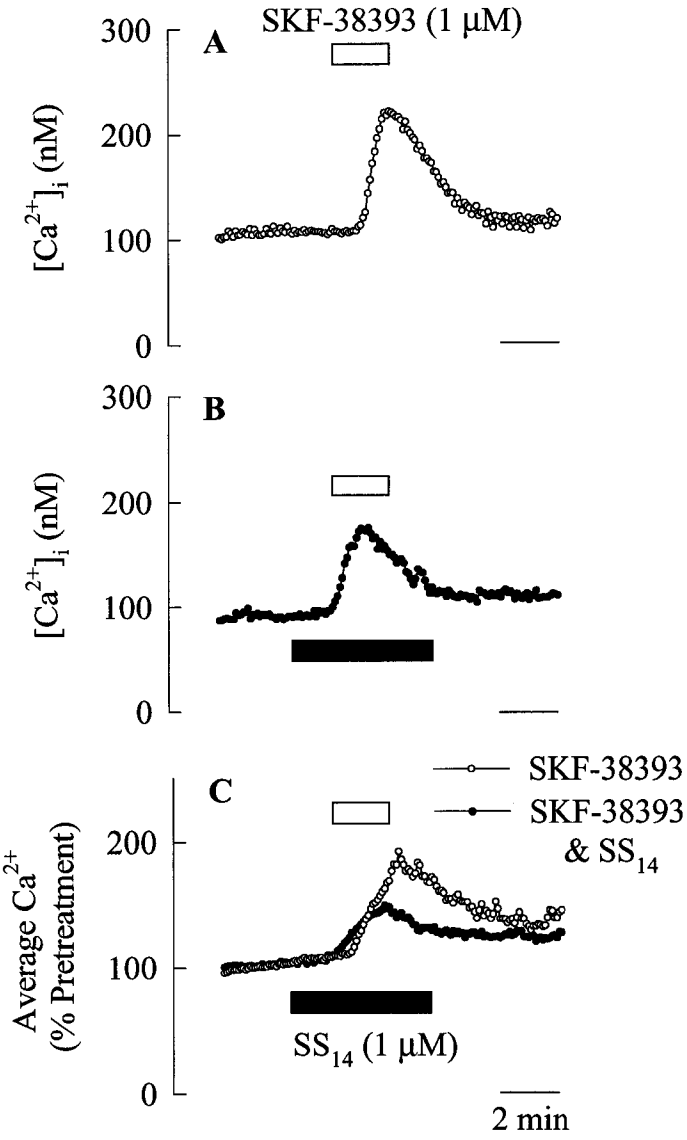

Fig. 4.2. SS_{14} Actions on SKF-38393-Evoked Ca^{2+} Signals.

Dispersed pituitary cells from goldfish at times of gonadal recrudescence (January) were used. (A) $[Ca^{2+}]_i$ trace of a single identified somatotrope responding to a 2-min application of SKF-38393 (open bar) with a >10% increase in average Ca^{2+}. Basal $[Ca^{2+}]_i$ for all cells treated with SKF-38393 was 116.51 ± 11.81 nM (n=22). (B) $[Ca^{2+}]_i$ trace of a single identified somatotrope responding to SKF-38393 with a >10% increase in average Ca^{2+} during a 5-min application of SS_{14} (black bar). Basal $[Ca^{2+}]_i$ for all cells treated with SKF-38393 plus SS_{14} was 99.37 ± 6.80 nM (n=21). (C) Data from all cells responding with >10% increases in average Ca^{2+} in response to SKF-38393, either alone or in the presence of SS_{14}, were pooled, and the resulting average Ca^{2+} profiles are presented (error bars have been omitted for clarity).

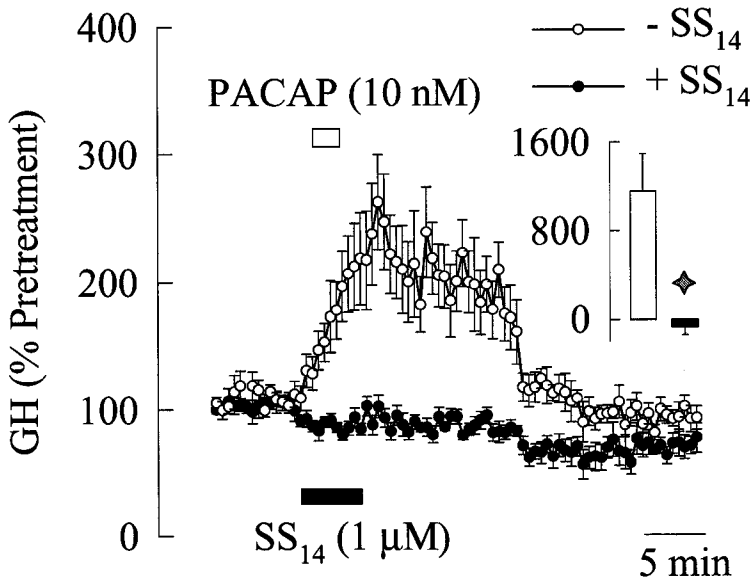

Fig. 4.3. SS_{14} Inhibits PACAP-Stimulated GH Release.

PACAP was applied for 2 min (open bar), while SS_{14} was applied for 5 min (black bar). Dispersed pituitary cells from goldfish at times of gonadal regression and recrudescence (June, November and December) were used. Basal GH levels were 60.22 ± 10.46 (n=10) and 61.38 ± 7.92 (n=8) ng/ml in the PACAP- and PACAP plus SS_{14}-treated columns respectively. Vertical bars (inset) represent net GH responses to either PACAP (open bar) or PACAP plus SS_{14} (closed bar). A star represents a significant difference between the two treatment groups.

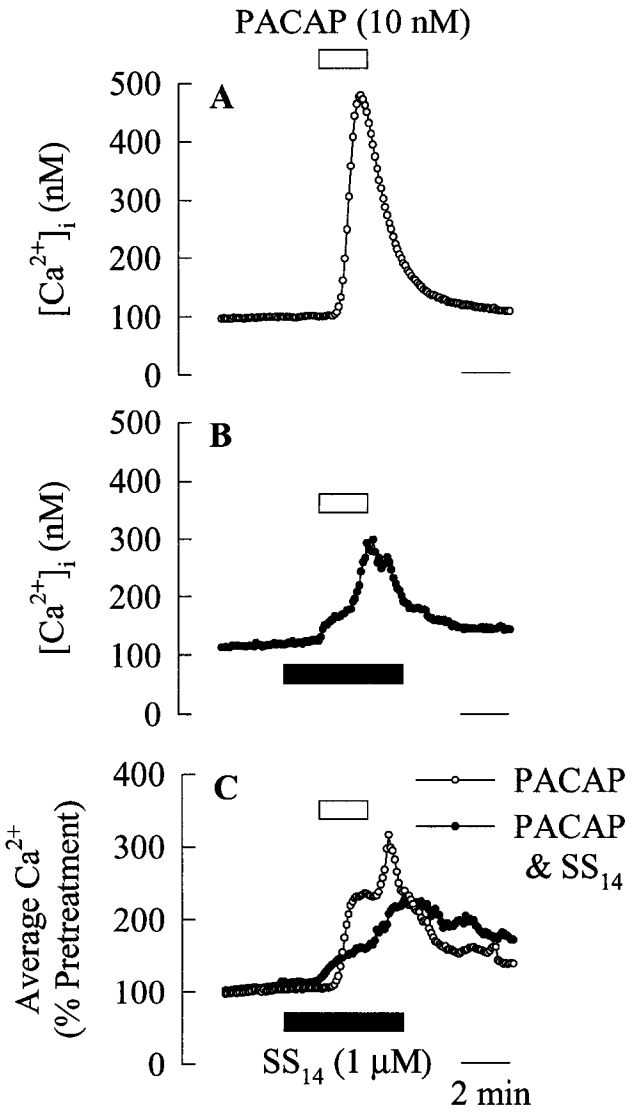

Fig. 4.4. SS_{14} Actions on PACAP-Evoked Ca^{2+} Signals.

