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CALCIUM AND CALCIUM-RELATED SIGNAL TRANSDUCTION MECHANISMS IN GONADOTROPIN RELEASING HORMONE-STIMULATED GONADOTROPIN AND GROWTH HORMONE SECRETION IN THE GOLDFISH

BY

RICHARD MARCEL JOBIN

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY**

DEPARTMENT OF ZOOLOGY

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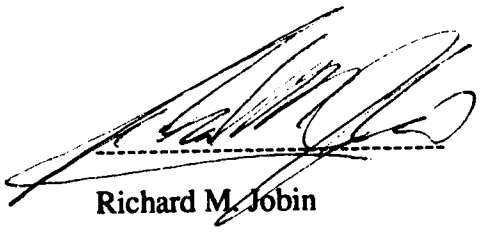

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Abstract

The signal transduction pathways mediating the gonadotropin (GtH) and growth hormone (GH) responses to salmon gonadotropin-releasing hormone (sGnRH) and chicken (c)GnRH II were investigated using cultured goldfish pituitary cells.

Both sGnRH and cGnRH II increase GtH release and levels of intracellular Ca^{2+} ($[Ca^{2+}]_i$), by stimulating entry of extracellular Ca^{2+} ($[Ca^{2+}]_o$) through voltage-sensitive Ca^{2+} channels. However, sGnRH- but not cGnRH II-stimulated increases in $[Ca^{2+}]_i$ and GtH release have components which are independent of $[Ca^{2+}]_o$. $[Ca^{2+}]_o$ plays a role in both prolonged and acute GtH secretion.

Activators of protein kinase C (PKC) mimic the Ca^{2+}-mobilizing and hormone-releasing abilities of these native GnRHs of the goldfish. Impairment of PKC activity (by use of inhibitors or PKC-depleted cells) attenuates the acute and sustained GtH responses as well as Ca^{2+} mobilization stimulated by PKC activators and the GnRHs. Additionally, GtH responses to sGnRH and PKC activators were not additive. LY171555, an agonist of the endogenous GtH-release inhibitor dopamine, decreased the GtH responses to PKC activators. These findings indicate that PKC mediates the modulation of GtH release by endogenous regulatory factors in the goldfish. Interestingly, results from experiments which examined the interactions between PKC and $[Ca^{2+}]_o$ entry suggest that PKC acts to initiate GtH release and the elevation of $[Ca^{2+}]_i$ amplifies this signal.

Involvement of calmodulin in sustained GtH responses to sGnRH and cGnRH II was indicated by the ability of calmidazolium to reduce GtH release in static incubation but not perifusion experiments.

Important roles for $[Ca^{2+}]_o$ entry and PKC activation in mediating sGnRH and cGnRH II stimulation of GH release were also indicated. In contrast to the GtH results, no differences in the $[Ca^{2+}]_o$ dependence of GH release stimulated by the two native GnRHs was found.

Although, both GnRHs stimulate GtH and GH release, the signal transduction mechanisms utilized by these GnRHs differs between GH and GtH cell types as well as within the GtH cell type.

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Chapter 1

General Introduction

I. Neuroendocrine Actions of GnRH in the Goldfish

Like in other vertebrates, gonadotropin (GtH) release in teleosts, including the goldfish (*Carassius auratus*), is under the stimulatory influence of gonadotropin releasing hormone (GnRH). In teleosts, a functional median eminence is absent and GnRH is delivered directly to the anterior pituitary by direct innervation [for reviews see refs. 1-4]. Unlike eutherian mammals which only possess one known form of GnRH (mammalian GnRH), goldfish along with most other vertebrates have at least two native GnRH peptides [5]. These two GnRH forms usually originate from separate branches of the GnRH gene family. One is derived from chicken GnRH II ([His^5, Trp^7, Tyr^8]-GnRH, cGnRH II) branch and the other comes from the branch as mammalian GnRH and salmon GnRH ([Trp^7, Leu^8]-GnRH, sGnRH). It has been proposed that cGnRH II does not function as a physiological regulator of pituitary hormone release in higher vertebrates. However, in the goldfish sGnRH and cGnRH II are both found in the pituitary and throughout the rest of the brain [6]. Both sGnRH and cGnRH II are released [7, 8] and are effective in eliciting gonadotropin (GtH) secretion *in vivo* [9] and *in vitro* [10, 8]. Both GnRH peptides compete for the same class of high affinity, low capacity receptors in membrane preparations of goldfish pituitaries; the association constants strongly agree with physiological levels of the releasing peptides and to their abilities to elicit GtH secretion [11, 12]. In *in vitro* studies, cGnRH II is more potent in releasing GtH than sGnRH [8, 10], suggesting that these two GnRHs may have different actions on GtH release in the

goldfish.

In the goldfish, GnRH neurons in the brain and pituitary apparently function as an integrated unit. The direct GnRH action on GtH release in the goldfish is modulated by other neuroendocrine factors [13, 14]. Briefly, GnRH-stimulated GtH release is blocked by the direct inhibitory action of dopamine (DA) via D_2 receptors [8, 1]. The increase in GnRH release, as well as the decrease in dopaminergic inhibition are both required for the inductoion of the preovulatory GtH surge in female goldfish [15, 16]. Gonadal steroids exert both positive and negative feedback actions on the GtH response to GnRH [reviewed in 13, 14]. More recently, gonadal inhibin/activin-like peptides have also been reported to stimulate basal GtH secretion and to potentiate the GnRH induced GtH response [17].

In addition to eliciting GtH release, GnRH also elevates serum growth hormone (GH) levels and increases body growth of goldfish; these GH-release responses can be inhibited by somatostatin [18]. Besides interacting with somatostatin, GnRH also interacts with other neuroendocrine regulators of GH secretion in the goldfish. GnRH stimulation of GH has been reported to be additive to that stimulated by GH-releasing hormone [19, 20], neuropeptide Y [21] and dopamine D_1 mechanisms [22, 23]. In contrast, the GH response to GnRH is attenuated by serotonin, another GH-release inhibitor [24]. These results suggest that GnRH is one of several important endogenous regulators of GH release and growth in the goldfish.

Like GtH release, sGnRH and cGnRH II directly stimulate GH secretion from goldfish pituitary cells [10]. GnRH stimulation of GH secretion in the goldfish is apparently mediated via receptors located on the somatotropes. Electronmicroscope studies demonstrate that avidin gold-labelled biotinylated sGnRH analog is displaced from the surfaces of immunohistochemically identified somatotropes and gonadotropes by both unlabeled sGnRH and cGnRH II [25]. These results indicate that within the somatotrope or gonadotrope cell type, both native GnRH peptides compete for same cell surface

receptors to induce GH secretion or GtH secretion, respectively. However, there appears to be differences between the GnRH receptors found on somatotropes as compared to gonadotropes. A mammalian GnRH antagonist selectively blocks GnRH-stimulated GH release while having no effect on GtH release in the goldfish [26].

From the above it is evident that GnRH is an important neuroendocrine regulator of reproduction and growth in the goldfish. Understanding the mechanism of action mediating GnRH stimulation of GtH and GH release is therefore, an essential step in the understanding the intracellular of events regulating the secretion of two important pituitary hormones in the goldfish. Moreover, goldfish pituitary release of GtH and GH provide a unique physiological model to compare the signal transduction mechanisms of two very similar native peptides (sGnRH and cGnRH II) on the same cell types as well as between cell types.

II. Mechanisms of GnRH Action at the Pituitary

A) A Review of the Mammalian Model

Although signal transduction of GnRH-stimulated gonadotropin secretion has been studied in numerous vertebrate systems including, cattle [27], sheep [28], chickens [reviewed by 29] and teleost fishes [30], the rat is by far the most thoroughly investigated model system [31]. In the rat, GnRH action on gonadotropes has been postulated to involve the generation of cyclic nucleotides [for a review see 31], arachidonic acid [for reviews see 32, 33]. increases in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$), activation of protein kinase C (PKC), hydrolysis of phosphoinositides [for reviews see 34, 31] as well as calmodulin activation [35, 36]. Of these second messenger pathways, increases in

cyclic nucleotides appear to be important in mediating luteinizing hormone (LH) synthesis but not the acute secretion response to GnRH [37]. In terms of eicosanoids, it has been shown that the metabolism of arachidonic acid by phospholipase A_2 and diacylglycerol lipase [38] and the subsequent metabolism of arachidonic acid through the lipoxygenase pathway [34] are components mediating GnRH-induced LH release. However, comparatively, the roles of Ca^{2+} and Ca^{2+}-related signaling pathways (PKC, calmodulin, phosphoinositide hydrolysis) in mediating the LH release response to GnRH have received much better characterization.

1) Calcium

In the rat, it has been well established that GnRH-stimulated LH secretion is a Ca^{2+} dependent process [31, 34]. It was originally believed that the entire LH response was mediated via extracellular Ca^{2+} entry [39, 40, 41]. However, results from perifusion experiments and use of the Ca^{2+}-sensitive fluorescent dyes indicate that Ca^{2+} from both intra- and extracellular stores are involved in LH release [42, 43]. Both the LH response and the elevation of cytosolic Ca^{2+} levels are biphasic in nature, consisting of an acute transient phase followed by a sustained phase of lower level response [43, 44, 45]. Dihydropyridine voltage sensitive Ca^{2+} channel (VSCC) antagonists fail to entirely block the initial, as well as the sustained, LH and intracellular Ca^{2+} responses [42, 43, 46]. Pretreatment with KCl also reduces subsequent GnRH-stimulated LH and Ca^{2+} responses [47]. These results suggest that part of the initial and sustained responses are not mediated by extracellular Ca^{2+} ($[Ca^{2+}]_o$) entry through dihydropyridine-sensitive VSCCs. Removal of $[Ca^{2+}]_o$ through the use of Ca^{2+} free medium containing EGTA blocked the sustained LH response to GnRH; when used in combination with A23187 (an ionophore), this treatment also abolished the entire LH response [43]. When repeatedly challenged with GnRH in the presence of Ca^{2+} free medium containing EGTA the subsequent but not the

first LH response was similarly abolished [42]. These results suggest that the sustained LH response is more dependent on $[Ca^{2+}]_o$ entry while the initial hormone-stimulated response is most likely more dependent on release of Ca^{2+} from intracellular stores.

Although the GnRH-induced LH response can be mimicked with ionophores [44] or potassium (K^+) stimulation [42, 46], GnRH-stimulated release is accompanied by only a modest increase in cytosolic Ca^{2+} concentrations (elevations of approximately 100nM) as compared to those elicited by ionophores or high KCl in populations of purified gonadotropes [47]. Furthermore, the K^+-stimulated, but not the GnRH-stimulated, LH release is abolished by dihydropyridine sensitive blockers [42, 46]. The above evidence further reinforces the hypothesis that GnRH-stimulated LH release is mediated by mechanisms different than those used by ionophores or K^+, which are "nonspecific" Ca^{2+} mobilization and $[Ca^{2+}]_o$ entry through VSCC, respectively.

In mammalian excitable cells, four types of VSCCs have been defined on the basis of their conductance, sensitivities to agonists and antagonists, and their kinetics; these are the N, T, L and P channels [48]. T- and L-channels are found in a large variety of cells including endocrine cells suggesting they play a role in general cell regulation [49]. L-type (long lasting) VSCCs have a prolonged open time, are opened by large changes in membrane potential and are slowly inactivated by both voltage- and Ca^{2+}-dependent mechanisms. These channels are affected by dihydropyridine VSCC agonists and channel blockers, and are involved primarily in the control of intracellular Ca^{2+} ($[Ca^{2+}]_i$) levels [50]. On the other hand, T (transient)-channels can be activated by small changes in membrane potential, have low conductances and are quickly inactivated in a voltage-dependent manner. These channels can be inhibited by $NiCl_2$ [50]. The T-channels may account for the dihydropyridine-insensitive portion of the sustained LH response in rat gonadotropes. Roughly 50% of $[Ca^{2+}]_o$ entry during the sustained response is mediated via the L-type channels while the remainder enter via dihydropyridine-insensitive channels

[45, 51]. These channels could include T-type VSCCs as well as inositol 1,4,5 trisphosphate ($InsP_3$) or PKC regulated channels [52]. $[Ca^{2+}]_o$ removal can reduce GnRH-stimulated increases in $[Ca^{2+}]_i$ in the initial phase by up to 40%; however, L-type channels play only a minor role in this initial component of Ca^{2+} entry, suggesting that dihydropyridine-insensitive Ca^{2+} entry occurs rapidly and contributes to the initial phase [53]. Overall, a close temporal relationship has been found to exist between $[Ca^{2+}]_i$ levels and LH release induced by GnRH in rat gonadotropes [54].

In patch-clamp recordings from single cells, both L- and T-channels undergo their characteristic time and voltage dependent inactivation in pituitary gonadotrophs [55]. Inactivation of the L-channels correlates with the characteristic GnRH-induced desensitization of these cells [56]. GnRH-induced desensitization cannot be entirely accounted for by receptor down regulation but is at least partly mediated by post-receptor modifications [53, 57]. Patch-clamp analysis has revealed that dihydropyridine-sensitive VSCCs are inhibited by repeated GnRH exposure [58]. This agonist-induced inactivation of L-channels may be part of the mechanism mediating the desensitization of LH response to prolonged or repeated challenges by GnRH.

Both L- and T-type channels play important roles in the generation and maintenance of spontaneous and GnRH-induced oscillations in $[Ca^{2+}]_i$ levels and plasma membrane voltage in rat gonadotropes. Currents carried by VSCC and Ca^{2+}-activated K^+ channels mediate firing of spontaneous and agonist-induced action potentials in the plasma membrane of gonadotropes [31]. Fluctuations in the membrane voltage and levels of $[Ca^{2+}]_i$ tend to be synchronized in rat gonadotropes. Spontaneous action potentials are totally dependent on $[Ca^{2+}]_o$ entry through VSCCs which contribute to the functioning of the plasma membrane oscillator [31]. Agonist-induced fluctuations in $[Ca^{2+}]_i$ levels are initially not dependent on membrane voltage or $[Ca^{2+}]_o$ entry, but can be inhibited by blockade of Ca^{2+}-ATPase suggesting involvement of release of Ca^{2+} from intracellular

stores. Conversely, maintenance of GnRH-induced oscillations of $[Ca^{2+}]_i$ concentrations and membrane potential fluctuation is dependent on both $[Ca^{2+}]_o$ entry and mobilization of Ca^{2+} from intracellular stores [31]. These two mechanisms serve as components of the cytoplasmic oscillator in the rat gonadotrope.

2) Protein Kinase C (PKC)

Elevation of $[Ca^{2+}]_i$ levels in cells is known to mediate the secretion of many hormones, including GnRH stimulation of GtH release in vertebrates (see previous section). Once $[Ca^{2+}]_o$ has entered the gonadotrope and increased the levels of $[Ca^{2+}]_i$, one of the possible sites of action for the free cytosolic Ca^{2+} is the enzyme protein kinase C (PKC). The PKC enzyme family consists of at least 8 subspecies, 2 of which (α and β-II) have been found in the pituitary of the rat [34]. Activation of PKC has requirements for both Ca^{2+} and phospholipid, phosphatidylserine in particular. In the presence of 1,2, diacylglycerol PKC has a greater sensitivity to Ca^{2+} activation [59]. Treatments of rat pituitary cells with synthetic diacylglycerols and 4β-phorbol esters, agents known to activate PKC, result in increased LH secretion [56, 60, 61]. Phorbol esters such as 12-O-tetradecanoyl phorbol-13-acetate (TPA) also increase PKC activity in rat pituitary cells [62, 63]. Addition of PKC isoforms, PKC-α and PKC-β, into preparations of permeablized rat pituitary cells also enhances LH release [64]. TPA-induced LH release is enhanced when tested in the presence of agents that increase Ca^{2+} entry into the cell but is decreased by those that inhibit Ca^{2+} entry [46, 52, 65]. Activators of PKC (TPA and synthetic diacylglycerols) have also been shown to increase levels of $[Ca^{2+}]_i$ via actions of VSCCs [66]. These results suggest that phorbol-induced LH release is partially dependent on $[Ca^{2+}]_o$ entry into gonadotropes.

There is also evidence which links PKC activity directly with GnRH action. GnRH

treatment increases PKC activity in rat pituitary cells preparations and induces the translocation of PKC enzyme activity from the cytosol to the membrane fractions of the cells [62, 63]. The PKC inhibitors retinal and H7 have also been found to decrease both TPA and GnRH-induced LH secretion [54, 57, 62].

Despite this overwhelming evidence implicating PKC's involvement in GnRH-stimulated LH release, its participation in GnRH action is still questioned. McArdle et al. [67] have reported that gonadotropes that had been pretreated with TPA, and showing a >95% loss of PKC activity (measured by incorporation of ^{32}P into histones), as well as a decrease in LH content, were not responsive to phorbol esters or synthetic diacylglycerols; however, these cells showed no inhibition of GnRH-stimulated LH release when release was expressed as a percentage of the initial LH content. Additionally, in column perifusion experiments, although PKC activity was found to increase during initial GnRH stimulation of LH secretion, PKC activity dropped to basal levels despite repeated subsequent GnRH applications and continued stimulation of LH secretion. These results suggest an uncoupling of the PKC pathway and LH secretion [68]. In contrast, others have found that under similar experimental conditions there is an inhibition of GnRH-stimulated LH secretion and point out that in normalizing the LH response to GnRH following TPA pretreatment, McArdle et al. [67] failed to account for LH synthesized during the GnRH challenge period [52, 69]. Recently, TPA pretreatment carried out at room temperature was found to deplete PKC levels with little loss of stored LH. Under such conditions the PKC-depleted cells showed attenuation of GnRH-stimulated LH release in perifusion experiments [70].

TPA has been found to have biphasic effects on $[Ca^{2+}]_i$ levels and LH release in rats. The nature, either stimulatory of inhibitory, of TPA's effects is dependent on existing $[Ca^{2+}]_i$ levels. Such results have lead to the development of the hypothesis that PKC acts to amplify the LH releasing signal initiated by elevations in $[Ca^{2+}]_i$ levels [34].

3) Calmodulin

Target sites other than PKC may be affected by elevations in levels of $[Ca^{2+}]_i$. In work done on the rat gonadotrope, calmodulin which is a ubiquitous Ca^{2+}-binding protein in vertebrate cells appears to be involved in GnRH-stimulated GtH release. Calmodulin is activated by the binding of Ca^{2+}, and when activated it alters the activity of other enzymes, among these are cytoskeletal proteins that appear to be involved in the secretory process [71]. Minutes following GnRH-stimulated Ca^{2+} entry in rat pituitaries calmodulin is found to redistribute from the cytosolic to membrane fractions of the cells [72]. Furthermore, calmodulin is found in association with clustered GnRH receptors; these micro-aggregations of receptors occur when GnRH or an agonistic ligand binds to the receptors [73]. Calmodulin antagonists (pimozide, penfluridol) also block GnRH-stimulated LH release in dispersed cells [74]. In perifusion studies penfluridol was found to strongly inhibit the sustained portion of the LH response but had little effect on the initial phase [43]. Together these results suggest that the $[Ca^{2+}]_o$ entering the gonadotropes uses calmodulin as its transducer. Recent work has attempted to locate the targets of the GnRH-activated calmodulin [75]. One such target has been found to be the vesicle binding protein, caldesmon, which may act to regulate interactions with secretory granules [35].

4) Phosphoinositide Hydrolysis

Stimulation of rat gonadotropes by GnRH leads to hydrolysis of polyphosphoinositides [for reviews see 76, 77]. The hydrolysis of membrane inositol containing phospholipids is carried out by the enzyme phospholipase C (PLC). This leads

to the creation of inositol phosphates and diacylglycerol (DG), which stimulates PKC activity. More specifically, there is a rapid increase in inositol 1,4,5,-trisphosphate (Ins-1,4,5-P_3, $InsP_3$) followed by the accumulation of Ins-P_4, P_5 , P_6 and DG [78, 79]. $InsP_3$ mobilizes $[Ca^{2+}]_i$ from $InsP_3$-sensitive store, these are presumed to be part of the endoplasmic reticular system or calciosomes [80, 81]. $InsP_3$ releases Ca^{2+} from intracellular stores by binding to specific $InsP_3$ receptors [82]. These $InsP_3$ receptors are Ca^{2+}-channel proteins [reviewed by [83]. Fractionation studies indicate that a plasma membrane associated vesicular system sequestering Ca^{2+} may also exist [84, 85]. Putney [86] suggests that the $InsP_3$-sensitive intracellular Ca^{2+} pool is refilled directly from the exterior of the cell. In agreement with this hypothesis refilling of depleted stores occurs with very little increase in cytosolic Ca^{2+} levels [87, reviewed by 88].

$InsP_3$ may also modulate Ca^{2+} entry at the plasma membrane [89]. $InsP_3$ has also been shown to modulate dihydropyridine VSCCs in skeletal muscle [90]. Similarly, other inositol phosphates, especially Ins-1, 3, 4, 5-P_4, have been proposed to regulate the entry of $[Ca^{2+}]_o$ [reviewed by 91].

Regardless of the possible roles of higher InsPs, the actions of $InsP_3$ on release of Ca^{2+} from intracellular stores is a central figure in all current theories regarding signal transduction mechanisms involving Ca^{2+} oscillations induced by ligand activation of by cytoplasmic oscillators [92, 93].

5) Summary of the Mammalian Model

GnRH-stimulated LH secretion from rat pituitaries is strongly dependent on Ca^{2+} in both the initial and sustained phases. Dihydropyridine-sensitive VSCCs play a minor role in the initial phase, a major role in the sustained response and is a possible mechanism involved in agonist-stimulated desensitization. Dihydropyridine-insensitive Ca^{2+} channels

are equally important to the sustained response, and relatively more important than their dihydropyridine-sensitive counterparts in mediating the initial phase of the GnRH-induced LH response. The initial phase appears to be mostly dependent on Ca^{2+} released from intracellular stores. $InsP_3$ appears to act to release Ca^{2+} from intracellular stores during the initial stages of GnRH-stimulated LH release. Release of Ca^{2+} from intracellular stores also plays an important role in the initiation of GnRH-induced Ca^{2+} oscillations. However, this released Ca^{2+} from intracellular stores does not appear to exert its effects through calmodulin or PKC and the method of its action on LH release remains unresolved. Both calmodulin and PKC appear to mediate the effects of $[Ca^{2+}]_o$ entry during the sustained phase. $[Ca^{2+}]_o$ entry via VSCC also plays roles in the mediation of spontaneous action potentials and maintenance of GnRH-induced oscillations of $[Ca^{2+}]_i$ levels and action potentials in rat gonadotropes.