Dispersed pituitary cells from goldfish at times of gonadal recrudescence (January) were used. (A) $[Ca^{2+}]_i$ trace of a single identified somatotrope responding to a 2-min application of PACAP (open bar) with a >10% increase in average Ca^{2+}. Basal $[Ca^{2+}]_i$ for all cells treated with PACAP was 127.07 ± 13.56 nM (n=24). (B) $[Ca^{2+}]_i$ trace of a single identified somatotrope responding to PACAP with a >10% increase in average Ca^{2+} during a 5-min application of SS_{14} (black bar). Basal $[Ca^{2+}]_i$ for all cells treated with PACAP plus SS_{14} was 134.46 ± 16.19 nM (n=24). (C) Data from all cells responding with >10% increases in average Ca^{2+} in response to PACAP, either alone or in the presence of SS_{14}, were pooled, and the resulting average Ca^{2+} profiles are presented (error bars have been omitted for clarity).

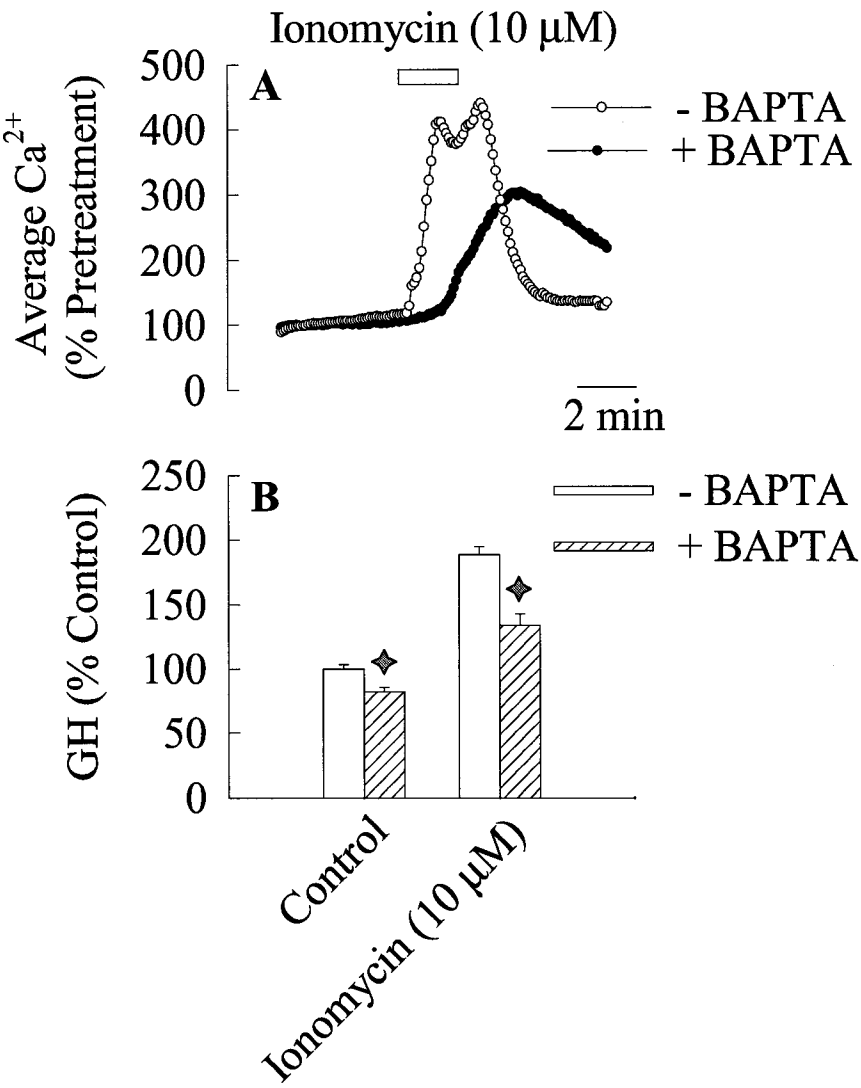

Fig. 4.5. BAPTA Inhibits Ionomycin-Evoked Ca^{2+} Signals and GH Secretion.

Dispersed pituitary cells from goldfish at times of gonadal recrudescence (January and December) were used for both the Ca^{2+} imaging and hormone release experiments. (A) Average Ca^{2+} traces assembled from all naïve or BAPTA-loaded cells exposed to a 2-min application of ionomycin (open bar). Basal $[Ca^{2+}]_i$ was 172.75 ± 25.72 nM (n=9) for the naïve cells and 136.74 ± 24.89 nM (n=10) for the BAPTA-preloaded cells. Error bars have been omitted for clarity. (B) BAPTA pretreatment inhibits ionomycin-stimulated GH secretion in 2-h static culture. Basal GH secretion was 625.58 ± 46.74 ng/ml (n=24). A star represents a significant reduction in GH release compared to the non-BAPTA exposed column of the pair.

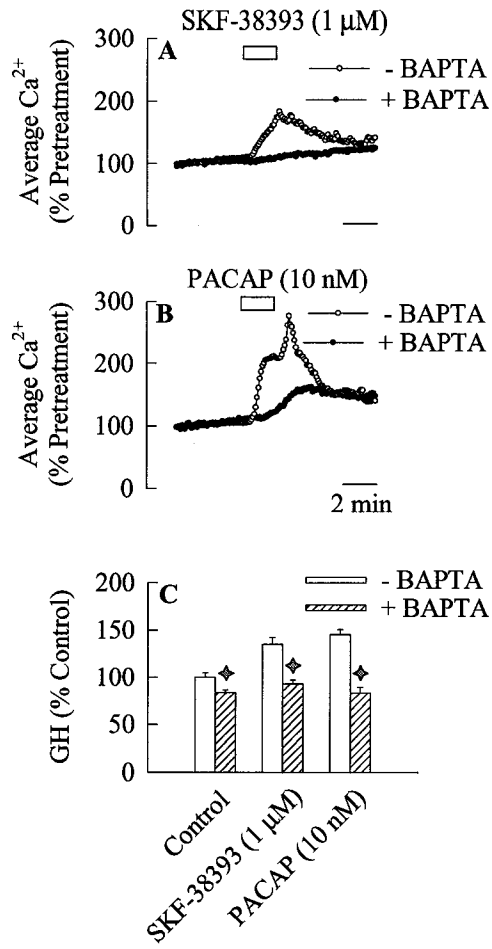

Fig. 4.6. BAPTA Prevents SKF-38393- and PACAP-Stimulated Ca^{2+} Signals and GH Secretion.

Dispersed pituitary cells from goldfish at times of gonadal recrudescence (December and January) were used for both the Ca^{2+} imaging and hormone release experiments. (A) Average Ca^{2+} trace assembled from all BAPTA-preloaded cells exposed to a 2-min application of SKF-38393 (open bar). Basal $[Ca^{2+}]_i$ for all BAPTA-loaded cells was 104.39 ± 11.89 nM (n=9). Data from all cells treated with SKF-38393 were pooled and the resulting average Ca^{2+} profile is presented for comparison. (B) Average Ca^{2+} trace assembled from all BAPTA-preloaded cells exposed to a 2-min application of PACAP (open bar). Basal $[Ca^{2+}]_i$ for all BAPTA-loaded cells was 125.79 ± 40.95 nM (n=10). Data from all cells treated with PACAP were pooled and the resulting average Ca^{2+} profile is presented for comparison. Error bars for panels A and B have been omitted for clarity. (C) BAPTA pretreatment prevents SKF-38393- and PACAP-stimulated GH secretion in 2-h static culture. Basal GH secretion was 780.72 ± 39.93 ng/ml (n=16). A star represents a significant reduction in GH release compared with the non-BAPTA exposed column of the pair.

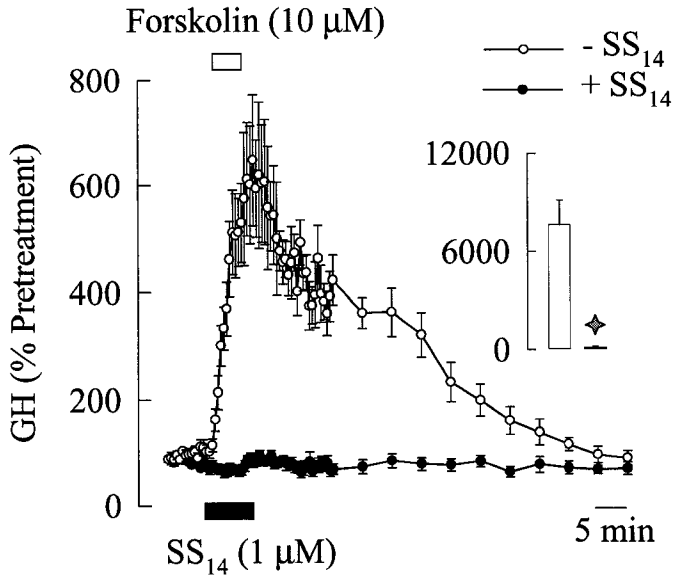

Fig. 4.7. SS_{14} Inhibits Forskolin-Stimulated GH Release.

Forskolin was applied for 5 min (open bar), while SS_{14} was applied for 8 min (black bar). Dispersed pituitary cells from goldfish with regressed gonads (June and September) were used. Basal GH levels were 45.15 ± 7.73 (n=8) and 36.25 ± 5.84 (n=10) ng/ml in the forskolin- and forskolin plus SS_{14}-treated columns respectively. Vertical bars (inset) represent net GH responses to either forskolin (open bar) or forskolin plus SS_{14} (solid bar). A star represents a significant difference between the two treatment groups.

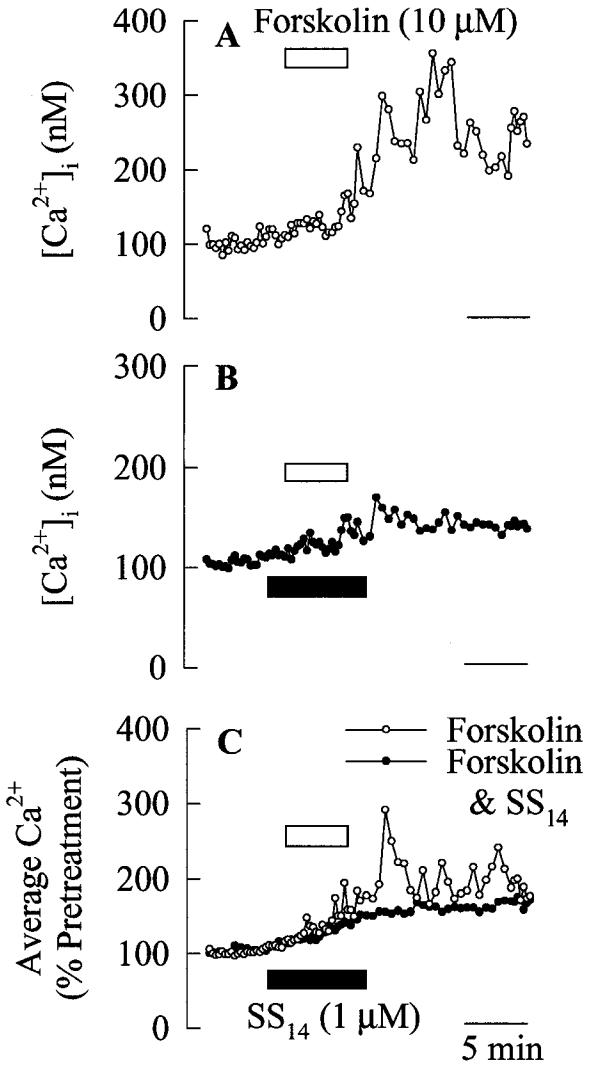

Fig. 4.8. SS_{14} Actions on Forskolin-Evoked Ca^{2+} Signals.

Dispersed pituitary cells from goldfish at times of gonadal regression and recrudescence (July, September and February) were used. (A) $[Ca^{2+}]_i$ trace of a single identified somatotrope responding to a 5-min application of forskolin (open bar) with a >10% increase in average Ca^{2+}. Basal $[Ca^{2+}]_i$ for all cells treated with forskolin was 265.75 ± 86.31 nM (n=17). (B) $[Ca^{2+}]_i$ trace of a single identified somatotrope responding to forskolin with a >10% increase in average Ca^{2+} during an 8-min application of SS_{14} (black bar). Basal $[Ca^{2+}]_i$ for all cells treated with forskolin plus SS_{14} was 117.01 ± 56.23 nM (n=12). (C) Data from all cells responding with >10% increases in average Ca^{2+} in response to forskolin, either alone or in the presence of SS_{14}, were pooled, and the resulting average Ca^{2+} profiles are presented (error bars have been omitted for clarity).

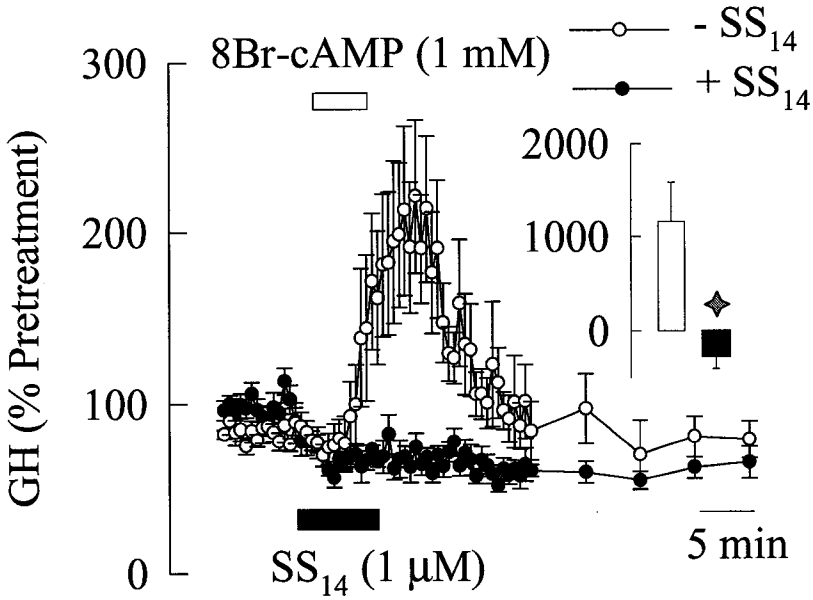

Fig. 4.9. SS_{14} Inhibits 8Br-cAMP-Stimulated GH Release.

8Br-cAMP was applied for 5 min (open bar), while SS_{14} was applied for 8 min (black bar). Dispersed pituitary cells from goldfish at times of gonadal regression (June) were used. Basal GH levels were 16.11 ± 1.89 (n=6) and 13.34 ± 1.14 (n=6) ng/ml in the forskolin- and forskolin plus SS_{14}-treated columns respectively. Vertical bars (inset) represent net GH responses to either 8Br-cAMP (open bar) or 8Br-cAMP plus SS_{14} (solid bar). A star represents a significant difference between the two treatment groups.