B) A Review of Second Messengers Mediating GnRH Action in Fish

1) Calcium

Several reports in the literature suggest that $[Ca^{2+}]_o$ may be involved in the release of GtH from the teleost pituitary. Using pituitary fragments from tilapia (*Tilapia sparrmanii*) the GtH release response to [D-Ala^6, des-Gly^{10}]-GnRH-ethylamide was attenuated by the removal of Ca^{2+} from the perifusion medium or the addition of cobalt chloride [94]. In pituitary fragments of the African catfish (*Clarias gariepinus*), Buserelin (another mammalian GnRH agonist) also induced GtH release; this GtH response was also inhibited by the removal of $[Ca^{2+}]_o$, the addition of EGTA or by the addition of nifedipine into the perifusion medium [95]. However, the addition of D600 (methoxyverapamil) to the

perifusion medium was not effective in reducing GtH release in these studies. In dispersed cells of the fresh water murrel (*Channa punctatus*) the GtH response to mammalian and partially purified *C. puctatus* GnRH was decreased by removal of extracellular Ca^{2+} (by addition of EGTA) and also by the addition of verapamil or lanthanum to the culture medium [96]. Dispersed pituitary cells of the goldfish (*Carassius auratus*) in static culture exhibited decreased sGnRH-stimulated GtH release when Ca^{2+} free medium was used as compared to control incubation with normal Ca^{2+}-containing medium [97]. In the presence of external Ca^{2+}, ionophores (such as A23187 and ionomycin) have also been shown to elicit GtH release from pituitary fragments of tilapia [94], the African catfish [95] and dispersed pituitary cells of the goldfish [98].

The above data suggest that $[Ca^{2+}]_o$ is involved in GnRH-stimulated GtH release. However, in the case of the studies using teleost pituitary fragments, indirect effects of extracellular Ca^{2+} availability cannot be excluded. Teleost pituitaries are directly innervated [4]; furthermore, GnRH release from goldfish preoptic/hypothalamic slices and pituitary fragments have been shown to be dependent on $[Ca^{2+}]_o$ entry [15].

2) PKC

In teleosts, PKC appears to be involved in mediating GtH secretion in teleosts. Involvement of PKC in mediating GtH release has been implicated in tilapia where high doses of the synthetic diacylglycerol 1-oleoyl-2 acetylglycerol elicits an increase in GtH secretion from pituitary fragments in perifusion [94]. Our preliminary work in the goldfish also suggests that PKC is involved in GtH release [99]. A variety of phorbol esters including TPA , 4β–phorbol 12,13 dibutyrate, 4β-phorbol 12,13 dibenzoate, 4β-phorbol 12,13 diacetate and the synthetic diacylglycerol dioctanoyl glycerol (DiC8), stimulate GtH release from dispersed goldfish pituitary cells in static culture. The ranked order of

potencies of these β-phorbols in stimulating goldfish GtH and GH release are similar to those observed in inducing LH release and PKC activation in mammals. However, the inactive phorbol, 4α-phorbol 12,13 didecanoate, is ineffective in stimulating hormone release. TPA-stimulated GtH release is partially dependent on $[Ca^{2+}]_o$ entry through VSCC [99]. Furthermore, the PKC inhibitor H7 attenuates the release of GtH stimulated by sGnRH, cGnRH II and TPA [99]. The above results suggest that PKC may be involved in the mediation of GnRH-induced GtH release in teleosts.

3) Other signal transduction pathways mediating GtH release in fish

In pituitary fragments of tilapia and dispersed cells of the goldfish, elevation of cAMP levels by the use of cAMP analogs, adenylate cyclase activator forskolin or phosphodiesterase inhibitor IBMX resulted in increased release of GtH [100, 101]. However, in the goldfish GnRH does not stimulate the release of cAMP into the medium and GtH release elicited by the adenylate cyclase stimulator forskolin or cAMP analogs are additive to sGnRH-stimulated release [101]. These data suggest that the cAMP pathway does not play a role in the mediation of GnRH-stimulated GtH release in the goldfish. Conversely, treatment with a mammalian GnRH analog led to the release of cAMP from tilapia pituitary fragments [100]; however the source of cAMP generation in these studies with pituitary fragments remains unknown.

Interestingly, despite evidence which suggests that sGnRH and cGnRH II occupy the same class of GnRH receptor on goldfish gonadotropes [25], metabolism of arachidonic acid appears to be involved in sGnRH- but not cGnRH II-stimulated release of GtH in goldfish [102]. This result supports the novel hypothesis that two closely related native peptides can occupy the same class of receptors and yet show differences in their subsequent activation of signal transduction mechanisms.

III Control and Signal Transduction of GH Release

In the goldfish, GnRH stimulates the release of GH as well as GtH [8]. This is not the case in mammals. However, much study has been directed towards signal transduction pathways that mediate growth hormone releasing factor (GRF)-stimulated release of GH in mammals. It is generally accepted that mobilization of Ca^{2+} and activation of the cAMP pathway are mostly responsible for the mediation of GRF-induced GH secretion [reviewed by 103]. Data collected indicate that activation of PKC and phosphoinositide hydrolysis do not play a role in the induction of GH release by GRF [104-108].

In the goldfish, GnRH stimulates GH release by binding to receptors on somatotropes [8, 25]. Treatments with Ca^{2+} ionophore and arachidonic acid elevate GH release in goldfish [97]. Use of Ca^{2+}-deficient medium, but not the addition of inhibitors of arachidonic acid metabolism, attenuate sGnRH-induced GH release [97]. Activators of PKC stimulate release of GH in goldfish in a manner which was partially dependent on $[Ca^{2+}]_o$ entry via VSCC [101]. The PKC inhibitor H7 reduced GH release stimulated by TPA, sGnRH and cGnRH II [101]. Taken together, the above results suggest that in the goldfish, activation of PKC and $[Ca^{2+}]_o$ entry through VSCC are involved in GnRH-stimulated GH release.

IV. Aims and Directions of this Thesis

As indicated by our preliminary results [101], Ca^{2+} and PKC appear to be important in mediating GnRH action in the goldfish. The involvement of Ca^{2+} and Ca^{2+}-related signal transduction mechanisms in the mediation of GnRH action on GtH and GH release

from the dispersed pituitary cells of the goldfish is further studied in detail in this thesis. In particular, the involvement, interaction and comparisons of second messenger pathways utilized by the two native GnRH peptides of the goldfish are investigated. Where possible, comparisons of pathways mediating GnRH-induced GtH and GH responses are also monitored. The goldfish pituitary cell hormone release model system presents a unique opportunity to study not only involvement and interactions of signal transduction pathways mediating GnRH action, but also, comparisons of the effects of the two native GnRHs on second messenger pathways mediating the release of the same as well as different hormones (GtH and GH).

Involvement of $[Ca^{2+}]_o$ entry in prolonged GtH and GH secretion stimulated by sGnRH and cGnRH II was studied in static culture. These results are presented in Chapter 2. In Chapter 3, the involvement of $[Ca^{2+}]_o$ entry in acute GtH secretion stimulated by sGnRH and cGnRH II was studied in perifusion using rapid fraction collection.

In Chapter 4, the involvement of PKC was examined by comparison of the GtH release response to stimulation by sGnRH, cGnRH II, activators of PKC, a Ca^{2+} ionophore, and a stimulator of the cAMP pathway in normal and PKC depleted cells. Data from both static culture and perifusion experiments are presented. In addition, the participation of PKC in GnRH action was further tested by examining the additivity between GtH release stimulated by GnRH, activators of PKC and the adenylate cyclase activator forskolin. Attenuation of GtH responses elicited by sGnRH, cGnRH II, and activators of PKC by an inhibitor of PKC were also examined. These data are presented in Chapter 5.

In Chapter 6, the Ca^{2+} mobilization responses to sGnRH, cGnRH II, activators of PKC and KCl and the dependence of these Ca^{2+} responses on $[Ca^{2+}]_o$ were examined with the use of the Ca^{2+}-sensitive fluorescent dye Fura 2. Differences between the $[Ca^{2+}]_o$-dependence of GtH release stimulated by TPA as compared to that induced by

DiC8 were also studied.

The roles of Ca^{2+} and PKC in the initiation and amplification of the release of GtH are assessed in Chapter 7. Effects of manipulations of Ca^{2+} flux and Ca^{2+} storage on GtH release, as well as the interactions between the Ca^{2+} and PKC pathways were studied in static culture and perifusion experiments.

In Chapter 8, the involvement of calmodulin in the prolonged and acute GtH release stimulated by sGnRH- and cGnRH II was studied with the use of calmodulin inhibitors. This chapter presents both static culture and perifusion data.

A summary of the general conclusions of the individual chapters and development of a hypothesized model for the signal transduction pathways mediating GnRH-stimulated GtH release in the goldfish is presented in the general discussion, Chapter 9. In addition other results from experiments using PKC-depleted cells to determine the involvement of PKC in the mediation of GnRH-stimulated GtH and GH release are presented in Appendix 1. In this appendix chapter, the attenuation of TPA- and DiC8-stimulated GtH release by the dopamine D_2 agonist LY171555 was used to determine involvement of the PKC pathway in the dopamine-elicited inhibition of GtH release in the goldfish.

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Chapter 2

Differences in Extracellular Calcium Involvement Mediating the Secretion of Gonadotropin and Growth Hormone Stimulated by two Closely Related Endogenous GnRH Peptides in Goldfish Pituitary Cells.[1]

Introduction

Gonadotropin (GtH) release in many teleosts, including the goldfish (*Carassius auratus*), is under the stimulatory influence of gonadotropin releasing hormone (GnRH) and the inhibitory effects of dopamine (DA) D_2 action, delivered directly to the anterior pituitary by direct innervation [for reviews see refs. 1-4]. In the goldfish two different forms of GnRH, [Trp^7, Leu^8]-GnRH (salmon GnRH, sGnRH) and [His^5, Trp^7, Tyr^8]-GnRH (chicken GnRH II, cGnRH II), have been found in the pituitary and throughout the rest of the brain [5]. Both sGnRH and cGnRH II are released [6,7] and are effective in eliciting gonadotropin (GtH) secretion *in vivo* [8] and from dispersed cells in static culture [9] and pituitary fragments in column perifusion *in vitro* [7]. Both GnRH peptides compete for the same class of high affinity, low capacity receptors in membrane preparations of goldfish pituitaries; the association constants strongly agree with physiological levels of the releasing peptides and to their abilities to elicit GtH secretion [10,11]. In *in vitro* studies, cGnRH II is more potent in releasing GtH than sGnRH

[1]A version of this chapter has been published, Jobin and Chang 1992, Neuroendocrinology 55:156-166.

[7,9].

In addition to eliciting GtH release, GnRH elevates serum growth hormone (GH) levels and increases body growth of goldfish, these GH-release responses can be inhibited by somatostatin, suggesting that GnRH is an endogenous regulator of growth [12]. sGnRH and cGnRH II directly stimulate GH release from goldfish pituitary cells [9]. GnRH stimulation of GH secretion in the goldfish is apparently mediated via receptors located on the somatotropes. Both native GnRH peptides displace membrane bound avidin gold-labelled biotinylated [D-Lys^6,Pro^9-N-ethylamide]-sGnRH analogue from immunohistochemically identified GH as well as cells in electron microscopic studies using dispersed goldfish pituitary cell populations (H. Cook, J. Berkenbosch, R.E. Peter, and J.P. Chang unpublished results). A GH releasing peptide (GRF) has been detected in teleosts [13], however, the respective roles of GRF and GnRH in modulation of growth remain unclear [for discussion see reference 14].

Second messenger systems mediating GnRH action on gonadotropin release have been much more thoroughly investigated in mammals than in any other vertebrate. A variety of signal transduction components have been found to be involved, including phosphoinositide turnover, protein kinase C (PKC), arachidonic acid, G proteins, as well as intra- and extracellular calcium ($[Ca^{2+}]_o$) [for reviews see refs 15-18]. At least two classes of voltage-sensitive Ca^{2+} channels (VSCC), resembling the "T" (low threshold, rapidly inactivating) and the "L" (high threshold, more slowly inactivating)-type, have been identified in rat anterior pituitary cells [19-21]. Although $[Ca^{2+}]_o$ entry may occur via VSCCs and other Ca^{2+}channels, dihydropyridine-sensitive "L"-type VSCCs have been demonstrated to participate in the GnRH stimulation of increases in intracellular Ca^{2+} concentration and luteinizing hormone release from rat gonadotropes [22-25].

In general, results from mammalian studies suggest that GH secretion is mediated by cyclic AMP as well as Ca^{2+}-dependent mechanisms [for a review see ref. 26]. More specifically, basal and GRF-induced GH secretion and changes in intracellular Ca^{2+} levels

are inhibited by several treatments that impede the entry of $[Ca^{2+}]_o$ into cells [27,28].

In contrast to the situation in mammals, information on the signal transduction pathways mediating GtH and GH release in teleost fishes is not readily available. Recent reports from studies conducted on fish pituitary fragments or dispersed cell preparations indicate that GnRH actions in teleosts, including sGnRH stimulation of GtH in the goldfish, are also dependent on $[Ca^{2+}]_o$ [for a brief review of the fish literature, see ref. 29]. Similarly, basal and sGnRH-induced GH release from dispersed goldfish pituitary cells are also dependent on $[Ca^{2+}]_o$ [29]. However, the involvement of $[Ca^{2+}]_o$ entry in mediating the actions of the other native GnRH peptide, cGnRH II, on GH and GtH release have not been investigated. Previous results suggest that the actions of sGnRH and cGnRH II on goldfish pituitaries may be mediated via slightly different signal transduction mechanisms. sGnRH and cGnRH II stimulation of GtH release from dispersed goldfish pituitary cells are inhibited by DA D_2 agonists with different sensitivities and efficacies [30]. Furthermore, the GtH response to these two native releasing peptides follow dissimilar dose-response characteristics [9].

In the present study the role of $[Ca^{2+}]_o$ in mediating both sGnRH- and cGnRH II-induced GtH and GH secretion was further examined using static incubations of dispersed goldfish pituitary cells. This model system allows one to study how a single secretagogue stimulates the release of different pituitary hormones and also how different, but closely related, secretagogues elicit the release of the same hormone from pituitary cells. Involvement of $[Ca^{2+}]_o$ was determined by three simple approaches: 1) altering Ca^{2+} levels in the bathing medium of the cells; 2) addition of cobalt to compete with Ca^{2+} present in the bathing medium; and 3) addition of VSCC blockers to impede the entry of $[Ca^{2+}]_o$ into the cells.

Materials and Methods

General. Common goldfish (8-12 cm in length), purchased from Ozark Fisheries Inc., Stoutland, Missouri and Grassyforks Fisheries, Martinsville, Indiana, were transferred to flow-through aquaria (1800 liters) immediately on arrival. The fish were held at 17-20 ^{o}C on a simulated natural (Edmonton, Alberta) photoperiod, and fed to satiation daily with commercial fish food. Fish of both sexes were acclimated to the above conditions for at least 7 days before use. Stock solutions of sGnRH and cGnRH II (Peninsula Lab. Inc., Belmont, CA) were dissolved in 0.1 N acetic acid. Nicardipine, nifedipine and verapamil (Sigma, St. Louis, MO) were dissolved in ethanol. Aliquots of stock solutions were stored at -20 ^{o}C until used.

Static cell culture and hormone response. Pituitaries from fish of both sexes were removed and enzymatically dispersed [for details see ref. 9]. The dispersed cells were cultured overnight in 24-well culture plates with medium 199 containing Earle's salts (Gibco, Grand Island, NY), 1% horse serum, 25 mM Hepes, 2.2 g/l sodium bicarbonate, 100 000 units/l penicillin, and 100 mg/l streptomycin at a density of 0.25 million cells/well/ml. The cells were incubated at 27 ^{o}C, 5% CO_2 and saturated humidity. The following day, the culture medium was replaced with testing medium (medium 199 containing Hank's Salts (Gibco), 25 mM Hepes, 2.2 g/l sodium bicarbonate, 100 000 units/l penicillin, 100 mg/l streptomycin, 0.1% bovine serum albumin). A special formulation of M199 containing Hank's salts made without $CaCl_2$ (Gibco) was utilized for experiments with altered Ca^{2+} concentrations. Test solutions diluted in the proper vehicle were then added (1 µl/ml to achieve desired final concentration). Inhibitors were usually added 10 minutes prior to application of the secretagogues. The concentration of vehicle was less than 0.1% of the final incubation volume and did not alter GtH nor GH release. Following a further 2 hr incubation the media were removed and stored at -20 ^{o}C until their

GtH and GH contents were measured using established radioimmunoassays [31,32]. All treatments were carried out in quadruplicate or sextuplicate. Experiments were repeated a minimum of three times and the GtH contents were measured in all replicate experiments. In some cases, GH contents were only measured in representative experiments due to the limited availability of radioiodinated GH tracer. Results from replicate experiments were similar and the data were usually pooled prior to statistical analysis by analysis of variance followed by Fisher's least significant difference test. GtH and GH values from replicate experiments were normalized by expressing the data as a percentage of the net hormone response to a maximal or near maximal (10 nM) stimulation by sGnRH or cGnRH II; net hormone response being hormone level for the treatment minus the hormone level in unstimulated controls. Analysis of pooled data produced a conservative estimate of statistical differences due to an increase in the variability of the data caused by interassay variability, as well as minor differences between cell preparations.

Results

Alteration of calcium levels in the testing medium. Removal of $[Ca^{2+}]_o$ from the incubation medium decreased the GtH and GH responses evoked by both sGnRH and cGnRH II. The amount of GtH secreted appeared to be proportional to the concentration of Ca^{2+} in the testing medium (Fig. 2.1). When the $[Ca^{2+}]_o$ concentration was lowered from 1.25 mM (standard M199) to 1 mM, GH secretion elicited by both GnRHs was significantly reduced, however, no such reduction was observed in GtH secretion until $[Ca^{2+}]_o$ concentrations were 0.5 mM or lower (Fig. 2.1). Reduction of $[Ca^{2+}]_o$ also significantly depressed basal GH but not unstimulated GtH values.

The influences of incubation with Ca^{2+}-depleted medium (Ca^{2+}-free with 0.1 mM EGTA) on GH and GtH responses were further examined in four replicated experiments

(Fig. 2.2). In these experiments, cGnRH II was significantly more effective in stimulating GtH secretion than sGnRH at an equimolar dose (10 nM). However, the GH responses to the two GnRH peptides were not significantly different from one another. Under $[Ca^{2+}]_o$-depleted conditions 10 nM sGnRH was still able to significantly stimulate GtH secretion while 10 nM cGnRH II was not. Similar to results in Fig. 2.1, basal GtH release remained unaffected by inhibition with Ca^{2+}-depleted medium. In contrast to GtH release, GH responses to both secretagogues was totally blocked and basal secretion was significantly decreased under $[Ca^{2+}]_o$-depleted conditions.

Addition of cobalt to the testing medium. When the competitive inhibitor of Ca^{2+} entry, $CoCl_2$, was added to the testing medium a suppression of GnRH-stimulated GtH and GH release was observed (Fig. 2.3). cGnRH II-induced GtH secretion was significantly inhibited by $CoCl_2$ at a concentration of 0.1 mM, while sGnRH-stimulated release was not affected until a dosage of 0.5 mM $CoCl_2$ was reached (Fig. 2.3). GH secretion stimulated by either sGnRH or cGnRH II was abolished (stimulated secretion was not significant when compared to $CoCl_2$ control) at concentrations of $CoCl_2$ between 100 μM to 10 mM were used. Decreases in the basal secretion of GtH and GH were only seen at the highest doses of $CoCl_2$ (Fig. 2.3).

Addition of VSCC blockers to the testing medium. All VSCC antagonists used inhibited GnRH-stimulated GtH and GH release (Figs. 2.4-2.6).

Verapamil (a D600 class phenylalkylamine VSCC blocker) significantly inhibited cGnRH II-stimulated GtH release at a 10 nM concentration while GtH secretion induced by sGnRH was not affected until a 1 μM dosage was reached (Fig. 2.4). GH secretion stimulated by both GnRH peptides was significantly depressed at the lowest dosage (1 nM) of verapamil tested and was abolished ($P>0.05$ vs verapamil control) at 10 μM

verapamil. Basal secretion rates of neither GH nor GtH were significantly affected by verapamil (Fig. 2.4). In these studies with verapamil, cGnRH II-induced GtH release was also significantly higher than that stimulated by sGnRH.

Nifedipine (a dihydropyridine VSCC blocker) significantly reduced cGnRH II-stimulated GtH release at a dosage of 1 nM, however, sGnRH-elicited GtH release was significantly inhibited only at dosages of 100 nM and higher (Fig. 2.5). Conversely, nifedipine significantly decreased sGnRH-stimulated GH secretion at a lower dosage (100 nM) than it did cGnRH II-stimulated GH release (10 μM) (Fig. 2.5). Basal secretions of neither GH nor GtH were affected by nifedipine.

Nicardipine (another dihydropyridine VSCC blocker) began to inhibit both sGnRH and cGnRH II-stimulated GtH release at a dosage of 10nM. However, at high concentrations (1&10 μM) cGnRH II-stimulated GtH release was more completely blocked than that released by sGnRH (Fig. 2.6). Similar to its effects on GtH release, nicardipine inhibited sGnRH and cGnRH II-stimulated GH secretion at concentrations of 100 nM and higher (Fig. 2.6). Basal GtH release tended to be elevated at low concentrations (1 nM) of nicardipine, while basal GH release remained unaffected by the drug.

Discussion

Unlike other studies of similar nature the present study is a comparison of the involvement, and extent of involvement, of $[Ca^{2+}]_o$ in the action of two native GnRH peptides (sGnRH and cGnRH II) on two different hormones (GtH and GH) from the pituitary gland of a teleost. Furthermore, these two native releasing peptides appear to bind to the same class of high affinity receptors in goldfish pituitary membrane preparations [10]. Since $[Ca^{2+}]_o$ entry is a mechanism involved in the release of many substances,

including sGnRH and cGnRH II from goldfish pituitary [6], it was essential to use a dispersed cell system. In studies using teleost pituitary fragments, manipulations of $[Ca^{2+}]_o$ availability may alter GtH release through indirect effects. As described earlier (see Introduction), neurons from the hypothalamus are known to innervate the pituitary in teleosts [for review see refs. 3,4]; in particular, some of these terminals are known to contain the major regulators of GtH release, DA [33] and GnRH [34]. The release of neurotransmitters from nerve terminals is dependent on $[Ca^{2+}]_o$. Furthermore, GnRH release from goldfish pituitary fragments is also dependent on $[Ca^{2+}]_o$ entry [6]. Using dispersed cells has the advantage of removing the possible confounding effects of nerve terminals in the pituitary that may have stimulatory or inhibitory effects [9].

The results from the present study indicate that extracellular Ca^{2+} entry is involved in pituitary hormone release induced by both GnRH peptides native to the goldfish. All treatments designed to impede $[Ca^{2+}]_o$ entry into the pituicytes inhibited GnRH-stimulated GtH and GH secretion, though the degrees of effectiveness between individual treatments varied. Reports of $[Ca^{2+}]_o$-dependence of GnRH action have similarly been reported for LH release in mammals and for GRF-stimulated GH secretion in mammals (see Introduction). The hypothesis that GnRH action in teleost invokes $[Ca^{2+}]_o$ entry is further supported by other recent findings. In pituitary fragments of tilapia (*Tilapia sp.*), [D-Ala^6,Pro^9-N-ethylamide]-GnRH-stimulated GtH release was attenuated by the removal of Ca^{2+} from the perifusion medium or by the addition of cobalt chloride [35]. In pituitary fragments of the African catfish (*Clarias gariepinus*), Buserelin (mammalian GnRH agonist)-induced GtH release was inhibited by the omission of $CaCl_2$ and addition of EGTA to the perifusion medium or by the addition of nifedipine, but not by the addition of D600 (methoxyverapamil) to the perifusion medium [36]. In dispersed pituitary cells of the fresh water murrel (*Channa punctatus*), the GtH responses to mammalian and partially purified *C. puctatus* GnRH peptides were decreased by the removal of $[Ca^{2+}]_o$ (by

addition of EGTA) and also by the addition of verapamil or lanthanum to the culture medium [37]. Dispersed pituitary cells of the goldfish in static culture exhibited decreased sGnRH-stimulated GtH release when Ca^{2+} free medium (prepared without the addition of Ca^{2+} salts) was used [9]. Ionophores (such as A23187 and ionomycin) also elicit GtH release in tilapia [35] and in an $[Ca^{2+}]_o$ dependent fashion in the African catfish [36] and the goldfish [30]. Furthermore, GH responses to sGnRH and ionophores in static cultures of dispersed goldfish pituitary cells were inhibited by incubation with Ca^{2+}-free medium [38].

The VSCC blockers verapamil, nifedipine and nicardipine reduced GtH and GH responses to both GnRH peptides in the present investigation. This suggests that GnRH-induced hormone release from goldfish pituitary cells is at least partially mediated by $[Ca^{2+}]_o$ entry through VSCCs. In mammals, sub-micromolar concentrations of phenylalkylamines (verapamil) and dihydropyridines (nifedipine, nicardipine) VSCC antagonists have a specificity for the "L-type" channel relative to the "T-type" channel [for a review see refs. 38,39]. The ability of nanomolar concentrations of verapamil, nifedipine and nicardipine to significantly reduce GtH and GH responses to sGnRH and cGnRH II suggests that the "L-type" channels are involved in the $[Ca^{2+}]_o$ entry mediating the hormone responses to these two native GnRH peptides. "L-type" VSCCs have been implicated in GnRH-induced LH secretion in the rat [24] and chicken [40]. These channels have also been implicated in the potassium [25] and GRF [11]-stimulated GH secretion from rat pituitary cells. However, definitive characterization of the VSCC channel types involved in GnRH peptide action on goldfish gonadotropes and somatotropes still requires further investigation.

As discussed earlier, the GH response to sGnRH and cGnRH II, as well as the GtH response to cGnRH II, were completely abolished by incubation with Ca^{2+} deficient medium, but these hormone responses were only partially inhibited by sub-micromolar concentrations of verapamil and dihydropyridine "L"-type VSCC antagonists. In addition

to other types of VSCC, $[Ca^{2+}]_o$ entry can also occur through Na^+/Ca^{2+} exchange or nonspecific cation channels. Na^+/Ca^{2+} exchange activity has been located on plasma membrane preparations of rat anterior pituitary cells [41]. However, McArdle et al. [42] recently reported that Na^+/H^+ but not Na^+/Ca^{2+} exchange participated in GnRH stimulation of LH release in rat gonadotropes. The possibility that Ca^{2+} channels other than the verapamil and dihydropyridine-sensitive "L"-type channels are also involved in GnRH action on goldfish pituitary hormone release requires further study.

Although both the GtH and GH responses to GnRH are dependent on $[Ca^{2+}]_o$, their relative dependence on $[Ca^{2+}]_o$ varied. sGnRH- and cGnRH II-induced GH secretion were inhibited by lower concentrations of verapamil, as well as smaller reductions in $[Ca^{2+}]_o$ concentrations than those required to significantly reduce the GtH response. Moreover, in $[Ca^{2+}]_o$ depleted incubation conditions, both sGnRH and cGnRH II failed to induce a GH response, whereas sGnRH was still able to significantly elevate GtH release above controls. Similarly, in the presence of 100-500 μM $CoCl_2$, both sGnRH and cGnRH II were able to stimulate GtH release but not GH release. These results suggest that GH secretion is more dependent on $[Ca^{2+}]_o$ than is GtH secretion when induced by the native GnRH peptides. These results further indicate that GH responses to both GnRH peptides may be entirely dependent on $[Ca^{2+}]_o$ availability, whereas the GtH response to sGnRH is partially independent of $[Ca^{2+}]_o$ entry. Although, the use of verapamil was able to reveal the greater $[Ca^{2+}]_o$ dependency possessed by the GH as compared to the GtH response, results with dihydropyridine VSCC antagonists used were not consistent in this respect. Thus the involvement of VSCCs in mediating the difference in $[Ca^{2+}]_o$ dependence between GtH and GH responses remains uncertain. These differences in the results with verapamil as compared to nicardipine and nifedipine may be due to verapamil and dihydropyridines modulating GnRH-induced changes in VSCC conductance differently. In mammals, phenylalkylamines and dihydropyridines alter VSCC activity by

binding to sites on the "L-type" channel which are distinct from each other [44].

In mammals basal LH secretion is not dependent on $[Ca^{2+}]_o$, however, basal GH secretion is inhibited by removal of $[Ca^{2+}]_o$ and the addition of nifedipine [27,45]. Results from the present study also indicate that basal GH and GtH release possess different dependence on $[Ca^{2+}]_o$. The ability of $[Ca^{2+}]_o$ depletion and 5 mM $CoCl_2$ to depress basal GH secretion suggests that this process is dependent on $[Ca^{2+}]_o$. The lack of effectiveness of both phenylalkylamine and dihydropyridine VSCC antagonists in altering basal GH secretion indicates that , unlike the mammalian model, channels sensitive to these compounds do not mediate basal GH release. In contrast, most treatments were ineffective in altering basal GtH secretion implying that it is a relatively $[Ca^{2+}]_o$-independent process. These results support the hypothesis derived from previous observations that basal GH, but not GtH release is dependent on $[Ca^{2+}]_o$ entry and verapamil sensitive VSCCs are not involved in mediating the basal secretions of GH or GtH [38]. However, in this study high mM doses of $CoCl_2$ inhibited basal GtH as well as GH release. Co^{2+} can enter cells and compete with intracellular Ca^{2+} for internal binding sites [46,47], therefore, the depression of basal hormone release at high (10 mM) $CoCl_2$ may not reflect an $[Ca^{2+}]_o$-dependence of basal GtH secretion. An elevation of basal GtH secretion was observed with 1 nM nicardipine, this effect may be due to dihydropyridine antagonists having agonistic action at low doses [48].

Throughout all the experiments conducted cGnRH II consistently, and often significantly (Figures 2.2 & 2.4), stimulated a greater release of GtH than did sGnRH. These results are consistent with the previous finding that cGnRH II is more effective than sGnRH (at equimolar doses) at stimulating GtH release [9]. In addition to being more efficient at stimulating GtH release from the dispersed goldfish pituitary cells, GtH release elicited by cGnRH II was also significantly inhibited by lower concentrations of $CoCl_2$, verapamil and nifidipine than those required to decrease GtH release stimulated by sGnRH.

Furthermore, high doses of nicardipine or the use of medium depleted of $[Ca^{2+}]_o$ abolished cGnRH II-stimulated release while sGnRH still evoked a significant response. In addition, when the GtH releasing ability of increasing concentrations of sGnRH and cGnRH II were compared in the presence of 1 μM verapamil, 1 nM sGnRH, but not 1 nM cGnRH II, significantly increased GtH release over basal secretion (JP Chang, G Freedman and RM Jobin, unpublished results). These data demonstrate that cGnRH II-induced GtH release is more sensitive to blockade of $[Ca^{2+}]_o$ entry into the cell as well as more dependent on $[Ca^{2+}]_o$ than GtH release stimulated by sGnRH. The different potencies of verapamil and dihydropyridines in inhibiting the GtH releasing abilities of sGnRH and cGnRH II suggests that the differential dependence on $[Ca^{2+}]_o$ of these two releasing peptides may in part be due to VSCCs. This differential dependence on $[Ca^{2+}]_o$ may also be related to the differential sensitivity of sGnRH and cGnRH II to perturbation of the PKC system. PKC activation requires both diacylglycerol and Ca^{2+} [48]. In previous studies, the PKC activating phorbol ester 12-O-tetradecanoyl-phorbol 13 acetate, enhanced GtH release from dispersed goldfish pituitary cells in a manner which was partially dependent on $[Ca^{2+}]_o$ [29]. The PKC inhibitor H7 was able to abolish the GtH response to low doses (0.01 and 0.1 nM) of cGnRH II whereas sGnRH remained effective at these concentrations [29]. Therefore, the cGnRH II-induced GtH release, which has been shown to be more effective than that induced by sGnRH [7], is also more dependent on $[Ca^{2+}]_o$ and Ca^{2+}-related mechanisms in its signal transduction processes.

The present study supports the previous finding [9] that the two native GnRHs do not differ in their GH releasing ability in static incubations of pituitary cells, which is in contrast to the effects that the two releasing peptides have on GtH release (see above discussion). No significant difference was found between the GH responses to sGnRH and cGnRH II. Furthermore, GH secretion elicited by both GnRHs was similarly affected by treatments with the competitive Ca^{2+} channel antagonist $CoCl_2$, the noncompetitive Ca^{2+} channel blockers verapamil and nicardipine, as well as by the reduction of $[Ca^{2+}]_o$

concentration. Although in one experiment nifedipine inhibited sGnRH and cGnRH II-stimulated GH release with different potencies, the overall results, nevertheless suggest that the dependencies on $[Ca^{2+}]_o$ of the GH responses to sGnRH and cGnRH II are very similar. Consistent with the lack of difference in the $[Ca^{2+}]_o$ dependence of the GH response elicited by sGnRH and cGnRH II, hormone response by goldfish somatotropes to both GnRHs show similar sensitivity to inhibition by the PKC inhibitor H7 [29]. These results suggest that sGnRH and cGnRH II-stimulated GH release differs in neither efficacy, $[Ca^{2+}]_o$ dependency nor in signal transduction systems related to $[Ca^{2+}]_o$.

The demonstration of differences in the $[Ca^{2+}]_o$ dependence of GnRH action on GH and GtH release in dispersed goldfish pituitary cells is not entirely surprising as two different cell types are involved. However, the differential involvement of $[Ca^{2+}]_o$ in sGnRH-induced, as opposed to cGnRH II-stimulated GtH secretion is both an unexpected and novel hypothesis. Both sGnRH and cGnRH II have been shown to bind to the same class of high affinity, low capacity receptors on goldfish pituitary membrane preparations [11]. The magnitude of the GtH response to the two peptides differs and accordingly one might expect that the signal transduction processes involved to be similar between the two peptides but of greater magnitude in the case of cGnRH II. Direct measurement of the $[Ca^{2+}]_o$ flux involved in sGnRH and cGnRH II stimulation of GtH release have not been performed, but results from the present study suggest that the difference in signal transduction mediating the actions of the two GnRH peptides is qualitative as well as quantitative. This difference is most clearly illustrated when depletion of $[Ca^{2+}]_o$ in the incubation medium led to the abolition of the stronger cGnRH II-generated GtH response, and leaving the weaker sGnRH response effective. An $[Ca^{2+}]_o$-independent component that mediated part of the action of sGnRH is apparently absent from the cGnRH II-induced GtH response. This qualitative difference begs the question: Why would an organism evolve two different ways of doing the same thing? If the two releasing peptides are

simply redundant and binding to the same receptor, why are the signals transduced differently? One possible explanation is that the two peptides have different physiological roles. Interestingly, the binding capacity of high affinity sites in preparations of goldfish pituitary membranes exhibits two distinct seasonal peaks, the first occurring during early recrudescence (Oct/Nov) the second at the time of ovulation (Mar/Apr) [11]. Perhaps one GnRH is more involved in gonadal recrudescence and the other with ovulation.

This study represents a case where two closely related endogenous releasing peptides that work on the same cells through common receptor sites to induce the same response do so via second messenger systems that are apparently at least slightly different. The $[Ca^{2+}]_o$ dependence of basal and stimulated GH release to either native GnRH peptide appears to be greater than that of GtH secretion. Although there is no apparent difference in the $[Ca^{2+}]_o$ dependence between the effects of the two GnRHs on GH release, cGnRH II-stimulated GtH release appears to have a greater $[Ca^{2+}]_o$ requirement than does sGnRH. Detection of other possible differences in the second messenger pathways mediating sGnRH and cGnRH II actions will require further investigation.

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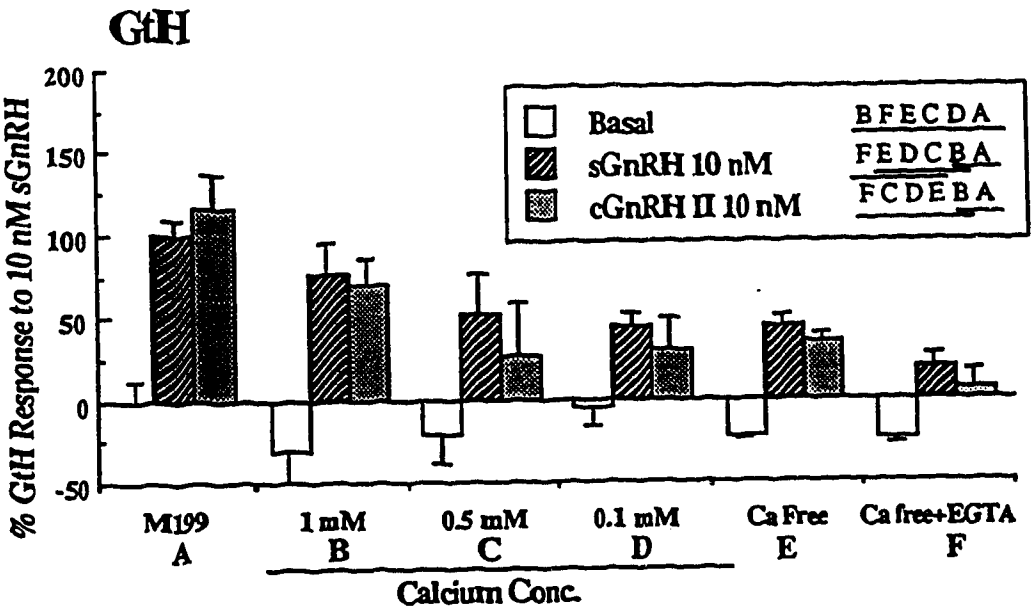
Figure 2.1. Dependence of sGnRH- and cGnRH II-stimulated GtH (upper panel), and GH release (lower panel) on extracellular Ca^{2+} concentration. Ca^{2+} free medium was prepared without the addition of Ca^{2+} salts but may not be totally devoid of Ca^{2+} (Ca^{2+} conc ≈ 10 μM). The addition of (0.1 mM) EGTA to the Ca^{2+} free medium (CF + EGTA) further reduces the Ca^{2+} concentration (Ca^{2+} conc.≈ 10-20 nM). Normal M199 contains 1.25 mM Ca^{2+}, other concentrations were produced by addition of $CaCl_2$. GtH results presented (mean ± SE) are pooled data from three replicate experiments. Treatments in individual experiments were carried out in sextuplicate. The average net GtH response to 10nM sGnRH and cGnRH II were 96.02 ± 3.12 and 113.43 ± 23.54 (ng/ml/0.25 million cells), respectively. In the inset, treatment groups are identified by letters and arranged in ascending order according to the average GtH response; letters not sharing the same underscore are statistically different from each other ($P<0.05$).


GtH
% GtH Response to 10 nM sGnRH
200
150
100
50
0
-50
Basal
sGnRH 10 nM
cGnRH II 10 nM
BFECDA
FEDCBA
FCDEBA
M199
A
1 mM
B
0.5 mM
C
0.1 mM
D
Ca Free
E
Ca free+EGTA
F
Calcium Conc.

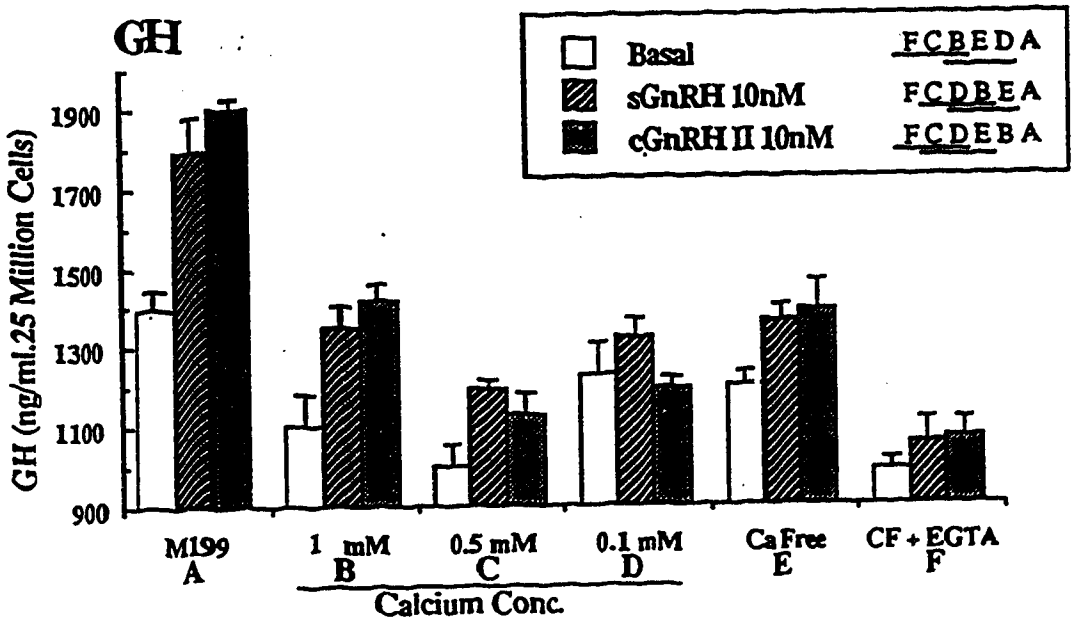

GH
GH (ng/ml.25 Million Cells)
1900
1700
1500
1300
1100
900
Basal
sGnRH 10nM
cGnRH II 10nM
FCBEDA
FCDBEA
FCDEBA
M199
A
1 mM
B
0.5 mM
C
0.1 mM
D
Ca Free
E
CF + EGTA
F
Calcium Conc.

Figure 2.2. Effect of removal of extracellular calcium on sGnRH- and cGnRH II-induced GtH (upper panel) and GH release (lower panel). Ca^{2+} depleted medium was prepared without the addition of Ca^{2+} and addition of (0.1 mM) EGTA. Average net GtH and GH response to 10nM sGnRH were 148.66 ± 13.37 and 269.0 ± 29.7 (ng/ml/0.25 million cells; N=4), respectively. In the inset, treatment groups are identified by numbers and arranged in ascending order according to the average GtH response; numbers not sharing the same underscore are statistically different from each other ($P<0.05$).