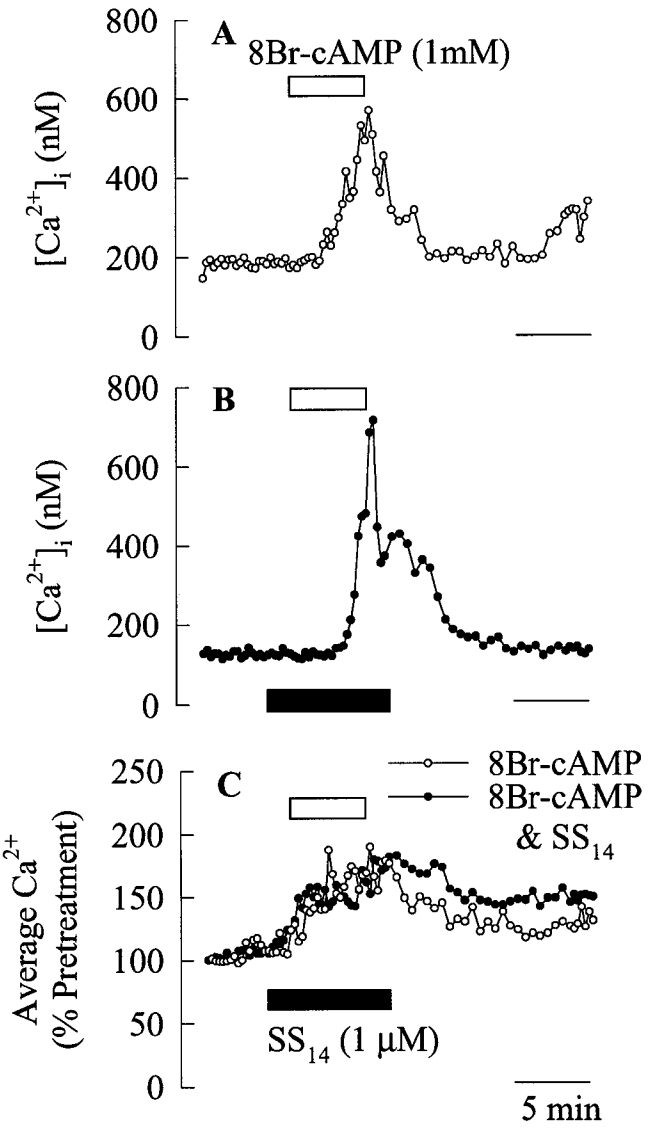

Fig. 4.10. SS_{14} Actions on 8Br-cAMP-Evoked Ca^{2+} Signals.

Dispersed pituitary cells from either sexually regressed goldfish or goldfish at times of early gonadal recrudescence (September to November) were used. (A) $[Ca^{2+}]_i$ trace of a single identified somatotrope responding to a 5-min application of 8Br-cAMP (open bar) with a >10% increase in average Ca^{2+}. Basal $[Ca^{2+}]_i$ for all cells treated with 8Br-cAMP was 264.59 ± 19.59 nM (n=28). (B) $[Ca^{2+}]_i$ trace of a single identified somatotrope responding to 8Br-cAMP with a >10% increase in average Ca^{2+} during an 8-min application of SS_{14} (black bar). Basal $[Ca^{2+}]_i$ for all cells treated with 8Br-cAMP plus SS_{14} was 248.19 ± 17.58 nM (n=35). (C) Data from all cells responding with >10% increases in average Ca^{2+} in response to forskolin, either alone or in the presence of SS_{14}, were pooled, and the resulting average Ca^{2+} profiles are presented (error bars have been omitted for clarity).

Table 4.1. Effects of SS_{14} on SKF-38393-, PACAP-, Forskolin-, and 8Br-cAMP-Stimulated Ca^{2+} Signals.

	% of cells[a]	Average Ca^{2+} During (% pretreatment)	Max Amplitude (% pretreatment)	Time to Max Amplitude (sec)
SKF-38393	86.4 (19/22)	142.0 ± 10.44	265.0 ± 30.49	143 ± 14.7
SKF-38393 & SS_{14}	76.2 (16/21)	136.7 ± 6.88	166.6 ± 13.77[b]	145 ± 28.5
PACAP	79.2 (19/24)	180.8 ± 15.70	404.8 ± 63.33	124 ± 11.7
PACAP & SS_{14}	87.5 (21/24)	150.2 ± 7.04	289.2 ± 40.35[b]	159 ± 22.2
Forskolin	64.7 (11/17)	151.8 ± 15.77	322.9 ± 57.26	400 ± 54.7
Forskolin & SS_{14}	75.0 (9/12)	127.2 ± 3.95	191.9 ± 12.06	550 ± 28.3[b]
8Br-cAMP	39.3 (11/28)	153.5 ± 9.64	254.1 ± 27.42	271 ± 42.0
8Br-cAMP & SS_{14}	48.6 (17/35)	150.7 ± 9.07	269.0 ± 34.15	356 ± 44.0

[a] % of cells responding with >10% pretreatment increases in average intracellular Ca^{2+} during secretagogue treatment. All of the Ca^{2+} parameters presented were calculated from cells that responded to a given treatment and are presented as mean ± SEM.

[b] Significantly different from non-SS_{14} treated control.

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Chapter 5 – General Discussion

5.1 General Summary

The objective of this thesis has been to examine the regulation of pituitary somatotropes by endogenous hypothalamic SS peptides in the goldfish, *Carassius auratus*. Chapter 2 demonstrated that multiple SS isoforms have the potential to reach the pituitary and differentially regulate basal cAMP production, as well as basal and stimulated GH secretion. Among the three endogenous SS isoforms examined, it was discovered that $gbSS_{28}$ was more potent than either SS_{14} or $[Pro^2]SS_{14}$ in reducing basal GH release, but was the least effective at reducing basal cellular cAMP levels. The ability of SS_{14}, $[Pro^2]SS_{14}$ and $gbSS_{28}$ to attenuate GH responses to GnRH were comparable. However, $gbSS_{28}$ was less effective than SS_{14} and $[Pro^2]SS_{14}$ in diminishing SKF-38393- and PACAP-stimulated GH release, as well as GH release resulting from the activation of their underlying signalling cascades.

The intent of chapters 3 and 4 was to determine whether the inhibition of Ca^{2+} signals was responsible for SS_{14} inhibition of basal and stimulated GH release from goldfish pituitary somatotropes. Chapter 3 examined SS_{14} actions on basal and GnRH-stimulated Ca^{2+} signals and GH release, while chapter 4 focused on the effects of SS_{14} on D1- and PACAP-stimulated Ca^{2+} signals and GH release. The data presented in these chapters establish that SS_{14} inhibition of basal GH secretion is not associated with changes in basal $[Ca^{2+}]_i$. Furthermore, it is apparent that SS_{14} does not abolish stimulated Ca^{2+} signals as a means of inhibiting GH responses to GnRH, PACAP and DA, as well as activators of their respective intracellular signalling cascades.

5.2 Ca^{2+} signals and GH secretion: A Kinetic Connection?

Ca^{2+} signals participate in the regulation of numerous cellular processes. In certain systems, the kinetic parameters of the Ca^{2+} signal, such as the rate of rise of $[Ca^{2+}]_i$, the frequency of Ca^{2+} oscillations, or the magnitude of the Ca^{2+} elevation, are

thought to participate in coupling Ca^{2+} signals to specific cellular events, such as secretion or cell division (1-5). Currently, the mechanism(s) responsible for coupling stimulated Ca^{2+} signals to stimulated GH release in goldfish somatotropes is unknown. However, based on the data presented in this thesis, the potential importance of some of these kinetic parameters can be evaluated.

In some systems, a rapid increase in $[Ca^{2+}]_i$ has been shown to be more effective than a slow increase in stimulating exocytosis (3, 4). However, the data presented in Chapter 4 indicate that the apparent rate of increase in $[Ca^{2+}]_i$ is not a predictor of the relative effectiveness of PACAP and SKF38393 in stimulating GH release from goldfish somatotropes. Another kinetic parameter that has been shown to couple Ca^{2+} signals to specific cell functions is the magnitude of the increase in $[Ca^{2+}]_i$ (1, 2). However, this also does not appear to be the case in goldfish somatotropes. For example, average Ca^{2+} during cGnRH-II (Chapter 3) and SKF-38393 (Chapter 4) treatment were nearly identical, yet the amount of GH secreted in response to SKF-38393 was considerably greater than that released in response to cGnRH-II. Taken together, it seems that neither the rate of rise, nor the magnitude of the Ca^{2+} signal, as measured at the whole cell level, is indicative of the secretory response of goldfish somatotropes.

However, this finding does not exclude the possibility that SS_{14} inhibits GH release by altering either the rate of increase or the magnitude of the Ca^{2+} signal. In the case of cGnRH-II-, sGnRH-, SKF-38393-, PACAP-, and forskolin-stimulated GH release, SS_{14} either lowered the maximum amplitude of Ca^{2+} response or delayed the time to maximum amplitude, or both. The result of which is a slowing in the apparent rate of rise in $[Ca^{2+}]_i$. However, altering the rate of rise in $[Ca^{2+}]_i$ cannot explain SS_{14} inhibition of 8Br-cAMP-stimulated GH release (Chapter 4). Furthermore, co-application of SS_{14} and DiC8 increased the apparent rate of increase in $[Ca^{2+}]_i$ while further reducing GH secretion (Chapter 3). As such, the ability of SS_{14} to inhibit stimulated GH release from goldfish somatotropes cannot be entirely attributed to changes in the apparent rate of increase in $[Ca^{2+}]_i$. The data also suggest that SS_{14} is not regulating GH release by altering the magnitude of the Ca^{2+} response. For example, average $[Ca^{2+}]_i$ during DiC8-

evoked signals was considerably higher in the presence of SS_{14} than when DiC8 was administered alone (Chapter 3). In addition, SS_{14} inhibited 8Br-cAMP-stimulated release without any significant changes in the corresponding Ca^{2+} signals (Chapter 4).

Although data from the present study do not support the hypothesis that alterations to the kinetic parameters of stimulated Ca^{2+} signals is an important determinant of GH secretory responses, further experiments are needed before any definitive conclusions can be made. Global or whole cell Ca^{2+} signals, as recorded in this study, are the sum of numerous elemental Ca^{2+} signals. An elemental Ca^{2+} signal is the microdomain of Ca^{2+} that results from the opening of a single Ca^{2+} channel, which may be situated on either an intracellular Ca^{2+} store or the plasma membrane (6). Elemental Ca^{2+} signals have a limited spatial range (nanometer), and can exhibit rapid (millisecond), high intensity fluctuations in Ca^{2+} concentration (micromolar) (reviewed in (6-9)). As such, the kinetics of global Ca^{2+} signals are not necessarily representative of the elemental Ca^{2+} signals occurring in close proximity to Ca^{2+} release or entry channels, and release-competent secretory granules. Additional studies, focusing on microdomain Ca^{2+} signals are required to more fully explore this question.

5.3 How does SS_{14} regulate GH release?

If SS_{14} inhibition of GH secretion cannot be attributed to alterations in either $[Ca^{2+}]_i$ or the kinetic parameters of the Ca^{2+} signal, is there an alternative explanation? Chapters 3 and 4 raised the possibility that SS_{14} is inhibiting GH secretion by uncoupling Ca^{2+} signals from GH release. This possibility is discussed below.

Studies in neuronal and neuroendocrine cells have demonstrated that stimulated secretion involves at least three distinguishable steps (reviewed in (10)). First is the recruitment and translocation of the secretory granule to the plasma membrane. Second, the secretory granule docks, either morphologically or biochemically, with the plasma membrane. Finally, the granule fuses with the cell surface, thereby releasing its contents. It is this last step, granule fusion, that is triggered by elevations in $[Ca^{2+}]_i$ (11). The

molecular machinery responsible for this fusion process, referred to as the SNARE hypothesis, is a complex interaction of numerous cytoplasmic and membrane bound proteins (reviewed in (12, 13)). One of these proteins, synaptotagmin I, is thought to function as the Ca^{2+} sensor responsible for initiating membrane fusion in the presence of elevated $[Ca^{2+}]_i$ (14). As the molecular mechanisms of membrane fusion are beyond the scope of this project the reader is referred to several excellent reviews of this material for more information (12, 13, 15, 16).

At any given time, only a small fraction of the docked granules are fusion-competent and capable of undergoing exocytosis in response to elevated $[Ca^{2+}]_i$ (10). Additional ATP-dependent steps, referred to as 'priming', are required to convey release competence to the majority of the docked vesicles. A number of ATP-dependent priming reactions have been characterized in neuroendocrine cells, including ATP-dependent synthesis of phosphoinositides and protein kinase-mediated protein phosphorylation of unidentified target proteins (reviewed in (10, 17, 18)). Assuming that there is a very limited number of primed and docked vesicles in goldfish somatotropes under basal conditions, this priming step would be an ideal target for SS_{14} action.

In several non-somatotrope secretory systems SS_{14} has been shown to regulate exocytosis through the activation of the protein phosphatase calcineurin (19, 20). In these systems, the activated calcineurin is thought to deprime release-competent secretory vesicles, thereby reducing the number of readily releasable granules capable of undergoing exocytosis in response to agonist-evoked increases in $[Ca^{2+}]_i$. It is conceivable that this is occurring in goldfish somatotropes. A hypothetical model of the regulation of secretory granule release from goldfish somatotropes in column perifusion is diagrammed in Fig. 5.1. In this model, secretory granules translocate to, and dock with the plasma membrane. There they remain, awaiting ATP-dependent vesicular priming and Ca^{2+}-triggered exocytosis. Stimulatory ligands, such as DA, PACAP and GnRH, activate intracellular signalling events capable of priming the docked vesicles, as well as generate the Ca^{2+} signals that trigger their subsequent fusion with the cell surface. SS_{14}

acts, possibly through a phosphatase(s), to deprime, or prevent the priming of, secretory granules and thus inhibit the exocytotic process.

An interesting aspect of this model is the possible identity of the intracellular process(es) responsible for vesicular priming. In Gromada et at. (2001), a study examining SS_{14} regulation of glucagon secretion in rats, the adenylate cyclase activator forskolin was used to increase the number of fusion-competent secretory granules (19). This suggests that, in rat α-cells, vesicular priming is PKA-dependent. This hypothesis is consistent with DA and PACAP stimulating GH release from goldfish somatotropes in a PKA-dependent manner (reviewed in Sections 1.8.1.2 and 1.8.1.3). However, in goldfish, PKC, and not PKA is required for GnRH stimulation of GH release ((21); see Section 1.8.1.1). As a result, it is reasonable to hypothesize that vesicular priming in goldfish somatotropes results from the activation of either PKA or PKC. Alternatively, GnRH-, DA-, and PACAP-stimulated GH release have all been shown to be dependent upon CaM KII (see Section 1.8.1), thus it is also possible that vesicular priming is the result of CaM KII activation.

The model depicted in Fig. 5.1 considers SS_{14} regulation of acutely stimulated GH release in column perifusion. However, this hypothesis can be easily adapted to also explain SS_{14} regulation of unstimulated GH release in column perifusion. In goldfish, basal GH secretion is reduced by the PKA inhibitor H89 (22, 23). In addition, treatment with the PKC inhibitor calphostine C also lowers basal GH secretion (WK Yunker, unpublished). These data suggest that, in goldfish, basal GH secretion has both PKA- and PKC-dependent components. It is conceivable that these kinases, are continuously priming a limited number of secretory granules, which are then undergoing exocytosis is response to unstimulated elemental Ca^{2+} signals occurring in close proximity to the plasma membrane and the docked secretory granule. Similar to SS_{14} regulation of stimulated GH secretion, application of SS_{14} would result in a depriming of secretory granules, and as a result, a reduction in basal GH secretion.

Interestingly, basal GH secretion is not totally abolished in the presence of SS_{14}, SS_{14} and NE (WK Yunker, unpublished), H89 (22, 23), or calphostine C (WK Yunker,

unpublished). This suggests that there are at least two secretory processes occurring during basal GH secretion in goldfish somatotropes. One that is susceptible to inhibition, and one that is not. This is not surprising and is consistent with what is known about other secretory systems. In general, secretory cells, such as pituitary cells, are believed to utilize at least two different secretory pathways (reviewed in (24, 25)). The first is the constitutive secretory pathway, which is not regulated by extracellular signals. In the present study, the constitutive pathway may be represented by the GH secretion that persists in the presence of SS_{14}, H89 or any other inhibitory factor. The second pathway is the regulated secretory pathway. This pathway, as the name implies, is susceptible to regulation by extracellular signals and intracellular messengers. According to this dual-path model, it is the regulated pathway that is inhibited by SS_{14}. The model depicted in Fig. 5.1 applies only to the regulated component of basal GH secretion.