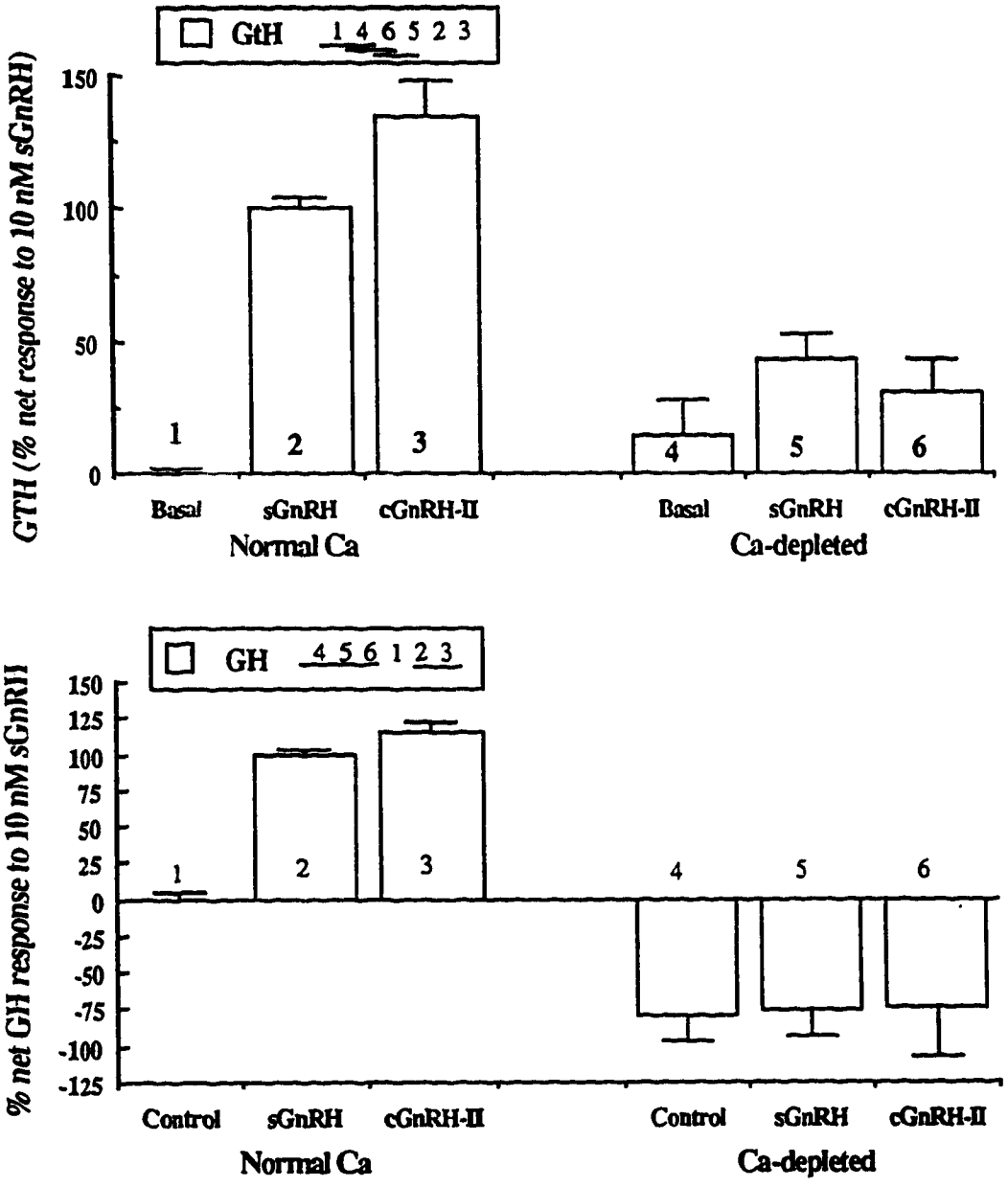
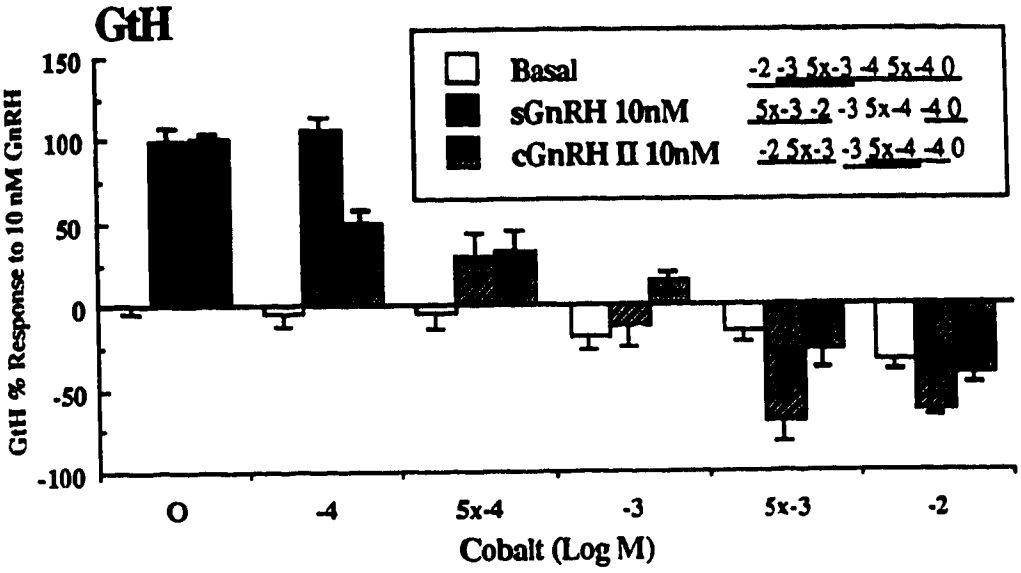

GtH 1 4 6 5 2 3
GTH (% net response to 10 nM sGnRH)
150
100
50
0
1
2
3
4
5
6
Basal
sGnRH
cGnRH-II
Basal
sGnRH
cGnRH-II
Normal Ca
Ca-depleted
GH 4 5 6 1 2 3
% net GH response to 10 nM sGnRH
150
125
100
75
50
25
0
-25
-50
-75
-100
-125
1
2
3
4
5
6
Control
sGnRH
cGnRH-II
Control
sGnRH
cGnRH-II
Normal Ca
Ca-depleted

Figure 2.3. Effect of cobalt on basal, sGnRH- and cGnRH II-stimulated GtH (upper panel), and GH (lower panel) release from static incubations of dispersed pituitary cells. Cobalt was added to M199 in the form of $CoCl_2$. . GtH data presented (mean ± SE) are data pooled from three replicate experiments. Treatments in individual experiments were carried out in quadruplicate. The average net GtH responses to 10nM sGnRH and cGnRH II were 86.4 ± 23.05 and 140.3 ± 28.15 (ng/ml/0.25 million cells), respectively. In the inset, concentrations of cobalt are arranged in ascending order according to the average GtH response; treatments not sharing the same underscore are statistically different from each other ($P<0.05$).


GtH
GtH % Response to 10 nM GnRH
150
100
50
0
-50
-100
Basal
sGnRH 10nM
cGnRH II 10nM
-2 -3 5x-3 -4 5x-4 0
5x-3 -2 -3 5x-4 -4 0
-2 5x-3 -3 5x-4 -4 0
O
-4
5x-4
-3
5x-3
-2
Cobalt (Log M)

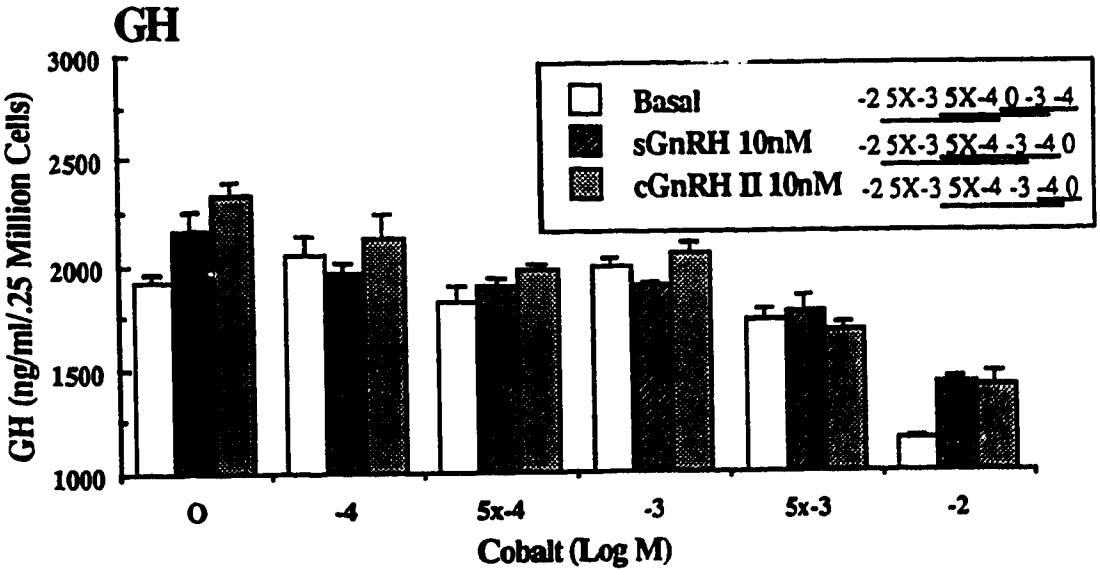
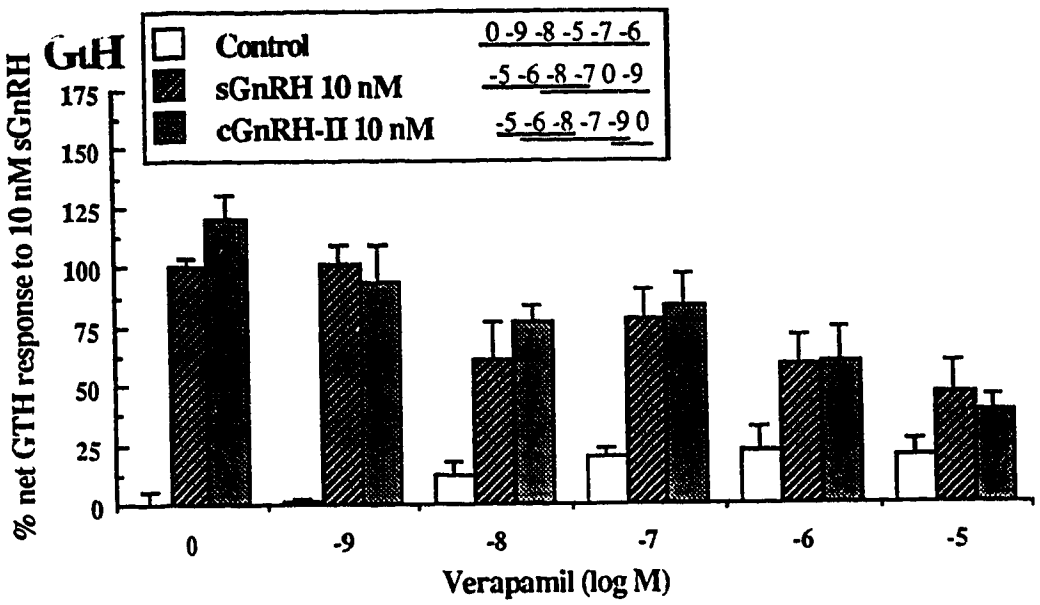

GH
GH (ng/ml/.25 Million Cells)
3000
2500
2000
1500
1000
Basal
sGnRH 10nM
cGnRH II 10nM
-2 5X-3 5X-4 0 -3 -4
-2 5X-3 5X-4 -3 -4 0
-2 5X-3 5X-4 -3 -4 0
O
-4
5x-4
-3
5x-3
-2
Cobalt (Log M)

Figure 2.4. Effect of verapamil on basal, sGnRH- and cGnRH II-stimulated GtH (upper panel) and GH release (lower panel). Results presented (mean ± SE) are pooled data from four replicate experiments. Treatments in individual experiments were carried out in quadruplicate. The average net GtH and GH responses to 10nM sGnRH were 86.75 ± 15.74 and 716.5 ± 30.7 (ng/ml/0.25 million cells), respectively. In the inset, concentrations of verapamil are arranged in ascending order according to the average GtH response; treatments not sharing the same underscore are statistically different from each other ($P<0.05$).


GtH
Control
sGnRH 10 nM
cGnRH-II 10 nM
0 -9 -8 -5 -7 -6
-5 -6 -8 -7 0 -9
-5 -6 -8 -7 -9 0
% net GTH response to 10 nM sGnRH
175
150
125
100
75
50
25
0
0
-9
-8
-7
-6
-5
Verapamil (log M)

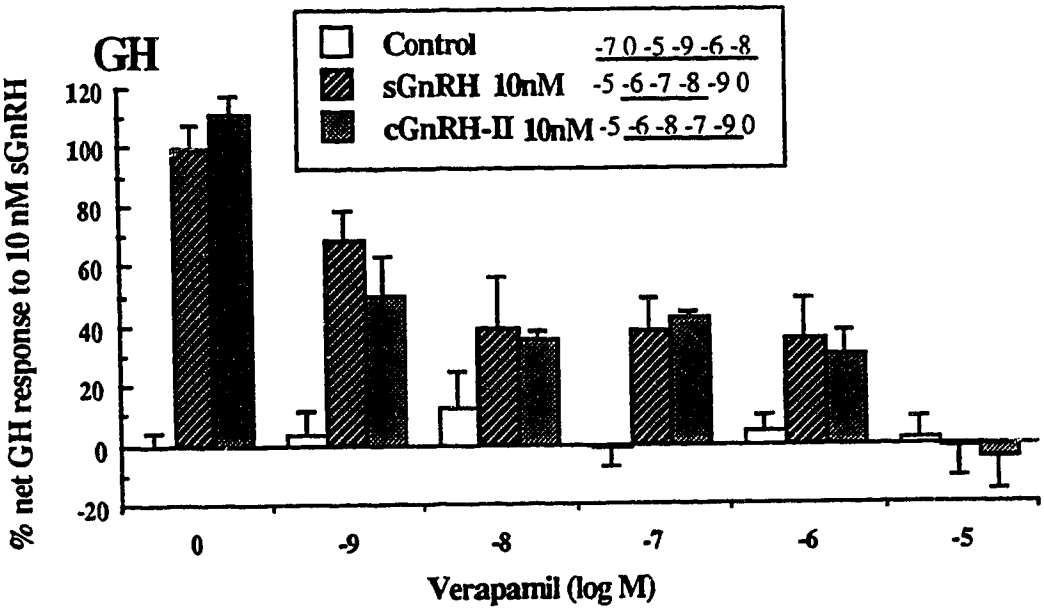

GH
Control
sGnRH 10nM
cGnRH-II 10nM
-7 0 -5 -9 -6 -8
-5 -6 -7 -8 -9 0
-5 -6 -8 -7 -9 0
% net GH response to 10 nM sGnRH
120
100
80
60
40
20
0
-20
0
-9
-8
-7
-6
-5
Verapamil (log M)

Figure 2.5. Effect of nifedipine on basal, sGnRH- (left) and cGnRH II-stimulated (right) GtH (upper panels) and GH (lower panels) release. GtH results presented (mean ± SE) are pooled data from three replicate experiments. GH results are data from one of the three replicate experiments. Treatments in individual experiments were carried out in quadruplicate. The average net GtH response to 10nM sGnRH and cGnRH II were 99.5 ± 13.8 and 133.4 ± 48.6 (ng/ml/0.25 million cells), respectively. In the inset, concentrations of nifedipine are arranged in ascending order according to the average GtH response; treatments not sharing the same underscore are statistically different from each other ($P<0.05$).

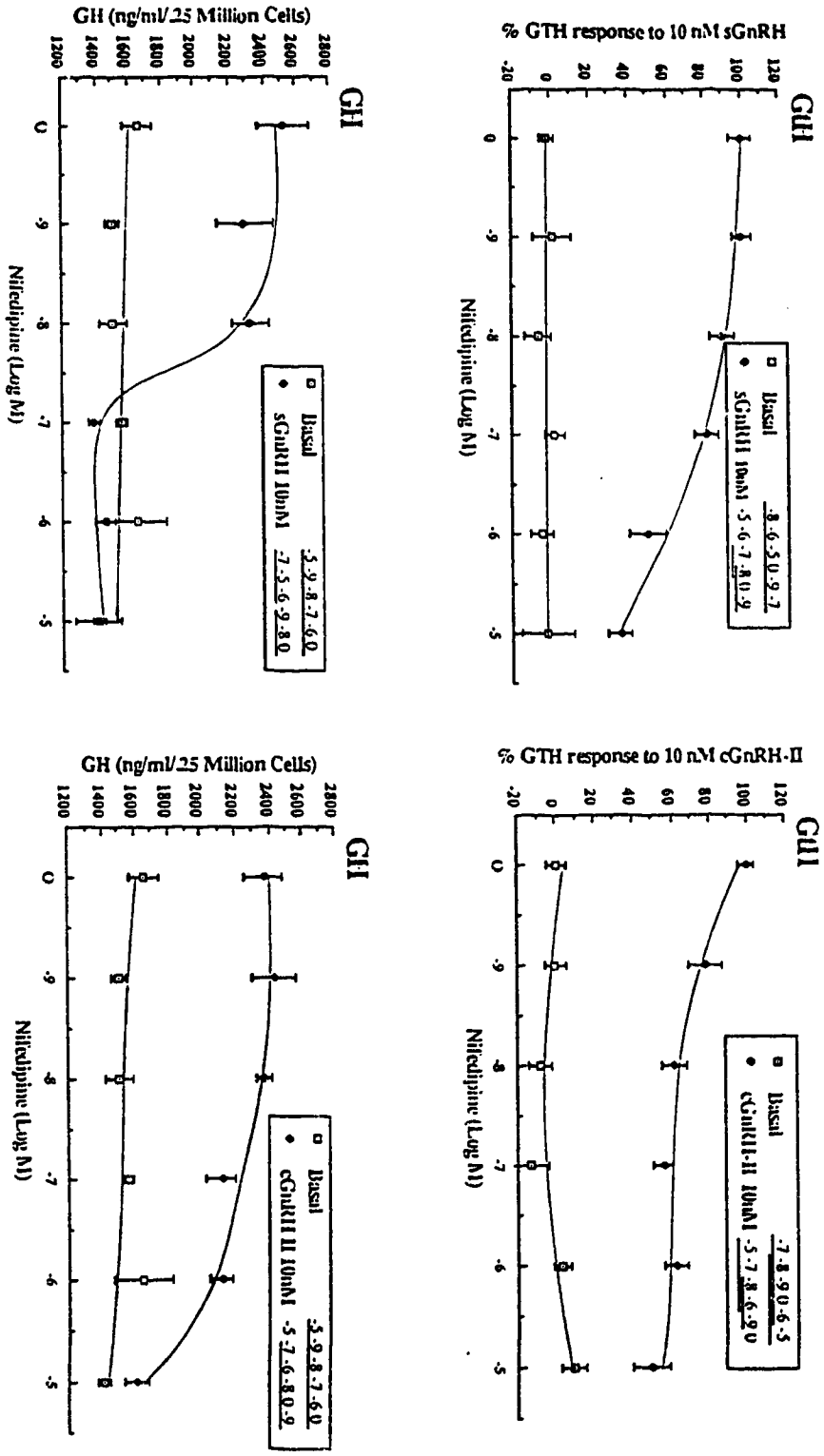
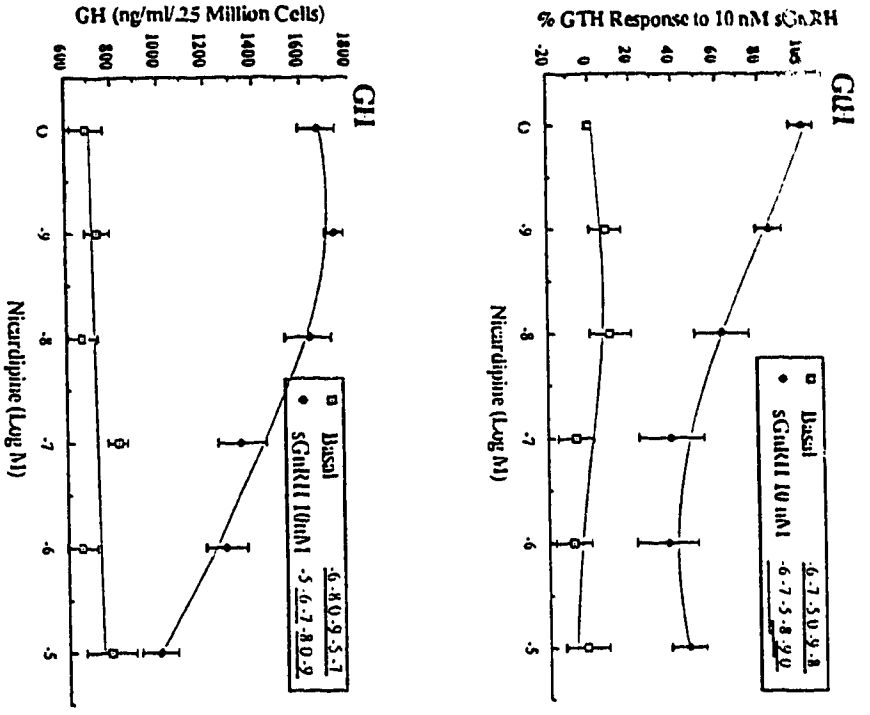
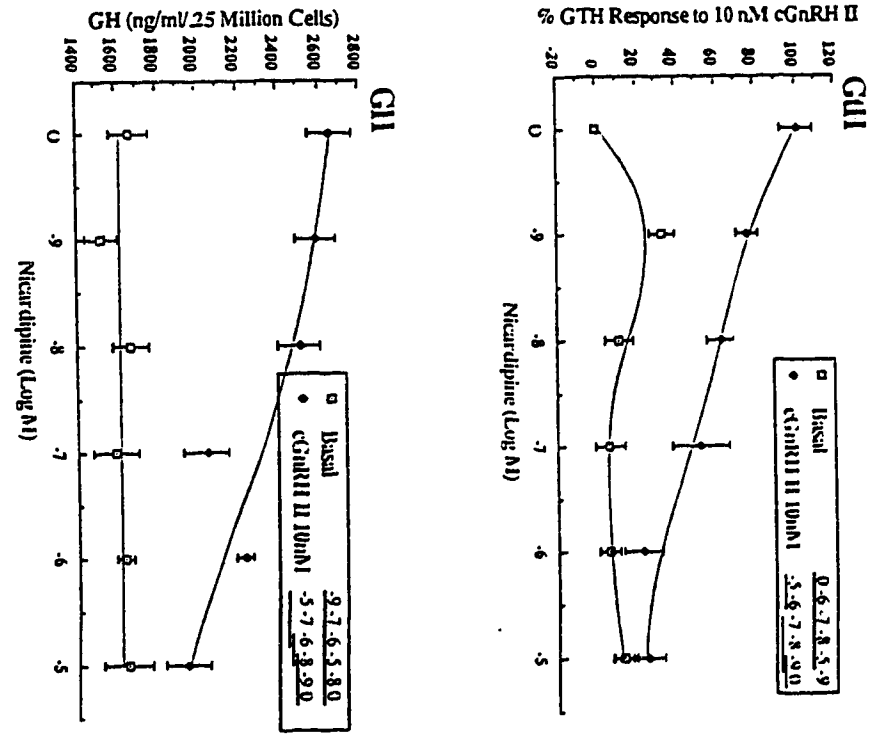

GtH
% GTH response to 10 nM sGnRH
Nitedipine (Log M)
Basal
sGnRH I 10nM
GtI
% GTH response to 10 nM cGnRH-II
cGnRH-II 10nM
GH
GH (ng/ml/.25 Million Cells)
sGnRH II 10nM
cGnRH II 10nM

Figure 2.6. Effect of nicardipine on basal, sGnRH- (left) and cGnRH II-stimulated (right) GtH (upper panels) and GH (lower panels) release from static incubations of dispersed pituitary cells. GtH results presented (mean ± SE) are pooled data from three replicate experiments. GH results are data from one of the three replicate experiments. Treatments in individual experiments were carried out in quadruplicate. The average net GtH response to 10nM sGnRH and cGnRH II were 100.2 ± 26.2 and 155.7 ± 48.6 (ng/ml/0.25 million cells), respectively. In the inset, concentrations of nicardipine are arranged in ascending order according to the average GtH response; treatments not sharing the same underscore are statistically different from each other ($P<0.05$).