In column perifusion, all of the GH-releasing compounds tested, sGnRH, cGnRH-II, DiC8, SKF-38393, PACAP, forskolin and 8Br-cAMP, were completely ineffective in the presence of SS_{14} (Chapters 3 and 4). Similarly, sGnRH, SKF-38393, PACAP, forskolin and 8Br-cAMP were completely ineffective in the presence of SS_{14} when tested in static incubation (26) (Table 2.1). However, in static incubation, GH responses to cGnRH-II and DiC8 were observed in the presence of SS_{14} (26) (Table 2.1). The model depicted in Fig. 5.1 deals with the acute regulation of GH release. In static incubation experiments, the somatotropes are subjected to regulatory ligands or pharmacological compounds for extended periods of time. Whether the intracellular processes occurring during this prolonged treatment are directly comparable to those occurring during acute regulation (column perifusion) are unknown. The discrepancy noted above suggests that they may not be.

5.4 Other GH release-inhibitors

Aside from the SS's, NE and 5-HT are also known to inhibit GH secretion from goldfish somatotropes (see Section 1.8.2). Like SS_{14}, $[Pro^2]SS_{14}$, and $gbSS_{28}$, NE is

capable of inhibiting GH responses to a wide variety of natural ligands, as well as activators of their respective intracellular signalling cascades (27) (Table 5.1). The actions of NE appear more similar to those of SS_{14} and $[Pro^2]SS_{14}$ than $gbSS_{28}$, as evidenced by the fact that NE is capable of inhibiting forskolin- and AA-stimulated GH release in static incubation (27) (Table 5.1). However, unlike SS_{14}, removal of NE results in transient GH and $[Ca^{2+}]_i$ rebounds (27, 28) (Table 5.1). As such, it seems unlikely that SS_{14} and NE are acting in an identical manner. It can be argued that the differences in GH and $[Ca^{2+}]_i$ rebounds are due to the fact that the effects of SS_{14} are not as readily reversible as those of NE. However, preliminary studies indicate that SS_{14} is capable of modulating GH secretion in the presence of NE (WK Yunker, unpublished), which suggests that NE and SS_{14} are acting independently of one another. Furthermore, NE, but not SS_{14} affects released cAMP levels (Table 5.1). Nevertheless, additional studies are required before any conclusions can be made.

Similar to the SS's and NE, 5-HT is also able to regulate ligand stimulated GH release *in vitro*. Interestingly, the initial data from this lab (JP Chang, unpublished) suggest that its mechanism(s) of action may be different from those of the other GH-release inhibitors studied (Table 5.1). It will be interesting to further explore this possibility by examining the effects of 5-HT on basal and stimulated cAMP levels and $[Ca^{2+}]_i$.

5.5 Why have multiple regulatory factors?

Precise control of somatotrope function is critical for the maintenance of homeostasis in vertebrates. In goldfish somatotropes, this is thought to arise through the complex interaction of multiple stimulatory and inhibitory neuroendocrine ligands (reviewed in (29-31)). It is hypothesized that each of these factors differentially regulates various aspects of somatotrope function, such as, GH release, GH mRNA levels and GH protein synthesis (31). *In vivo*, when all of these factors are present, the result is the

highly coordinated regulation of somatotrope physiology, thus allowing the organism to adapt to constantly changing physiological demands.

Selective modulation of different somatotrope functions would require differences in the intracellular mechanisms of the various neuroendocrine regulators. In accordance with this hypothesis, differences in the signalling pathways of various stimulatory factors have been documented (Section 1.8.1). The data from the present study, in conjunction with previous studies, establish that differences in the intracellular mechanisms of the inhibitory neuroendocrine factors SS_{14}, $[Pro^2]SS_{14}$, $gbSS_{28}$, and NE (Table 5.1) also exist. However, whether the differences apparent in Table 5.1 allow these inhibitory factors to differentially control basal GH release, stimulated GH secretion, GH mRNA levels and GH protein synthesis *in vivo* is currently unknown. Nevertheless, documenting such differences provides a starting point for such experimentation.

Regardless of how different signalling pathways participate in the regulation of somatotrope homeostasis, it seems likely that Ca^{2+} signals constitute a vital component of the intracellular mechanisms that enable various cellular processes to be differentially controlled by the same neuroendocrine factor. It has been shown, in goldfish, that perturbations in Ca^{2+} homeostasis dissociate GH secretion from hormone gene expression and synthesis (32). If $[Ca^{2+}]_i$ is involved in regulating other cellular functions, such as mRNA transcription and translation, then SS_{14} inhibition of GH release, independent of changes in $[Ca^{2+}]_i$, should allow for the selective control of secretion without altering mRNA levels. Consistent with this hypothesis, it has been shown that SS_{14} suppresses GH release but does not alter GH mRNA in tilapia (33, 34). It would be interesting to further test this hypothesis by examining the effects of the different inhibitors on agonist-evoked Ca^{2+} signals, GH secretion, GH mRNA levels and GH content.

5.6 Seasonal regulation of GH release by SS

In goldfish, the regulation of GH release is subject to seasonal variation (reviewed in (35, 36)). In addition, seasonal variations in GH release are likely accompanied by

variations in intracellular Ca^{2+} store involvement. In particular, Ry-sensitive stores, which selectively participate in cGnRH-II signalling, seem to do so only in sexually regressed fish (37). This suggests that intracellular Ca^{2+} homeostasis may be seasonally dependent. In the current study, Ca^{2+} signalling was studied in somatotropes obtained from fish at different stages of the reproductive cycle. This enabled the yearly changes in basal $[Ca^{2+}]_i$ to be assessed. Basal $[Ca^{2+}]_i$ varied significantly throughout the year, being at its lowest between January and July, and peaking in December (Fig. 5.2A). This pattern appears somewhat similar to the significant seasonal changes in basal GH secretion measured from cells in culture (Fig. 5.2B). This suggests that changes in basal $[Ca^{2+}]_i$ may be related to changes in basal GH secretion *in vitro*. However, this *in vitro* pattern of change in basal GH release is out of phase with the seasonal variations in serum GH seen in intact goldfish (38). This suggests that, in addition to the inherent basal release state, neuroendocrine regulation has an important role to play in determining the amount of GH secretion from the pituitary *in vivo*.

Previous data have shown that brain irSS and PSS-I mRNA content vary inversely with serum GH on a seasonal basis, suggesting that the brain SS system may be involved in establishing the seasonal variations in circulating GH levels (reviewed in Section 1.3.2). Given that the mRNA levels of the three PSS's do not change synchronously on a seasonal basis, whether all three SS genes are involved in the seasonal regulation of serum GH levels, and how they interact, is unclear. Seasonal fluctuations in serum GH may also be due to changes in the responsiveness of the somatotrope. The sensitivity of the GH release response to various neuroendocrine factors changes along a seasonal time line. In particular, DA, cholecystokinin, and GHRH are most effective in sexually regressed fish, whereas PACAP acquires prominence during sexual recrudescence. In comparison, it is during the latter stages of recrudescence, maturation and spawning that GnRH, thyrotropin-releasing hormone and neuropeptide Y are at their most effective (31). Similarly, Cardenas et al. (2003) demonstrated that GH release from pituitary fragments obtained from sexually mature female goldfish are less responsive to SS_{14} inhibition than pituitary fragments from sexually regressed females (39). In contrast, the

data presented in the current study reveal no significant change in SS_{14} inhibition of basal GH from dispersed pituitary cells throughout the course of the year (Fig. 5.2C). Both the present study and Cardenas et al. (2003) employed overnight culture prior to experimentation; however, in the current study, dispersed pituitary cells from both males and females, rather than pituitary fragments from females were used. Whether these differences in the seasonal effects of SS_{14} are the result of the inclusion of male pituitaries in the current study, or the existence of hypothalamic nerve terminals within the pituitary fragment preparations of Cardenas et al. (2003) is unknown. However, the data presented in this study suggest that upon removal from the organism and subsequent overnight culture, somatotropes are, to some extent, released from seasonal control. This must be considered when relating *in vitro* cell culture results to seasonal regulation within the intact animal.