% GTH Response to 10 nM sGnRH
GII
Basal
sGnRH II 10nM
Nicardipine (Log M)
GH (ng/ml/.25 Million Cells)
GH
Basal
sGnRH II 10nM
Nicardipine (Log M)


% GTH Response to 10 nM cGnRH II
GII
Basal
cGnRH II 10nM
Nicardipine (Log M)
GH (ng/ml/.25 Million Cells)
GII
Basal
cGnRH II 10nM
Nicardipine (Log M)

Chapter 3

Involvement of Extracellular Calcium in the Mediation of Acute and Sustained GnRH-Stimulated Gonadotropin Secretion in the Goldfish.

Introduction

In the goldfish, as in most teleosts, the secretion of gonadotropin (GtH) is under the stimulatory control of gonadotropin-releasing hormone (GnRH) [for review see Peter et al., 1]. The goldfish has two native GnRH peptides. These two GnRH forms, salmon GnRH (sGnRH) and chicken GnRH II (cGnRH II), have been found in the pituitary and throughout the rest of the goldfish brain [2]. Both forms are released and found in blood plasma [3, 4]. sGnRH and cGnRH II are both able to stimulate secretion of GtH *in vivo* and *in vitro* in the goldfish [4, 5]. In static incubation experiments, cGnRH II is often more effective than sGnRH in releasing GtH [Chapter 2, 6, 7]. In pituitary membrane preparations sGnRH and cGnRH II compete for the same class of high affinity/low capacity receptors [8, 9]. Electronmicroscope studies demonstrate that avidin gold-labelled biotinylated sGnRH analog is displaced from the surfaces of immunohistochemically identified gonadotropes by both unlabeled sGnRH and cGnRH II [10]. These results indicate that both native GnRH peptides bind to the same cell surface receptors to induce GtH secretion in the goldfish.

Signal transduction of GnRH-stimulated GtH release in the goldfish has been shown to involve extracellular Ca^{2+}, intracellular Ca^{2+}, protein kinase C, and arachidonic acid metabolism [7, 11, 12, 13]. Differences between signal transduction pathways utilized

by sGnRH and cGnRH II have been identified [7, 11, 13]. In particular, the sustained GtH response to cGnRH II appears to be more dependent on extracellular Ca^{2+} ($[Ca^{2+}]_o$) entry than is the response to sGnRH [Chapter 2, 7]. Furthermore, elevations of intracellular Ca^{2+} levels by sGnRH have a component that is independent of $[Ca^{2+}]_o$, this component is absent in cGnRH II-stimulated Ca^{2+} mobilization [Chapter 6, 11]. A difference in the abilities of inhibitors of the lipoxygenase enzyme to reduce sGnRH- and cGnRH II-stimulated GtH release has also been reported, suggesting that sGnRH, but not cGnRH II action involves arachidonic acid metabolism [13].

In perifusion studies, GnRH-induced GtH secretion in the goldfish, like luteinizing hormone (LH) release in the rat, is biphasic, consisting of a peak phase which is acute and of high magnitude and a plateau phase which is more sustained and of lower magnitude [14]. In the rat, these two phases show differences in their dependencies on signal transduction pathways [reviewed by Catt and Stojilkovic, 15]. Interestingly, $[Ca^{2+}]_i$ levels in rat gonadotropes mirror LH secretion profiles in a time and concentration dependent manner, suggesting that Ca^{2+} plays a crucial role in mediating GnRHs signal during both phases of LH release. In particular, $[Ca^{2+}]_o$ entry has been found to play roles in the mediation of GnRH-induced LH release in both the peak and plateau phases. However, mobilization of Ca^{2+} from intracellular pools appears to play a large role in the initiation of the LH response [reviewed by Stojilkovic and Catt, 16].

In the present study, the involvement of $[Ca^{2+}]_o$ during the peak and plateau phases of the acute sGnRH- and cGnRH II-stimulated GtH release was examined in the goldfish. To clearly elucidate the two phases of GnRH-stimulated GtH release a 1-minute fraction collection time was used in perifusion experiments as previously described [14]. To determine the involvement of $[Ca^{2+}]_o$, treatments which impede entry of $[Ca^{2+}]_o$ were used. These treatments included use of nifedipine, a dihydropyridine inhibitor of voltage-sensitive Ca^{2+} channels (VSCC), Ca^{2+}-deficient medium and Ca^{2+}-deficient medium

supplemented with $CoCl_2$.

Materials and Methods

General. Common goldfish (8-12 cm in length), purchased from Ozark Fisheries Inc., Stoutland, Missouri and Grassyforks Fisheries, Martinsville, Indiana, were transferred to flow-through aquaria (1800 liters) immediately on arrival. The fish were held at 17-20 ^{o}C on a simulated natural (Edmonton, Alberta) photoperiod, and fed to satiation daily with commercial fish food. Fish of both sexes were acclimated to the above conditions for at least 7 days before use. sGnRH and cGnRH II (Peninsula Lab. Inc., Belmont, CA) were dissolved in distilled deionized water. Nifedipine (Sigma, St. Louis, MO) was dissolved in ethanol. Aliquots of stock solutions were stored at -20 ^{o}C and diluted with testing medium just prior to use. Ca^{2+}-deficient medium was a formulation of medium 199 containing Hank's Salts, 25 mM Hepes, 2.2 g/l sodium bicarbonate, 100 000 units/l penicillin, 100 mg/l streptomycin, 0.1% bovine serum albumin with no added $CaCl_2$ (Gibco, Grand Island, NY-special order). $CoCl_2$ (Sigma, St. Louis, MO) was dissolved in Ca^{2+}-deficient medium. In all perifusion experiments, collected fractions were stored at -20 ^{o}C until their GtH contents were measured using an established radioimmunoassay [17].

Use of nifedipine and $CoCl_2$ in column perifusion with rapid fraction collection. Dispersed goldfish pituitary cells were prepared by trypsin/DNase treatment as previously described [18]. Dispersed cells were cultured on Cytodex-I beads and loaded onto perifusion columns as described by [19]. The mixture of cells and beads were perifused with testing medium (medium 199 containing Hank's Salts, (Gibco), 25 mM Hepes, 2.2 g/l sodium bicarbonate, 100 000 units/l penicillin, 100 mg/l streptomycin, 0.1% bovine

serum albumin). After 4 hours of perifusion with testing medium, a relatively low basal secretion rate was achieved and the experiment commenced with the collection of perifusate in 10-minute fractions. 30 minutes after commencement of the experiment, a 10-minute pulse of 100 nM of sGnRH or cGnRH II was applied (pre-pulse). During the GnRH application and for 20 minutes following GnRH treatment, 1-minute fractions of perifusate were collected, following which the collection interval was returned to 10 minutes. 1 hour after administration of the GnRH pulse the cells were perifused with medium containing 1 μM nifedipine. 1 hour after commencement of the nifedipine treatment another 10-minute GnRH pulse was given (test-pulse) in the presence of nifedipine during which time, fractions were again collected once per minute for the next 30 minutes. The fraction collection time was then returned to 10 minutes and the nifedipine treatment continued for an additional 30 minutes, making the entire duration of the treatment 2 hours. The cells were then perifused with normal testing medium for one hour following which a final 10-minute pulse of GnRH was given (post-pulse) while one-minute fractions were collected for the next 30 minutes. For the remainder of the experiment, the perifusion continued with normal testing medium and fractions were collected every 10 minutes. In control columns, the GnRH test-pulse was not given during the nifedipine treatment and both the pre-pulse and post-pulse were tested using sGnRH.

GtH response to sGnRH and cGnRH II treatment were divided into two phases, peak and plateau. The peak response was calculated as being the sum of the first 12 fractions of the GtH response to the GnRHs, while the plateau response was the sum of the subsequent 12 fractions. Total GtH response to GnRH was taken as the sum of the peak and plateau responses. The size of the responses was measured as the amount of GtH release above basal levels and expressed as a percentage of the total pre-pulse response elicited by the sGnRH pre-pulse in the control column. Basal release was calculated as the average release during the 5 fractions preceding and 5 fractions following the GtH response elicited by GnRH. A total of 3 nifedipine experiments (6 columns/ treatment) were performed and

the pooled results were presented.

The protocol for perifusion experiments conducted with 1.26 mM $CoCl_2$ was identical to that of the nifedipine perifusions, except that the cells were given 90 minute pretreatment with $CoCl_2$ before the test pulse was given, making the total treatment time with $CoCl_2$ 2.5 hours, instead of the 2-hour treatment used with nifedipine. A total of 3 cobalt experiments (6 columns/ treatment) were performed and the pooled results were presented.

Use of nifedipine in column perifusion of cell populations with enriched gonadotrope content. To enhance the absolute GtH responses, a perifusion experiment was performed using cell populations that were enriched with gonadotropes. Dispersed goldfish pituitary cells were separated by density using a discontinuous percoll density gradient. The protocol used was a modified version of that used by de Leeuw et al. [20]. Briefly, percoll (Pharmacia, Piscataway, NJ) was diluted with Dulbeco's Ca^{2+}-free phosphate buffered saline with 0.5% BSA so that solutions of 40, 50, 60, 70 and 80% percoll (by volume) were obtained. The percoll gradient was built in a 50-ml centrifuge tube and the dispersed cells were layered at the top of the gradient. The gradient and cells were centrifuged at 1400 x g for 25 minutes at 17 ^{o}C. Following centrifugation, a band of cells was seen at each density interface and one at the bottom of the tube. The 5th fraction, the band of cells found between the 80% and 70% percoll solutions, was enriched with gonadotropes and had approximately 2.5 times the concentration of gonadotropes as that found in mixed cell populations [21]. These cells were harvested, cultured onto Cytodex-I beads overnight and used for perifusion the following day as described by Chang et al., [19] using 1.35 million cells per column. The protocol used in these experiments was identical to that used in the nifedipine experiments conducted on mixed pituitary cell populations (see preceding section).

Use of Ca^{2+}-deficient medium in perifusion experiments with 5-minute fraction collection time. Due to the lack of observed differences in sGnRH and cGnRH II-

stimulated GtH release in previous experiments and the cost associated with collection and analysis of repeated rapid fraction collection studies, a 5-minute fraction collection time was used in studies which utilized Ca^{2+}-deficient medium. As a result of the slower fraction collection the GnRH-induced GtH responses were not divided into peak and plateau phases.

Cells were prepared as described by Chang et al., [18]. Populations of mixed pituitary cells were loaded onto columns and perifused for 4 hours to achieve a relatively low and stable rate of unstimulated GtH release. The experiment commenced with the collection of perifusate in 5-minute fractions. After 30 minutes a 5-minute pulse of 100 nM sGnRH or cGnRH II was administered. This was followed by the continued perifusion with normal testing medium for an additional 35 minutes. The cells were then perifused with Ca^{2+}-deficient medium for 75 minutes before being challenged with another 5-minute sGnRH or cGnRH II pulse, followed by an additional 35 minutes of exposure to the Ca^{2+}-deficient medium. The cells were then once again perifused with normal testing medium for 35 minutes, given a final 5-minute GnRH pulse and then perifused for an additional 35 minutes with normal testing medium. Control columns were not treated with Ca^{2+}-deficient medium, but were given three successive sGnRH pulses in the presence of continuous perifusion with normal testing medium. The magnitude of the GtH response was measured as the amount of GtH release above basal levels and expressed as a percentage of the total pre-pulse response elicited by sGnRH in control columns. Basal release was calculated as the average GtH release measured during the 3 fractions preceding and 3 fractions following the GtH response elicited by GnRH.

Results

Effects of $CoCl_2$ in perifusion. Applications of sGnRH or cGnRH II induced an acute GtH release response in perifusion studies with mixed populations of dispersed goldfish pituitary cells (Fig. 3.1). In the pre-pulse GtH responses to 10-minute pulses of sGnRH and cGnRH II, peak GtH values accounted for approximately 70% of the GtH release, while the remaining 30% was accounted for by the plateau phase (Fig. 3.2). No significant differences were observed between the sGnRH- and cGnRH II-induced GtH release, however the cGnRH II-stimulated total release was slightly higher than that stimulated by sGnRH (Fig. 3.2).

$CoCl_2$ (1.26 mM) was added to Ca^{2+}-deficient medium to maintain the osmolarity of the medium and to compete with the remaining free Ca^{2+} in the medium. The effects of perifusion with $CoCl_2$ on basal GtH release were examined in the control columns. Initial exposure to $CoCl_2$-containing medium resulted in a transient increase in the release of GtH (Fig. 3.1). This was followed by a decrease in basal GtH release to levels below those observed prior to $CoCl_2$ treatment. Upon return to perifusion with normal medium, no recovery of basal secretion rate was observed. Prior exposure to $CoCl_2$ also decreased the subsequent GtH response to GnRH (compare pre-pulse and post-pulse values, Fig. 3.2). In the post-pulse response in control columns, the plateau phase was absent.

The sGnRH- and cGnRH II-stimulated GtH responses were also tested in the presence of $CoCl_2$. Treatment with $CoCl_2$ significantly reduced both the sGnRH- and cGnRH II-induced GtH response (Fig. 3.1). The peak as well as the total GtH responses were all greatly reduced by $CoCl_2$ as compared to the corresponding pre-pulse values (Fig. 3.2). In addition, the plateau responses to sGnRH and cGnRH II were abolished in the presence of $CoCl_2$. During the same time interval as these test responses were measured, no significant GtH-responses were observed in the control columns (Fig. 3.2, lower panel). This clearly indicated that the reduction in GnRH-induced responses in the presence of $CoCl_2$ was not an artifact of the mathematical technique used to assess the size of the GtH responses.

In columns receiving sGnRH or cGnRH II test-pulses during $CoCl_2$ treatment, the post pulse GtH responses to these GnRHs were also reduced compared to the corresponding prepulse responses. This occurred despite a slight although not significant recovery in both the peak and total GtH responses. However, the post-pulse GtH responses in these sGnRH and cGnRH II test columns were not significantly different from those observed in the control columns. Noticeably, the plateau response was absent in all test and post-pulse GtH responses.

Effects of nifedipine on populations of mixed pituitary cells. As was observed in the previous section sGnRH- and cGnRH II-stimulated GtH release during the pre-pulse (Fig. 3.3), the peak and plateau GtH values also accounted for approximately 70% and 30% of the total GnRH response, respectively (Fig. 3.4). There were no significant differences between the sGnRH and cGnRH II-stimulated GtH responses but the cGnRH II response was slightly larger than that elicited by sGnRH (Fig. 3.4).

Exposure to nifedipine (an inhibitor of VSCC) alone caused a transitory increase in GtH release followed by a decrease in basal release rate. Following perifusion with nifedipine, the post-pulse GnRH-induced response in the control columns were also decreased compared to the control pre-pulse response (Fig. 3.3). Nifidipine also attenuated the total GtH released by the sGnRH and cGnRH II test-pulses (Fig. 3.3). Both the peak and plateau phases of GtH release were reduced in the presence of nifedipine (Fig. 3.4). Following nifedipine application, there was an apparent slight recovery of sGnRH and cGnRH II responses in the post-pulse response (Fig. 3.3). Although there appeared to be a recovery of the plateau phase, no significant differences between the post-pulse and test-pulse responses could be demonstrated (Fig. 3.4). The post-pulse GtH release characteristics in the sGnRH and cGnRH II test columns were not different from responses observed in control columns (Fig. 3.4).

Effect of nifedipine on populations of cells enriched with gonadotropes. The cell

enrichment technique was adopted to increase the absolute size of the GnRH-induced GtH response so that any differences between the sGnRH- and cGnRH II-induced responses, as well as the effects of nifedipine on these response could be more easily discerned. The GtH response elicited by GnRH was approximately 4 times greater from the populations of cells enriched in gonadotrope contents than in populations of mixed pituitary cells (Fig. 3.5 versus Figs. 3.1 and 3.3). In control columns, the GtH release stimulated by exposure to nifedipine alone was of lower magnitude and longer duration than that observed in populations of mixed cells (Fig. 3.5 versus Fig. 3.3). No decrease in basal GtH release was apparent (Fig. 3.5). In these enriched gonadotrope cells populations the plateau phase constituted a larger part (40%) of the GnRH stimulated GtH release with peak GtH release accounting for approximately 60% of the GtH response (Fig. 3.6).

In general, the effects of nifedipine treatment on the GnRH-induced GtH responses in gonadotrope enriched cells were similar to those obtained from populations of mixed pituitary cells. No obvious differences were seen between GtH secretion elicited by sGnRH and cGnRH II (Figs. 3.5 and 3.6A&B). Since the data from enriched cells represent only 1 experiment (2 columns per treatment) the data obtained from stimulation with sGnRH and cGnRH II were pooled before statistical analysis was carried out (Fig. 3.6D). The pooled data showed an attenuation of the peak, plateau and total GnRH-stimulated GtH release in the presence of nifedipine (Fig. 3.6D). No significant increase in the release of GtH was recorded during the test-pulse collection time period in the control columns, suggesting that the technique used for estimation of the magnitude of GtH response was accurate (Fig. 3.6, lower left hand panel).

Effects of the use of Ca^{2+}-deficient medium. Due to the lack of observed differences in sGnRH and cGnRH II-stimulated GtH release in the previous experiments with nifedipine and $CoCl_2$, and the cost associated with collection and analysis of repeated rapid fraction collection studies, a 5-minute fraction collection time was used in these studies

which utilized Ca^{2+}-deficient medium. As a result of the slower fraction collection the GnRH-induced GtH responses were not divided into peak and plateau phases. A summary of the results obtained from these perifusions is presented in Figure 3.7.

Both sGnRH- and cGnRH II-stimulated GtH release were greatly attenuated by treatment with Ca^{2+}-deficient medium (Fig. 3.7). Interestingly, with return to normal medium, there was a 100% and nearly 100% recovery of the cGnRH II- and sGnRH-stimulated response during the post-pulse GtH release, respectively (Fig. 3.7). In control columns, repeated administration of sGnRH (pre-, test and post-pulse) in the presence of normal testing medium resulted in GnRH-stimulated GtH release which showed no signs of desensitization (Fig. 3.7).

As seen in other treatments that were designed to impede $[Ca^{2+}]_o$ entry into cells, exposure of cells to Ca^{2+}-deficient medium also lead to a transient increase in the release of GtH followed by a decrease in basal GtH release (see Chapter 7 for more details). However, in contrast to the effects of nifedipine and $CoCl_2$, there was a partial recovery of basal GtH release rate in cells treated with Ca^{2+}-deficient medium upon return to normal testing medium (data not shown).

Discussion

The present results confirm the biphasic nature of the acute GnRH-stimulated GtH secretion in the goldfish [14]. The peak and plateau phases generated by a 10-minute administration of sGnRH or cGnRH II in mixed populations of dispersed goldfish pituitary cells were found to be 70% and 30% of the total GtH response, respectively. Similarly, in cell preparations enriched with gonadotropes, the peak and plateau phases accounted for 60 and 40 % of the total response, respectively. Treatment with $CoCl_2$ or nifedipine

attenuated both the peak and plateau phases. Both of these treatments, and the use of Ca^{2+}-deficient medium caused a significant reduction of total GtH response elicited by both native GnRH peptides of the goldfish. These data suggest that entry of $[Ca^{2+}]_o$, in part through VSCC, into cells is involved in the acute release of GtH stimulated by sGnRH and cGnRH II in the goldfish. These data support and further extend previous findings from static culture experiments indicating the involvement of VSCC in mediating prolonged (2 hr) GnRH-induced GtH release [7; Chapter 2]. In the present study, both the peak and plateau phases of the acute GtH release-response showed a dependence on $[Ca^{2+}]_o$. The plateau phase of GtH release was particularly dependent on $[Ca^{2+}]_o$ entry, as shown by the abolition of this phase of the GtH response by treatment with $CoCl_2$. The $[Ca^{2+}]_o$ independent portion of the peak phase may be due to the release of Ca^{2+} from intracellular stores [11; Chapters 2&6], arachidonic acid metabolism [13], or activation of PKC (Chapter 4). Release of Ca^{2+} from intracellular stores and arachidonic acid metabolism appear to be only involved in sGnRH-stimulated GtH release [11, 13]. However, PKC may act as the initiator of both sGnRH- and cGnRH II-stimulated GtH release (Chapter 7). Furthermore, activation of PKC appears to be involved in both phases of GtH release (Chapter 4) and GtH release stimulated by activators of PKC is partially independent of the presence of $[Ca^{2+}]_o$ [11, Chapter 6].

These results are also similar to those from studies on rat gonadotropes. In the rat model system the peak and plateau phases of GnRH-stimulated GtH secretion show some degree of dependence on entry of $[Ca^{2+}]_o$ with the plateau phase being more dependent on $[Ca^{2+}]_o$ than the peak phase. Part of the $[Ca^{2+}]_o$ entering the cells does so through VSCCs [reviewed by Catt and Stojilkovic, 15].