Another important area to consider in the seasonal regulation of GH release (as well as modulation of release under other physiological conditions) is local factors within the pituitary, which may be acting in a paracrine/autocrine fashion. Previously, cDNA for the GH-releasing peptide activin (40), as well as activin immunoreactivity (41), have been detected in the goldfish pituitary. In addition, PSS-I mRNA has been previously detected within freshly excised pituitaries from goldfish of unspecified gonadal state(s) (42). In the present study, the presence of PSS-I and -II mRNA were demonstrated in pituitary cells. Although the presence, in the pituitary, of the corresponding SS_{14} and $gbSS_{28}$ peptides awaits confirmation, the ability of these peptides to alter GH secretion has been firmly established in this thesis. The absence of PSS-III mRNA within cultured pituitary cells taken from sexually recrudescing animals does not exclude the possibility that this transcript is expressed at other times of the year. PSS-I and -III mRNA levels in the forebrains of both male and female goldfish are known to be upregulated by estradiol (reviewed in Section 1.3.2). Whether PSS-III is expressed in pituitaries obtained from more sexually mature fish warrants further examination.

5.7 Conclusion

The present study examined the regulation of pituitary somatotrope GH secretion by endogenous hypothalamic SS peptides in goldfish. Evidence that multiple SS isoforms are capable of reaching the pituitary and differentially regulating somatotrope function was presented. These data suggest that perhaps these peptides fulfill different roles pertaining to the regulation of somatotrope physiology. A more detailed examination of the mechanisms of action of one the SS isoforms, SS_{14}, was subsequently conducted. The results establish that SS_{14} does not abolish stimulated Ca^{2+} signals as a means of inhibiting stimulated GH responses. This would allow for the differential regulation of secretion and other Ca^{2+}-dependent cellular responses by SS_{14}. To conclude, by comparing the effects of three closely related peptide isoforms and subsequently examining more closely the effects of one these peptides, this thesis provides evidence for differential regulation of cellular functions at both the extracellular and intracellular levels.

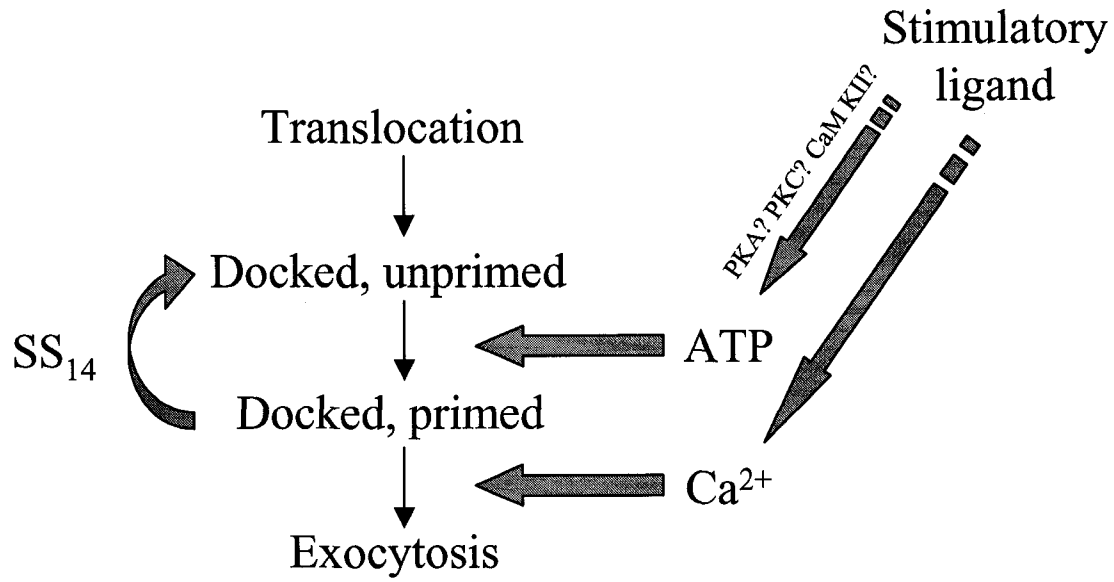

Fig. 5.1. A Hypothetical Model of the Regulation of Acutely Stimulated GH Release from Goldfish Somatotropes by SS_{14}.

Secretory granules translocate to, and dock with the plasma membrane. There they remain, awaiting ATP-dependent vesicular priming and Ca^{2+}-triggered exocytosis. Stimulatory ligands activate intracellular signalling mechanisms capable of priming the docked vesicles and generating Ca^{2+} signals that trigger their exocytosis. The mechanism(s) responsible for vesicular priming is unknown, as is its relationship to the Ca^{2+} signal. For simplicity these two events have been diagrammed independently of one another. SS_{14} is hypothesized to inhibit GH release by depriming release-competent secretory granules, thereby uncoupling Ca^{2+} signals and exocytosis.

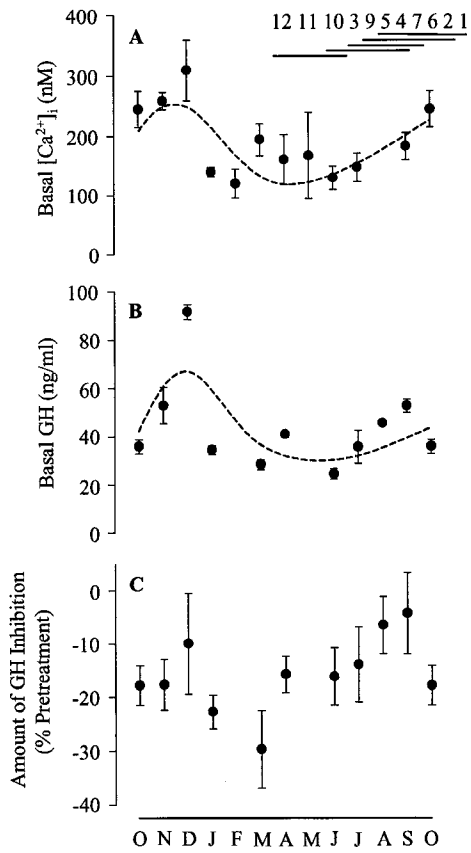

Fig. 5.2. Basal $[Ca^{2+}]_i$, Basal GH Secretion, and SS_{14} Inhibition of Basal GH Release from Goldfish Somatotropes Throughout the Year.

Months are represented by their first letter. (A) Basal $[Ca^{2+}]_i$ (defined as the average $[Ca^{2+}]_i$ from 0 to 2 min) from all cells examined in this study were pooled according to the month in which the experiment was conducted. Data were analyzed using a one-way ANOVA followed by LSD comparisons. Differences were considered significant when $P<0.05$. Underscores identify months whose basal $[Ca^{2+}]_i$ are not significantly different from one another. A trend line has been superimposed. (B) Basal GH release (ng/ml) (defined as the average GH from time 0 to 30 min) from all perifusions presented in this study were pooled according to the month in which the experiment was conducted. Data were analyzed using a Kruskal-Wallis test followed by pair-wise Mann-Whitney tests. Differences were considered significant when $P<0.0011$ (Bonferroni adjusted P-value for multiple comparisons). Results from the pair-wise comparisons are presented in Appendix A. A trend line has been superimposed. (C) Amount of SS_{14} inhibition of basal GH release (defined as the average GH release for the 3 fractions immediately following SS_{14} application minus the average GH release for the 3 fractions immediately prior to SS_{14} application) from all perifusions presented in this study were pooled according to the month in which the experiment was conducted. Data were analyzed using a one-way ANOVA. No significant differences (i.e. $P>0.05$) were detected.

Table 5.1. Summary of the Effects of Different GH Release Inhibitors on Basal and Stimulated Goldfish Somatotropes.

	SS_{14}	$[Pro^2]SS_{14}$	$gbSS_{28}$	NE	5-HT
Basal GH Secretion					
lowers basal secretion?	Yes	Yes	Yes	Yes[3]	Yes[4]
IC_{50} (nM)	1.73	6.69	0.16	100[3]	0.75[5]
GH rebounds?	No	No	No	Yes[3]	No[4]
Basal $[Ca^{2+}]_i$					
lowers basal $[Ca^{2+}]_i$?	No			No[3]	
Ca^{2+} rebounds?	No			Yes[3]	
Basal cAMP levels					
lowers released cAMP?	No	No	No	Yes[3]	
lowers cellular cAMP?	Yes	Yes	Yes	Yes[3]	
Stimulated GH Secretion					
GnRH Cascade					
sGnRH	X[1]	X	X	X[3]	↓[4]
cGnRH-II	↓[1]	X	X	X[3]	↓[5]
DiC8	↓[1]	↓	↓	↓[3]	↔[5]
TPA	↓[1]	↓	↔	↓[3]	↓[5]
DA/PACAP Cascade					
PACAP	X[2]	X	↓		
SKF-38393	X[1]	X	↓	X[3]	↓[4]
Forskolin	X[1]	X	↔	↓[3]	↓[5]
8Br-cAMP	X[1]	X	↔		↔[5]
AA	↓	↓	↔	X[3]	
NO Cascade					
SNP	X	X	↔		
Ca^{2+} Ionophores					
A23178	↓[1]	↓	↔	X[3]	
Ionomycin	↓[1]	↓	↓	↓[3]	↔[5]
Depolarization					
30 mM KCl	X	X	X		

X = abolished ↓ = reduced ↔ = not affected

[1] taken from (26), [2] taken from (43), [3] taken from (27), [4] taken from (36), [5] unpublished (JP Chang)

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Appendix 1

Table A.1. Significant Differences in Seasonal Basal GH Release. [1]

January vs.	March	n. s.	**April vs.**	December	s. d.
	April	n. s.	**June vs.**	July	n. s.
	June	n. s.		August	s. d.
	July	n. s.		September	s. d.
	August	s. d.		October	n. s.
	September	s. d.		November	s. d.
	October	n. s.		December	s. d.
	November	n. s.	**July vs.**	August	n. s.
	December	n. s.		September	n. s.
March vs.	April	s. d.		October	n. s.
	June	n. s.		November	n. s.
	July	n. s.		December	s. d.
	August	s. d.	**August vs.**	September	n. s.
	September	s. d.		October	s. d.
	October	n. s.		November	n. s.
	November	n. s.		December	s. d.
	December	s. d.	**September**	October	s. d.
April vs.	June	s.d.		November	n. s.
	July	n. s.		December	s. d.
	August	n. s.	**October vs.**	November	n. s.
	September	s. d.		December	s. d.
	October	n. s.	**November**	December	s. d.
	November	n. s.			

n. s. = not significantly different s. d. = significantly different.

[1] Basal GH release (ng/ml) for all perifusions presented in this study were pooled according to the month in which the experiment was conducted. Data were analyzed using a Kruskal-Wallis test followed by pair-wise Mann-Whitney tests. Differences were considered significant when $P<0.0011$ (Bonferroni adjusted P-value for multiple comparisons).

Curriculum Vitae

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Doctor of Philosophy (Ph.D.)
Physiology and Cell Biology
University of Alberta
2003

Bachelor of Science (B.Sc.)
Honours Environmental Biology (First Class Honours)
University of Alberta
1998

Graduate Academic Awards

Canada Graduate Scholarship, 2003 (Declined)

Natural Sciences and Engineering Resource Council of Canada (NSERC) Postgraduate Scholarship B (PGS B), 2003 (Declined)

University of Alberta Graduate Intern Tuition Supplement, 2001-2002

Jimmie Dodd Memorial Prize for Best Student Presentation at the 14th International Congress of Comparative Endocrinology, Sorrento, Italy, 2001

Alberta Heritage Foundation for Medical Research (AHFMR) MD/PhD Studentship, 2000, 2001, 2002, 2003

Natural Sciences and Engineering Resource Council of Canada (NSERC) Postgraduate Scholarship A (PGS A), 1998, 1999

Walter H Johns Graduate Fellowship, 1998, 1999

University of Alberta Graduate Studies Entrance Scholarship, 1998

Undergraduate Academic Awards

Jason Lang Scholarship, 2001

University of Alberta Dean's Silver Medal in Science, 1998

Alberta Heritage Foundation for Medical Research (AHFMR) Summer Studentship, 1998

Louise McKinney Post-Secondary Scholarship, 1997

University of Alberta Undergraduate Scholarship, 1997

Dr. WM A (Bill) Preshing Scholarship, 1996

University of Alberta Undergraduate Scholarship, 1995

University of Alberta Entrance Scholarship, 1994

Research Experience

September 1998 to Present – Graduate Studies

Title: "Regulation of Pituitary Somatotropes by Endogenous Somatostatins"

Cell Biology and Physiology Research Interest Group
Department of Biological Sciences, University of Alberta
Supervisor: Dr. John P. Chang

May 1998 to August 1998 – Summer Research Assistant

Title: "Adrenergic Receptors and the Associated Intracellular Signaling Mechanisms Mediating Norepinephrine Regulation of Growth Hormone Release"

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Cell Biology and Physiology Research Interest Group
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September 1997 to May 1998 – Honours Undergraduate Research Project

Title: "Norepinephrine Mediates Growth Hormone release from Dispersed Goldfish (*Carassius auratus*) Pituitary cells in Static Culture"

Undergraduate Independent Research Course (Biology 499)
Cell Biology and Physiology Research Interest Group
Department of Biological Sciences, University of Alberta
Supervisor: Dr. John P. Chang

September 1997 to December 1997 -- Research Project

Title: "Conditioned Alarm Behavior in Fathead Minnows (*Pimephales promelas*) Resulting from Association of Chemical Alarm Pheromone with a Nonbiological Visual Stimulus."

Undergraduate Course in Behavioral Ecology and Sociobiology (Zoology 371)
Department of Biological Sciences, University of Alberta
Course Instructor: Dr. Brian Wisenden

Academic Teaching Experience

September 1999 to December 1999 -- Graduate Student Marking Assistant

Grade midterms and finals for Biology 201 (Cellular Biology)

September 1998 to December 1998 -- Graduate Student Teaching Assistant

Teaching assistant for Zoology 241 (Introductory Animal Physiology I)
Conduct seminars based on lecture material
Develop practice questions, mark assignments and exams

Scientific Society Memberships

Canadian Society of Zoologists – 1998 to 2000

Refereed Publications

Published (Total = 11)

Yunker, W.K., Wein, D.E., Wisenden, B.D. 1999. Conditioned alarm behavior in fathead minnows (*Pimephales promelas*) resulting from association of chemical alarm pheromone with a nonbiological visual stimulus. *Journal of Chemical Ecology* 25, 2677-2686

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Yunker, W.K., Smith, S., Graves, C., Davies, P. J., Unniappan, S., Rivier, J. E., Peter, R. E., Chang, J. P. 2003. Endogenous hypothalamic somatostatins differentially regulate growth hormone secretion from goldfish pituitary somatotropes *in vivo*. *Endocrinology* 144:4031-4041

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Yunker, W. K., Chang, J. P. Somatostatin-14 uncouples dopamine- and pituitary adenylate cyclase-activating polypeptide-evoked Ca^{2+} signals from growth hormone secretion. Submitted to the *Journal of Neuroendocrinology*, April 16, 2003 (36 ms pages, 10 figures).

Non-Refereed Publications

Abstracts (Total = 9)

Wong, C.J.H., Johnson, J.D., **Yunker, W.K.**, Goldberg, J.I., Chang, J.P. 1998. Role of extracellular Ca^{2+} entry in caffeine-induced GTH-II release. *Bulletin* 28: 98.

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Yunker, W.K., Smith, S., Graves, C., Davies, P.J., Rivier, J.E., Peter, R.E., Chang, J. P. 2001. Three endogenous somatostatin isoforms differentially inhibit growth hormone release. *14th International Congress of Comparative Endocrinology* P3-64

Chang, J.P., **Yunker, W.K.**, Uretsky, A.D., Wong, C.J.H., Sawisky, G., Johnson, J.D. 2002. Signal transduction in neuroendocrine regulation of somatotropes in goldfish. *Signal Transduction in Health and Disease Symposium (STADY) III Symposium, October 1-4, 2002.*

Extracurricular Activities

Student Representative on the University of Alberta MD/PhD Committee, 2002-2003

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Workplace Hazardous Materials Information Services (WHMIS)

University of Alberta Radiation Safety Course

University of Alberta Care and Use of Animals in Research, Teaching and Testing Course