In this study, the recovery of GnRH-stimulated GtH release during the post-pulse was often incomplete, especially in the $CoCl_2$- or nifedipine-treated columns, This may be due to $CoCl_2$ or nifedipine being absorbed by the Cytodex-I beads during the testing period and then being released slowly during the remainder of the experiment. This would result in

the continued inhibition of GnRH action. This idea is supported by several observations. Prior exposure to $CoCl_2$ or nifedipine alone also decreased the GtH response to GnRH in the post-pulse period; in contrast, no such inhibitory effects were observed with treatments with Ca^{2+}-deficient medium alone. Furthermore, cells challenged with GnRH during treatments with with Ca^{2+}-deficient medium showed a much more complete recovery during subsequent GnRH challenge in normal medium. Treatments with Ca^{2+}-deficient medium did not include an inhibitor or competitor of Ca^{2+} entry and would not have a substantial residual inhibitory effect on GnRH action as as would the subsequent re-release of $CoCl_2$ or nifedipine from the Cytodex-I beads. In static culture relatively low doses of $CoCl_2$ and nifedipine significantly reduced sGnRH- and cGnRH II-stimulated GtH responses [7; Chapter 2]. In future studies, perhaps omission of the Cytodex-I beads and use of extra filter paper to more securely contain the dispersed cells would alleviate these problems.

The experimental results obtained using dispersed goldfish pituitary cells and cell populations with enriched gonadotrope contents are qualitatively similar. However, differences in the absolute size and relative proportions of the peak and plateau phases of the GnRH-stimulated GtH secretion were observed between experiments conducted these two types of cell with populations (Figs. 3.5 and 6). The difference in absolute size of the GnRH-stimulated GtH release might be partially due to the increase in the concentration of gonadotropes , which was roughly 2.5 times greater in the enriched cell populations than in mixed cell populations [21]. However, this increase in gonadotrope concentration cannot fully account for the observed differences in the GtH responses. Despite the fact that fewer cells were used (1.35 vs 2 million; an increase of 1.7 times the number of gonadotropes as in mixed cell populations), the GtH response was more than 4 times greater in the enriched cells (Fig. 3.5). The larger than expected GtH response and difference in relative sizes of the peak and plateau phases of the GtH response suggest that a particular subset of

gonadotropes was isolated by the cell enrichment process. The GtH release properties of these gonadotropes isolated by enrichment appear to be slightly different from those observed from mixed cell populations.

In summary, the present study provides evidence for the involvement of $[Ca^{2+}]_o$ entry in the mediation of acute GtH release stimulated by both native GnRH peptides of the goldfish. This entry of Ca^{2+} is at least partially dependent on the activation of VSCCs. The plateau phase of GtH release is heavily if not totally dependent on $[Ca^{2+}]_o$ entry. This entry of Ca^{2+} may be required for the continued stimulation of PKC, which has been shown to be involved in the mediation of this phase of the GtH response (Chapter 4). The peak phase is partially dependent on $[Ca^{2+}]_o$ entry with the remaining signal transduction probably being accounted for by other signal transduction pathways such as, release of Ca^{2+} from intracellular stores, arachidonic acid metabolism, and activation of PKC.

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Figure 3.1. Effects of $CoCl_2$ on sGnRH- and cGnRH II-stimulated GtH release in perifusion. Horizontal bar indicates the administration of 1.26 mM $CoCl_2$. Arrow heads indicate beginning of a ten-minute application of 100 nM sGnRH (A) or cGnRH II (B). In the control columns (C) no GnRH was added during the $CoCl_2$ treatment and sGnRH was used before and after the treatment. Representative data from one of three experiments with similar results are shown. All data are expressed as mean ± SE (n=2).

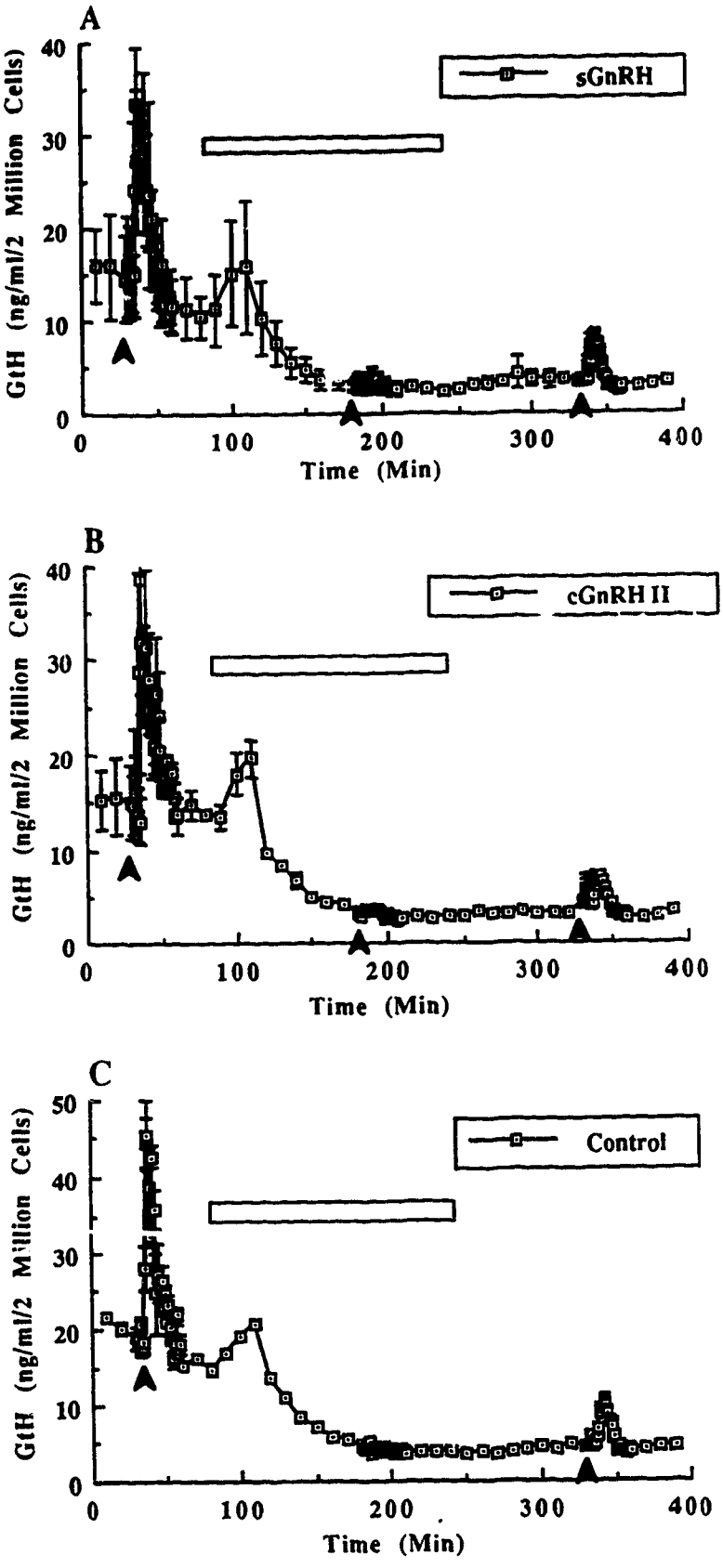

A
sGnRH
GtH (ng/ml/2 Million Cells)
Time (Min)
B
cGnRH II
GtH (ng/ml/2 Million Cells)
Time (Min)
C
Control
GtH (ng/ml/2 Million Cells)
Time (Min)

Figure 3.2. Summarized results on the effects of $CoCl_2$ on sGnRH- and cGnRH II-stimulated GtH release in perifusion. The GtH responses to the three GnRH pulses, before (pre-pulse), during (test-pulse) and after (post-pulse) 1.26 mM $CoCl_2$ treatment, were divided into peak and plateau phases. The magnitude of these two phases as well as the total GtH response were compared using analysis of variance followed by Fisher's least significant difference test. Values not sharing the same underscore are significantly different from each other ($p<0.05$). GtH values were expressed as a percentage of the total GtH response to pre-pulse GnRH (156.73 ± 29.37) in the control columns. Data presented represent pooled results of 3 separate experiments (mean±SE, n=6).

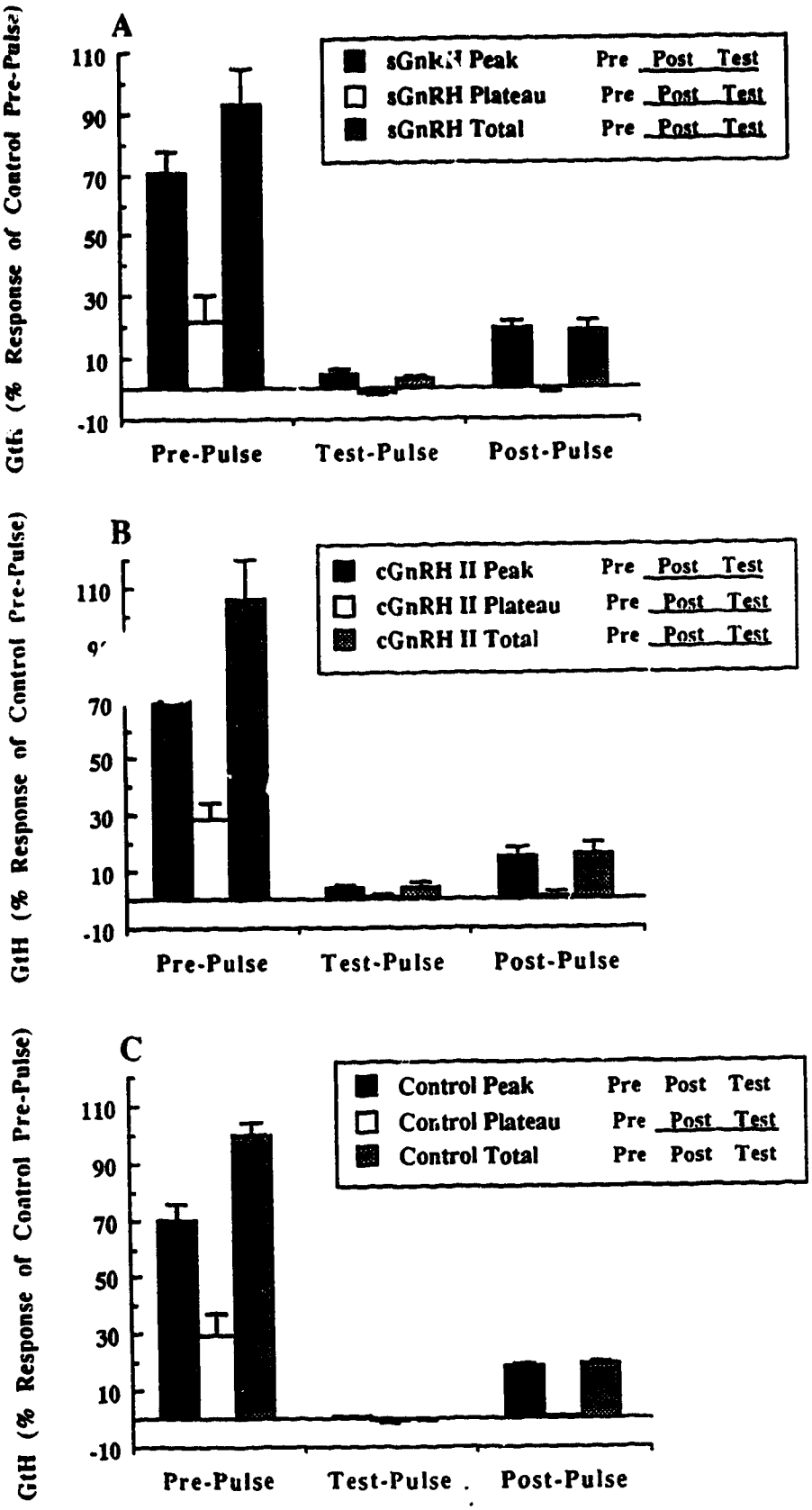

A
sGnRH Peak Pre Post Test
sGnRH Plateau Pre Post Test
sGnRH Total Pre Post Test
GtH (% Response of Control Pre-Pulse)
110
90
70
50
30
10
-10
Pre-Pulse
Test-Pulse
Post-Pulse
B
cGnRH II Peak Pre Post Test
cGnRH II Plateau Pre Post Test
cGnRH II Total Pre Post Test
GtH (% Response of Control Pre-Pulse)
110
70
50
30
10
-10
Pre-Pulse
Test-Pulse
Post-Pulse
C
Control Peak Pre Post Test
Control Plateau Pre Post Test
Control Total Pre Post Test
GtH (% Response of Control Pre-Pulse)
110
90
70
50
30
10
-10
Pre-Pulse
Test-Pulse
Post-Pulse

Figure 3.3. Effects of nifedipine on sGnRH- and cGnRH II-stimulated GtH release in perifusion. Horizontal bar indicates the administration of 1 μM nifidipine. Arrow heads indicate beginning of a ten-minute application of 100 nM sGnRH (A) or cGnRH II (B). In the control columns (C) no GnRH was added during the nifedipine treatment and sGnRH was used before and after the treatment. Representative data from one of three experiments with similar results are shown. All data are expressed as mean ± SE (n=2).

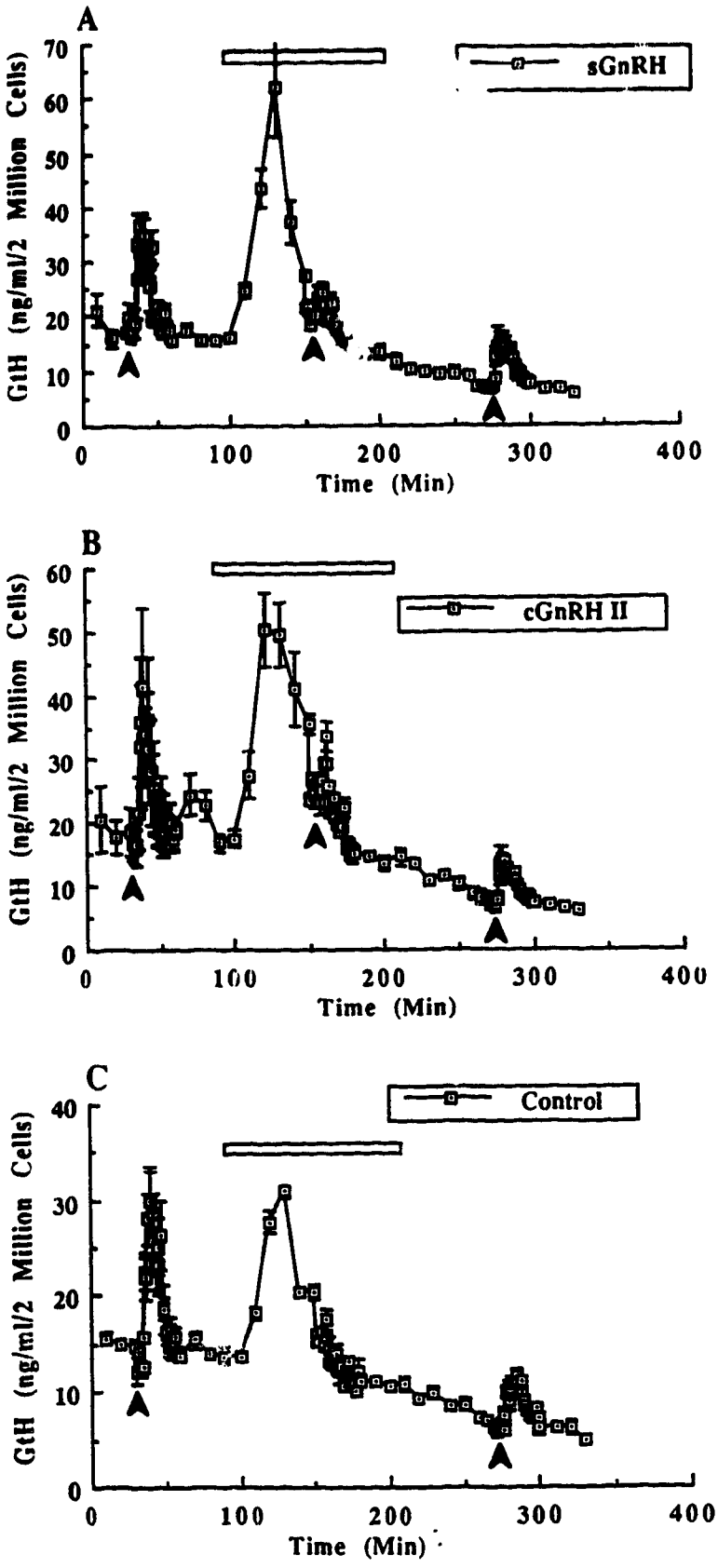

A
sGnRH
GtH (ng/ml/2 Million Cells)
Time (Min)
B
cGnRH II
GtH (ng/ml/2 Million Cells)
Time (Min)
C
Control
GtH (ng/ml/2 Million Cells)
Time (Min)

Figure 3.4. Summarized results on the effects of nifedipine on sGnRH- and cGnRH II-stimulated GtH release in perifusion. The GtH responses to the three GnRH pulses, before (pre-pulse), during (test-pulse) and after (post-pulse) 1 μM nifedipine treatment, were divided into peak and plateau phases. The magnitude of these two phases as well as the total GtH response were compared using analysis of variance followed by Fisher's least significant difference test. Values not sharing the same underscore are significantly different from each other ($p<0.05$). GtH values were expressed as a percentage of the total GtH response to pre-pulse GnRH (163.74 ± 34.06) in the control columns. Data presented represent pooled results of 3 separate experiments (mean ± SE, n=6).

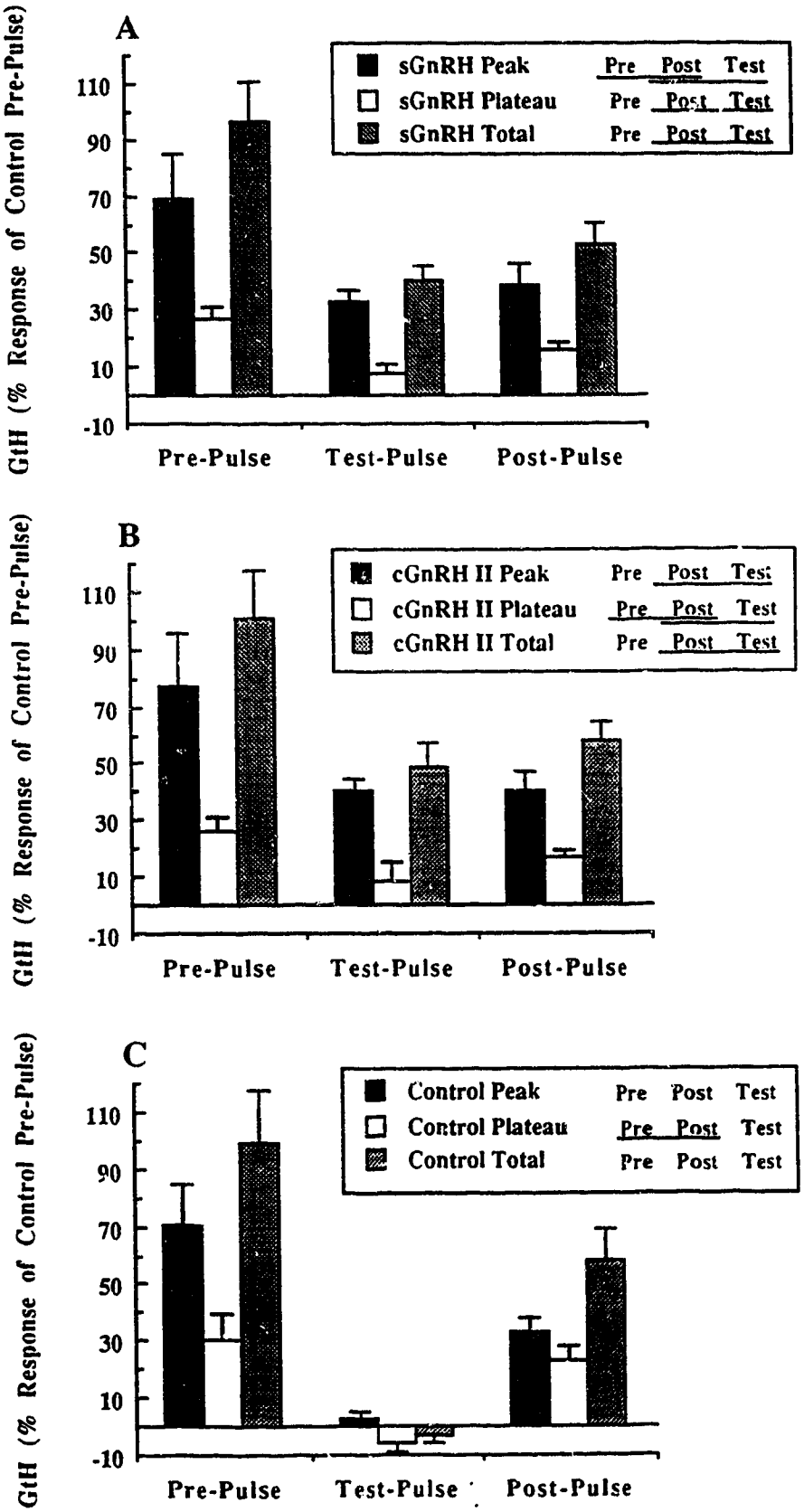

A
sGnRH Peak
sGnRH Plateau
sGnRH Total
Pre Post Test
GtH (% Response of Control Pre-Pulse)
110
90
70
50
30
10
-10
Pre-Pulse
Test-Pulse
Post-Pulse
B
cGnRH II Peak
cGnRH II Plateau
cGnRH II Total
Pre Post Test
GtH (% Response of Control Pre-Pulse)
110
90
70
50
30
10
-10
Pre-Pulse
Test-Pulse
Post-Pulse
C
Control Peak
Control Plateau
Control Total
Pre Post Test
GtH (% Response of Control Pre-Pulse)
110
90
70
50
30
10
-10
Pre-Pulse
Test-Pulse
Post-Pulse

Figure 3.5. Effects of nifedipine on sGnRH- and cGnRH II-stimulated GtH release in perifusion of pituitary cell populations enriched with gonadotropes. Horizontal bar indicates the administration of 1 μM nifidipine. Arrow heads indicate beginning of a ten-minute application of 100 nM sGnRH (A) or cGnRH II (B). In the control columns (C) no GnRH was added during the nifedipine treatment and sGnRH was used before and after the treatment. Representative data from one or two experiments with similar results are shown. All data are expressed as mean ± SE (n=2).

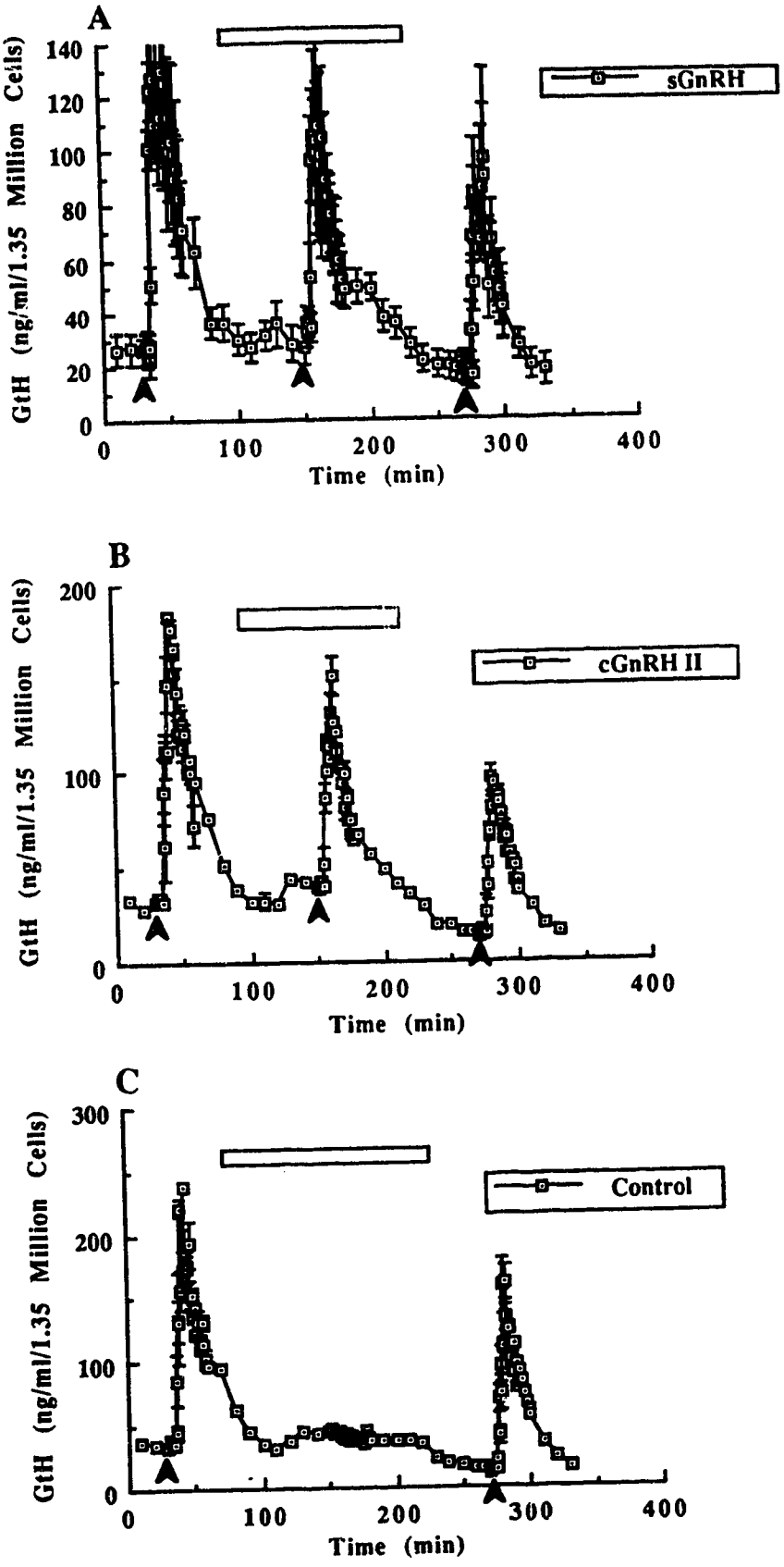

A
GtH (ng/ml/1.35 Million Cells)
140
120
100
80
60
40
20
0
0
100
200
300
400
Time (min)
sGnRH
B
GtH (ng/ml/1.35 Million Cells)
200
100
0
0
100
200
300
400
Time (min)
cGnRH II
C
GtH (ng/ml/1.35 Million Cells)
300
200
100
0
0
100
200
300
400
Time (min)
Control

Figure 3.6. Summarized results on the effects of nifedipine on sGnRH- (A) and cGnRH II-stimulated (B) as well as unstimulated (C) GtH release in perifusion of pituitary cell enriched with gonadotropes. The GtH responses to the three GnRH pulses, before (pre-pulse), during (test-pulse) and after (post-pulse) nifedipine treatment, were divided into peak and plateau phases. When the results from sGnRH and cGnRH II were pooled (D), the magnitude of the two phases as well as the total GtH response were compared using analysis of variance followed by Fisher's least significant difference test. Values not sharing the same underscore are significantly different from each other ($p<0.05$). GtH values were expressed as a percentage of the total GtH response to pre-pulse GnRH (511.93 ± 2.13) in the control columns (mean $\pm$ SE, n=4).

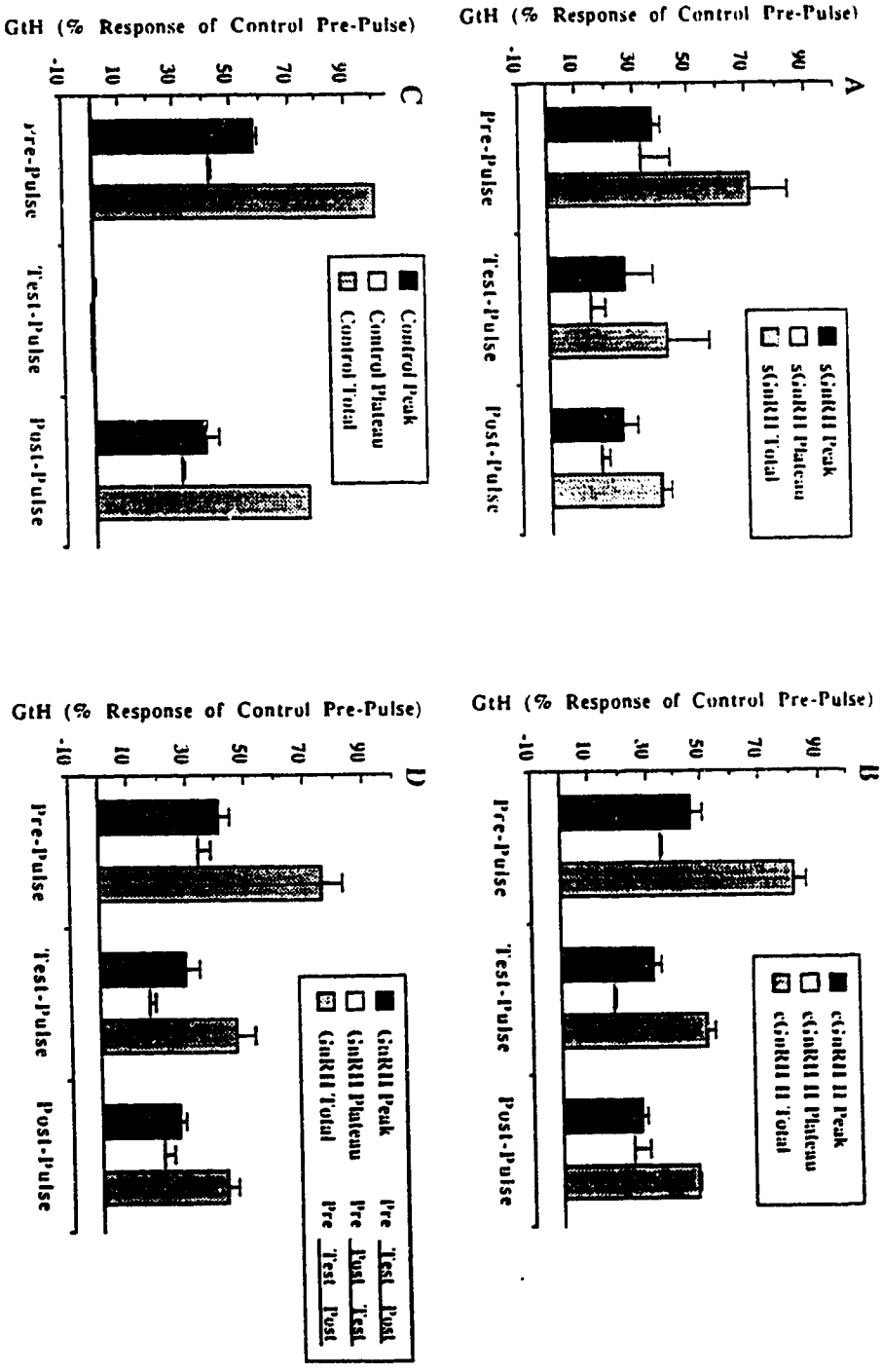
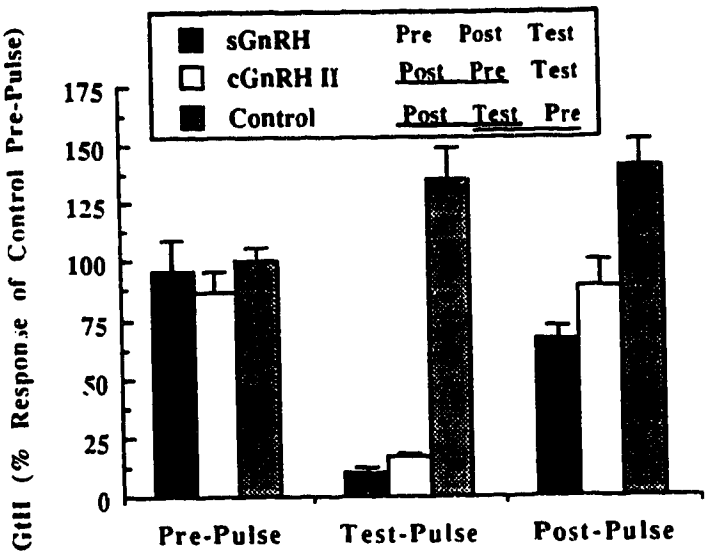

GtH (% Response of Control Pre-Pulse)
A
sGtnRH I Peak
sGtnRH I Plateau
sGtnRH I Total
Pre-Pulse
Test-Pulse
Post-Pulse
GtH (% Response of Control Pre-Pulse)
C
Control Peak
Control Plateau
Control Total
Pre-Pulse
Test-Pulse
Post-Pulse
GtH (% Response of Control Pre-Pulse)
B
cGtnRH II Peak
cGtnRH II Plateau
cGtnRH II Total
Pre-Pulse
Test-Pulse
Post-Pulse
GtH (% Response of Control Pre-Pulse)
D
GtnRH Peak Pre Test Post
GtnRH Plateau Pre Post Test
GtnRH Total Pre Test Post
Pre-Pulse
Test-Pulse
Post-Pulse

Figure 3.7. Summarized results on the effects of Ca^{2+}-deficient medium on sGnRH- and cGnRH II-stimulated GtH release in perifusion. In control colums, three 100 nM sGnRH pulses were administered in normal Ca^{2+} containing medium. In the sGnRH or cGnRH II test columns, the second of the three 100 nM GnRH pulses (test-pulse) were applied during perifusion with Ca^{2+}-deficient medium. The GtH responses to the three GnRH pulses, before (pre-pulse), during (test-pulse) and after (post-pulse) Ca^{2+}-deficient medium treatment, were compared using analysis of variance followed by Fisher's least significant difference test. Values not sharing the same underscore are significantly different from each other ($p<0.05$). GtH values were expressed as a percentage of the total GtH response to pre-pulse sGnRH (72.36 ± 8.90). Data presented represent pooled results of 3 separate experiments (mean ± SE, n=6).


sGnRH
cGnRH II
Control
Pre Post Test
Post Pre Test
Post Test Pre
GtH (% Response of Control Pre-Pulse)
175
150
125
100
75
50
25
0
Pre-Pulse
Test-Pulse
Post-Pulse

Chapter 4

Down Regulation of PKC Levels Leads to Inhibition of GnRH-Stimulated Gonadotropin Secretion From Dispersed Pituitary Cells of Goldfish.[2]

Introduction

In the goldfish (*Carassius auratus*), as well as many other teleosts, the secretion of gonadotropin (GtH) is under the stimulatory control of gonadotropin releasing hormone (GnRH) that originates from the hypothalamus [for review see 1]. The goldfish possesses two different forms of GnRH, these being [Trp^7, Leu^8]-GnRH (salmon GnRH, sGnRH) and [His^5, Trp^7, Tyr^8]-GnRH (chicken GnRH II, cGnRH II) [2]. These native GnRH forms have been found in the pituitary and throughout the rest of the brain of the goldfish [2]. Both forms are released from hypothalamic and pituitary preparations and are effective in eliciting GtH responses from goldfish *in vivo* and *in vitro* [3, 4, 5]. Signal transduction mechanisms responsible for mediating GnRH-stimulated GtH secretion in the goldfish are currently under investigation.

In the rat, which has been extensively used as a mammalian model system, the second messenger systems mediating luteinizing hormone (LH) secretion elicited by GnRH have been more thoroughly studied than those of the goldfish. In the rat model system, extracellular Ca^{2+}, intracellular Ca^{2+}, calmodulin, PKC and inositol phosphates have been

[2]A version of this chapter has been submitted for publication. Jobin, Ginsberg, Matowe and Chang. Neuroendocrinology, November 4th, 1992.

implicated in the mediation of LH secretion elicited by GnRH [6, 7, 8]. However, there has been some controversy over the involvement of PKC in the LH secretory response to GnRH. Although stimulators of PKC mimic the LH releasing and Ca^{2+} mobilizing abilities of GnRH [9, 10] and inhibitors of PKC have been shown to antagonize GnRH actions [11], experiments based on the down regulation of PKC levels by pretreatment with TPA have yielded conflicting results. While some researchers found that reduction of PKC levels by TPA pretreatment caused an attenuation of GnRH-stimulated LH release [12, 13], others found that this treatment had no effect on LH secretion [14]. Additionally, in column perifusion experiments, although PKC activity was found to increase during initial GnRH stimulation of LH secretion, PKC activity dropped to basal levels despite repeated subsequent GnRH applications and continued stimulation of LH secretion. These results suggest an uncoupling of the PKC pathway and LH secretion [15].

In the goldfish, several lines of evidence support the hypothesis that the PKC pathway participates in the mediation of GtH secretion elicited by GnRH. Activators of PKC (TPA and DiC8) and both forms of native GnRH stimulate secretion of GtH in an extracellular Ca^{2+} dependent manner [5, 16]. TPA, DiC8, sGnRH and cGnRH II also elevate levels of intracellular Ca^{2+} from dispersed cultured pituitary cells [17]. Furthermore, inhibitors of PKC (H7, staurosporine) attenuate both the GtH secretory response and the increases in intracellular Ca^{2+} levels elicited by either a PKC activator (TPA) or GnRH (sGnRH and cGnRH II) [17, 18].

To further examine the role of PKC in the GtH response to GnRH in the goldfish, we have studied the effects of GnRH and PKC activators on pituitary cells that were pretreated with TPA to deplete PKC levels. GtH release responses were monitored under static incubation and perifusion conditions to examine the effects of PKC-down regulation on prolonged and acute GtH secretion, respectively. If PKC plays a role in GnRH-stimulated GtH release in the goldfish, then a reduction in the ability of GnRH to elicit a GtH response should be observed in the cells with reduced PKC levels. Stimulation of

GtH release with the adenylate cyclase stimulator forskolin was utilized to assess the viability of the cells and the size of the releasable pool of GtH. The cAMP pathway does not appear to be involved with GnRH action on GtH release; no detectable elevation of cAMP levels were observed during GnRH stimulation of GtH secretion [19].

Materials and Methods

General. Common goldfish (8-12 cm in length), purchased from Ozark Fisheries Inc., Stoutland, Missouri and Grassyforks Fisheries, Martinsville, Indiana, were transferred to flow-through aquaria (1800 liters) immediately on arrival. The fish were held at 17-20 ^{o}C on a simulated natural (Edmonton, Alberta) photoperiod, and fed to satiation daily with commercial fish food. Fish of both sexes were acclimated to the above conditions for at least 7 days before use. sGnRH and cGnRH II (Peninsula Lab. Inc., Belmont, CA) were dissolved in distilled deionized water. TPA, forskolin (Sigma, St. Louis, MO) and DiC8 (Calbiochem, San Diego, Ca) were dissolved in dimethylsulfoxide. Ionomycin (Calbiochem, San Diego, Ca.) was dissolved in ethanol. Aliquots of stock solutions were stored at -20 ^{o}C until used.

Dispersed goldfish pituitary cells were used in all experiments in this study. Pituitaries from fish of both sexes were removed and the cells dispersed with a trypsin/DNAse procedure as previously described [20]. Dispersed pituitary cells were cultured overnight prior to all experiments. GtH released and cellular GtH contents were quantified using a radioimmunoassay validated for maturational GtH (GtH II) [21, 22].

PKC immunoblots. Dispersed goldfish pituitary cells were cultured overnight in petri dishes (5 million cells/dish) as previously described for cells being prepared for loading with Fura 2 dye [17]. The cells were exposed to 10 nM TPA for varying amounts of time (0- 240 min). After TPA exposure, 10 million cells from each treatment were

harvested. The cells were then homogenized in a buffer containing 10% NP_4O and centrifuged (100 000 g for 45 min.) for extraction of protein. The supernatant was then subjected to SDS-PAGE (stacking gel 3% acrylamide, separating gel 10 % acrylamide) overnight at 7.5 mA and 200 volts. The following day the protein was transferred to a nitrocellulose membrane at 220 mA and 250 volts for 5 hours. The PKC bands were then detected using a monoclonal antibody (MC5, Amersham, Il), that recognized α and β forms of PKC, at a 1:500 dilution. The PKC was then visualized using a second antibody (mouse Ig from sheep, Amersham, Il) at a 1:500 dilution and a chemiluminescent system (Amersham, Il).

TPA-desensitization in static culture. For static incubation studies, dispersed goldfish pituitary cells were cultured in 24-well plates as previously described [20]. The experimental protocol for this section is summarized in Figure 4.1. Cells were incubated for 4 hours during the pretreatment period with testing medium in the absence (control) or presence (TPA-pretreated group) of 10 nM TPA. The medium from the pretreatment period was then collected and replaced with fresh testing medium for a 1 hour rest period. Medium from the rest period was collected and replaced with fresh testing medium for a 2 hour test period. During the testing period the cells were exposed to 10 nM sGnRH, 10 nM cGnRH II, 10 nM TPA, 100 μM DiC8, 10 μM ionomycin or testing medium alone. All treatments were performed in triplicate. Following the test period, medium was again collected. The cells were then lysed using 1 ml distilled water, freezing and thawing, after which the hormone contents (cell contents) were assessed. For each individual treatment, total hormone measurable was calculated by adding the amount of hormone released during the pretreatment, rest and test periods as well as the remaining cell contents. Data from two individual experiments were pooled and statistical analysis was performed by analysis of variance followed by Fisher's least significant difference test.

TPA-desensitization in cell column perifusion. A perifusion system using

dispersed cells cultured on Cytodex-I beads as described by Chang et al. [5] was used. Prior to loading onto the columns, half of the preparations of pituitary cells were exposed to 10 nM TPA for 6 hours (TPA-pretreated groups); the remaining control cells received no such treatment. After 4 hours of perifusion with testing medium, a relatively low basal secretion rate was achieved and the experiment commenced with the collection of perifusate in 10 minute fractions. 30 minutes after commencement of the experiment, a 10-minute pulse of 100 nM of sGnRH, cGnRH II or a 5-minute pulse of 10 nM TPA was applied. During the GnRH application and for 20 minutes following GnRH treatment, 1-minute fractions of perifusate were collected, following which the collection interval was returned to 10 minutes. To evaluate the general GtH-releasing ability of the pituitary cells, a 5-minute pulse of 10 μM forskolin was then applied 80 minutes after the termination of the GnRH or TPA pulse. Activation of adenylate cyclase by forskolin has previously been shown to increase GtH release independent of GnRH in the goldfish [19]. Perifusate from the columns was frozen at -20 °C until their GtH contents could be determined by radioimmunoassay. Results from each individual column were expressed as a percentage of the average rate of hormone secretion obtained from the five fractions prior to the first GnRH or TPA response in TPA-pretreated (desensitized) preparations. Data presented are pooled results from three control and three TPA-pretreated columns obtained from three separate experiments.

Results

Down regulation of PKC levels. Results from PKC immunoblots clearly demonstrate a marked decrease in detectable PKC levels in pituitary cells exposed to 10 nM TPA for 30 or 240 minutes (Fig. 4.2). The photograph represents one of three experiments, all of which yielded similar results. These results indicated that pre-exposure

to 10 nM TPA consistently decreased cellular PKC levels such that this treatment could be used to down regulate PKC-dependent pathways in goldfish pituitary cells.

GtH-release responses of PKC depleted cells in static culture. During the four-hour pretreatment period, exposure to 10 nM TPA lead to an increase in GtH release. This increase persisted, although at a much lower level, in the rest and test periods (see legend Fig. 4.3). During the test period, all of the secretagogues used elicited a significant increase in the secretion of GtH from M199 pretreated (control) cells (Fig. 4.3 top panel). In desensitized cells, the GtH responses elicited by sGnRH, cGnRH II and ionomycin were totally abolished; responses to TPA and DiC8 were greatly reduced, but by comparison, the forskolin-stimulated response was only slightly reduced (Fig. 4.3 top panel).

TPA pretreatment generally resulted in a reduction of cellular contents of GtH by approximately 50 percent of that contained in control cells (Fig. 4.3 center panel). Only TPA pretreated cells receiving a challenge of DiC8 or forskolin, during the test period, exhibited significantly lower cellular contents of GtH when compared to the cells receiving the TPA pretreatment alone.

In control cells (M199 pretreated), challenges of TPA, DiC8, ionomycin and forskolin given during the test period also resulted in a lowering of the cellular contents of GtH compared to cells receiving no challenge during the test period (Fig. 4.3 center panel). In contrast, challenges of sGnRH and cGnRH II presented during the test period did not alter the cellular GtH content in the control cells (Fig. 4.3 center panel).

Changes in the total GtH measured during the entire experiment mirrored those of the cellular contents closely (compare Fig. 4.3 center and lower panels). These results indicate that the differences observed in cellular contents of cells receiving different treatments are not merely due to differing abilities of secretagogues to stimulate GtH release into the medium during the pretreatment, rest and test periods. If this were true, the total

GtH measured should be equal in all treatment groups. This is clearly not the case, therefore the different treatments appear to be affecting the synthesis and/or degradation of GtH in the gonadotropes.

GtH-release responses in PKC depleted cells in column perifusion studies. In addition to the static culture studies, effects of PKC down regulation on GnRH-stimulated GtH secretion were also examined in column perifusion. Perifusion experiments allow for the examination of changes of hormone secretion with respect to time. In the rat, LH secretion from pituitary cells in perifusion is biphasic. The LH response to GnRH displays a distinct peak and a plateau phase; these two phases appear to be mediated differently by signal transduction pathways [for a review see 23]. In addition, the secretion of GnRH is pulsatile in mammals. Treatments with a transient pulse of GnRH, as permitted in perifusion studies, are a better approximation of the natural condition than a chronic administration of GnRH, as is the case in static culture experiments. Furthermore, with the high resolution afforded by rapid fraction collection, second messenger systems responsible for mediating peak versus plateau phases of the GtH response to GnRH can also be examined.

The use of rapid fraction collection (1 min/fraction) facilitated the resolution of both sGnRH- and cGnRH II-stimulated GtH secretion into two distinct phases. The initial phase consisted of a rapid rise in GtH release of high magnitude (peak phase), this was followed by a longer lasting period of elevated hormone secretion of lower magnitude (plateau phase) (Fig. 4.4). These phases are similar to those observed in LH secretion stimulated by GnRH from rat gonadotropes [23].

Compared to control cells, the total GtH secretion stimulated by sGnRH and cGnRH II were reduced by about 85-90 percent by TPA pretreatment. This was achieved by a large reduction (80%) in the peak GtH response and an almost total abolition (90%) of the plateau response (Table 4.1). Similarly, the GtH response to a 5-minute pulse of TPA

was reduced by roughly 75 % in the PKC depleted cells. In contrast to the inhibitory effects of prior exposure to TPA on GnRH-stimulated GtH response, TPA pretreatment did not affect basal secretion of GtH (Fig. 4.4).

Following GnRH application, subsequent administration of pulses of forskolin stimulated the release of GtH in both control and PKC-depleted cells. However, the GtH responses elicited by forskolin in TPA desensitized cells were generally twice as great as responses obtained from control cells. The forskolin- stimulated GtH response in control cells which received a 5-minute TPA pulse prior to forskolin exposure (Fig. 4.4 lower panel, Table 4.1) was 4 times larger than those control cells receiving GnRH pulses (Fig. 4.4 top and center panels, Table 4.1).

In perifusion experiments, the cells were given a 6 hour TPA pretreatment as opposed to the 4 hour treatment that the cells received in static culture. This procedure was adopted because, subsequent to the loading of cultured cells onto columns, a 4-hour washing period is required to achieve a relatively low and stable rate of GtH secretion. To guard against the recovery of PKC activity, a longer TPA pretreatment was given to the cells used for perifusion. To assess the effects of this longer 6-hour pretreatment on cellular GtH contents, hormone contents of the cells exposed to 10 nM TPA for 6 hours and given a 4 hour rest (M199) were measured in static culture (Fig. 4.5). This tactic was adopted due to the difficulty in reliably collecting cells in a reproducible manner from columns following perifusion experiments. Similar to the results obtained using 4-hour TPA exposure (Fig. 4.3), cells receiving 6 hours of exposure to TPA (as out lined above) also had cellular contents of GtH that were approximately 50 percent of that found in control cells (Fig. 4.5).

Discussion

The ability of TPA pretreatment, to visibly reduce PKC levels as measured by immunoblot, as well as to greatly reduce the GtH releasing ability of PKC activators in static culture and perifusion strongly indicate that that this treatment is effective in impairing the PKC activity in goldfish gonadotropes.

The reduced ability of PKC deficient cells to respond to sGnRH and cGnRH II in both static culture and column perifusion supports the hypothesis that PKC is involved in the secretion of GtH when stimulated by GnRH. These results are consistent with results obtained from previous experiments examining the involvement of PKC in GnRH action in the goldfish (see Introduction). Furthermore, the reduction of GtH release under both constant and transient application of GnRH suggests that PKC plays a role in prolonged as well as acute GtH secretion when stimulated by GnRH. In perifusion, the plateau phase of the GtH response is reduced to only 10 % of control levels. This may explain the total abolition of sGnRH and cGnRH II-stimulated GtH release from TPA pretreated cells in static culture; where the plateau phase likely constitutes a major part of the GtH response to chronic GnRH exposure.

The magnitude of the reduction in GnRH-stimulated GtH release caused by TPA-pretreatment appears to be greater in the goldfish than in the rat. Pretreatment with 10 nM TPA reduced GtH response to both native GnRHs by 85-90% in perifusion and by greater than 95% in static incubation experiments in this study. In comparison, a 10 nM TPA pretreatment decreased the absolute LH release elicited by GnRH by only 65% in a study that used rat gonadotropes [9]. Similarly, pretreatment with 15 nM TPA reduced absolute LH secretion by approximately 50% in another study which also used rat pituitary cells [14]. These results imply that PKC may play a larger role in mediating GnRH-stimulated GtH release in the goldfish than in the rat. Alternatively, prolonged TPA pretreatment may be relatively more effective in down-regulating PKC activity in the goldfish that in the rat.

In the present study, cell contents of GtH were reduced 50% by TPA pretreatment.

However, this decrease in GtH content alone does not appear to be the cause of the diminished response to GnRH. The relative reduction in cellular GtH content (50%) is far smaller than the relative reduction in the GtH release induced by either sGnRH or cGnRH II (greater than 85%). Additionally, forskolin is effective in stimulating GtH release in PKC depleted cells with lowered cellular GtH content in both static culture and perifusion studies. These results with forskolin also imply that TPA pretreatment has not lead to a general impairment of the release process or cellular function; in fact, the cells are quite capable of a full if not potentiated GtH responses. Therefore, the decreased GtH response to GnRH is most likely due to a specific impairment of PKC function resulting from TPA pretreatment and not some sort of general cellular impairment or damage. It would appear that the potentiation of the forskolin response, caused by PKC down regulation, is limited to only the acute response because only the first few fractions of the forskolin response show potentiated levels of GtH release in perifusion and no potentiation of response is observed in static culture. The nature of this interaction between the PKC and cAMP pathways requires further study for proper characterization.

Interestingly, application of TPA during pretreatment or application of TPA, DiC8, ionomycin or forskolin during the test period reduced the cellular GtH contents of goldfish gonadotropes. Reduction in total hormone measured during the experiment in cells receiving the above treatments suggest that the reduction of cell contents may be due to alteration of the synthesis and or degradation of GtH (See Results Section). Conversely, the native secretagogues (sGnRH, cGnRH II) did not reduce cellular contents or total GtH measured in static culture. Therefore, it appears that the two GnRHs stimulate replacement of the secreted hormone via a mechanism not mimicked by the direct stimulation of Ca^{2+}, PKC or cAMP pathways.

The ability of TPA pretreatment to abolish ionomycin-stimulated GtH secretion in static culture suggests that prolonged GtH secretion elicited by this secretagogue is largely

mediated via PKC. This result is not surprising, because ionomycin is a Ca^{2+} ionophore and activation of some forms of PKC are known to be dependent on elevation of of intracellular Ca^{2+} levels.

Blockade of the GtH-releasing abilities of the PKC activators (TPA, DiC8) served as another indicator that PKC activity was indeed impaired by the TPA pretreatment. However, the abilities of additions of DiC8 and forskolin, given during the test period, to lower cellular contents of GtH were additive to that of the TPA pretreatment. These results suggest that DiC8 and forskolin may stimulate intracellular pathways different from those used by TPA to affect cellular GtH content. This is not surprising in the case of forskolin which is an adenylate cyclase stimulator and has no known direct effects on PKC. Although TPA and DiC8 are both PKC activators, they have been shown to possess actions that differ in the goldfish pituitary, notably, the mobilization of Ca^{2+} from intracellular sources [17]. This difference in the actions of the PKC activators may explain the additivity of their effects on cellular GtH content.

In summary, the present study confirmed that PKC is involved in the prolonged secretion of GtH elicited by both native GnRHs and further implicates the involvement of PKC in mediating acute GnRH action on GtH release in the goldfish. The difference in the abilities of PKC activators and forskolin to stimulate release of GtH from TPA pretreated cells exhibiting lowered cell contents strongly suggests that a compartmentalization of the releasable pools of GtH exists. Therefore, depending on the manner in which the levels of GtH are reduced in a gonadotrope, use of the cellular GtH content of the cell as an indicator of the amount of GtH available for release may not be prudent. Results from the current study also illustrate that GnRH-stimulation of GtH release is normally accompanied by the replenishment of cellular stores of GtH; however, intracellular pathways leading to hormone release and those leading to hormone synthesis are at least partially dissociated. The mechanisms mediating the GnRH induction of synthesis in goldfish gonadotropes

remains to be identified.

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Table 4.1. Effects of PKC desensitization, induced by TPA pretreatment, on GtH responses to 100 nM sGnRH, 100 nM cGnRHII, 10 nM TPA and 10 μM forskolin in perifusion studies. Peak values are taken from fractions which show the highest GtH response to GnRH. The plateau values are taken as the average response from fractions 23-28 (50-56 minutes). These fractions were considered to be part of the second phase of the GnRH response. Total responses were calculated by determining the net elevation in GtH secretion over basal release for the entire response to a secretogogue. Only total values were calculated for the TPA and forskolin responses. Amounts of GtH were expressed as a percentage of the initial unstimulated secretion rates (first 5 fractions) from the TPA pretreated (desesitized) cells.

Test pulse	Peak GtH Response		Plateau GtH Response		Total GtH Response	
	Control	Desensitized	Control	Desensitized	Control	Desensitized
A) sGnRH Column						
sGnRH	396.7 ± 57.1	70.2 ± 10.6	156.0 ± 20.5	11.8 ± 8.8	5639.0 ± 411.7	674.5 ± 154.8
Forskolin					805.6 ± 208.3	1415.0 ± 183.0
B) cGnRH II Column						
cGnRH II	499.8 ± 94.9	80.8 ± 8.6	159.4 ± 18.6	20.3 ± 14.9	5924.8 ± 880.7	892.6 ± 243.5
Forskolin					1031.0 ± 90.5	1647.8 ± 175.3
C) TPA Column						
TPA					11530.3 ± 1078.0	2725.6 ± 741.8
Forskolin					3910.8 ± 181.7	2211.3 ± 293.8

Fig. 4.1. Experimental protocol for PKC down regulation by pretreatment with TPA for static culture experiments.

Experimental protocol for desensitization experiments with phorbol ester TPA. PKC is downregulated by 4-h pretreatment with TPA.

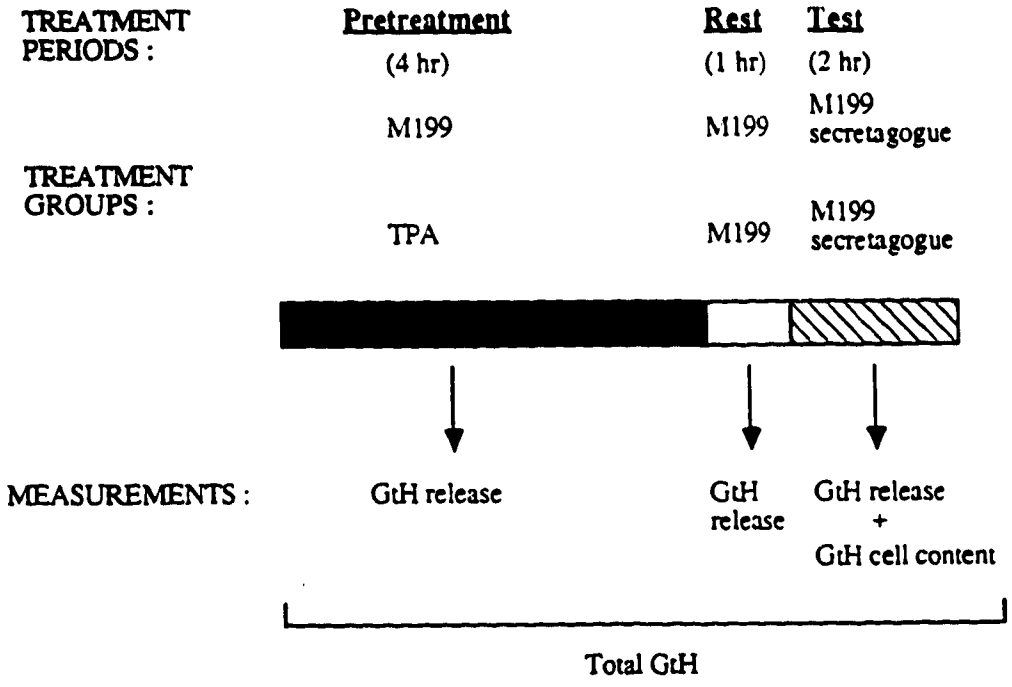
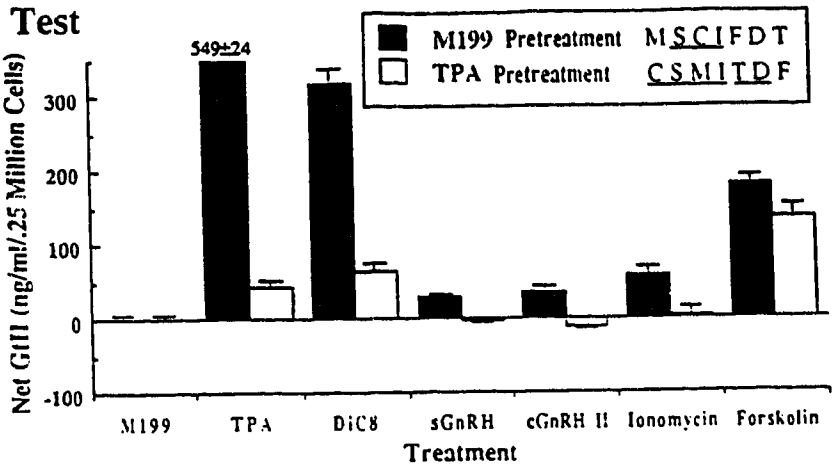
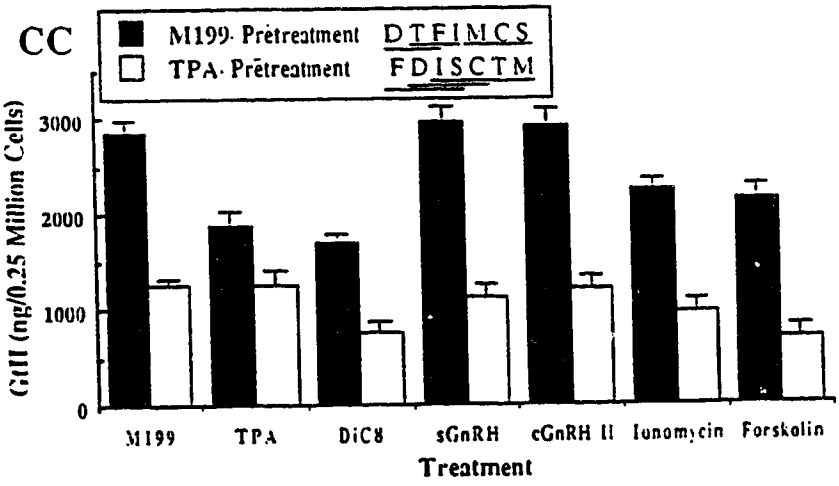


Fig. 4.2. PKC immunoblot visualized from a nitrocellulose membrane utilizing a chemiluminescent system. Three lanes are presented representing (A) 0, (B) 30 min and (C) 240 min exposure to 10 nM TPA. Each lane contains the protein extracted from 10 million cells.

A B C

Fig. 4.3. Effects of PKC down regulation by pretreatment with 10 nM TPA on subsequent GtH responses in static culture. During the pretreatment stage the GtH released by control and TPA pretreated cells were 290±7 and 1108±18 (ng/ml/0.25 million cells), respectively. This difference in secretion was greatly reduced during the rest stage where GtH release by control cells was 143±5 (ng/ml/0.25 million cells), while that of the pretreated cells was 218±4 (ng/ml/0.25 million cells). The upper panel represents the results from the test stage; results are expressed as net GtH secreted. Net secretion values were determined by subtracting the amount of GtH secreted by cells treated with M199 during the testing period from the amount of GtH released by exposure to the various secretagogues during the test period. Basal release during the test period from the M199 and TPA pretreated cells was 99±6 and 129±5 (ng/ml/0.25 million cells) respectively. In the center panel, results from the measurements of cell contents (CC) of GtH are represented. Comparisons of the effects of the different treatments on the total GtH secreted during the entire experiment are given in the lower panel. In all panels, results presented (Mean±SE) are pooled data from two separate experiments. Treatments in individual experiments were performed in triplicate. Treatment groups are identified by the first letter in their name (M199=M), and groups not sharing the same underscore are significantly different from each other ($p<0.05$).


Test
M199 Pretreatment MSCIFDT
TPA Pretreatment CSMITDF
549±24
Net GtII (ng/ml/.25 Million Cells)
300
200
100
0
-100
M199
TPA
DiC8
sGnRH
cGnRH II
Ionomycin
Forskolin
Treatment


CC
M199 Pretreatment DTFIMCS
TPA Pretreatment FDISCTM
GtII (ng/0.25 Million Cells)
3000
2000
1000
0
M199
TPA
DiC8
sGnRH
cGnRH II
Ionomycin
Forskolin
Treatment

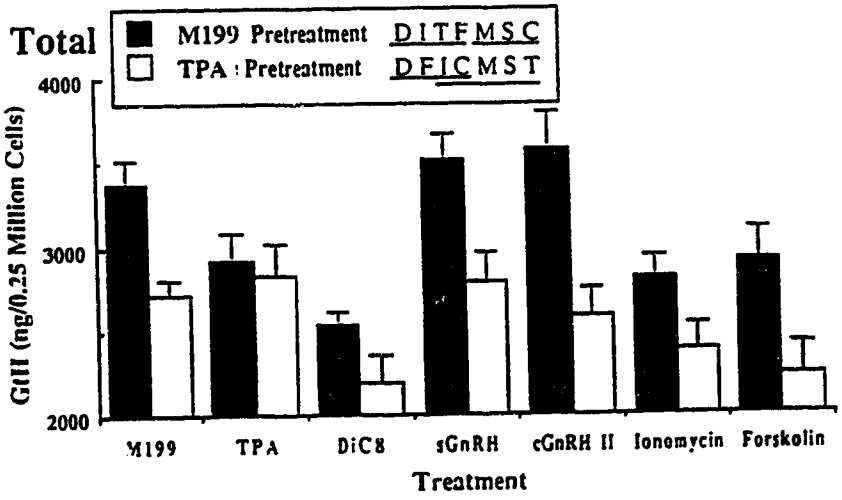
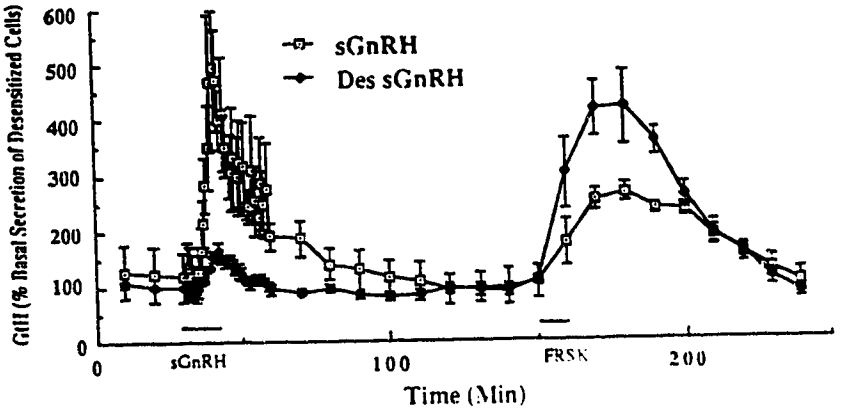
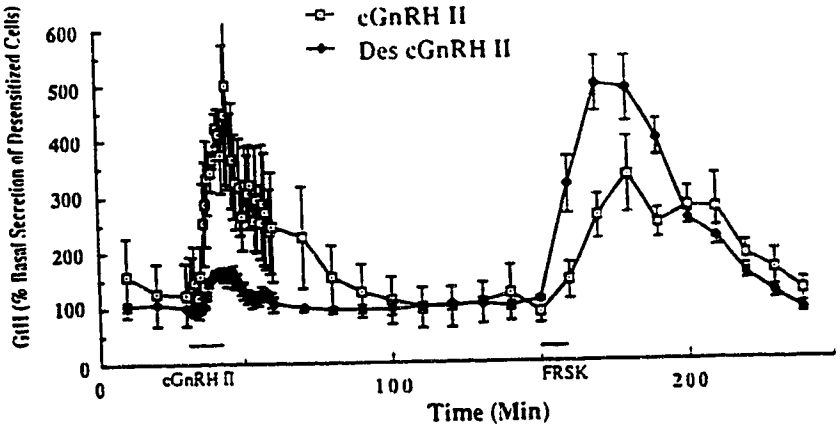

Total
M199 Pretreatment DITFMSC
TPA Pretreatment DFICMST
GtII (ng/0.25 Million Cells)
4000
3000
2000
M199
TPA
DiC8
sGnRH
cGnRH II
Ionomycin
Forskolin
Treatment

Fig. 4.4. Effects of TPA pretreatment on the GtH responses to 100 nM sGnRH (top panel), 100 nM cGnRH II (center panel), 10 nM TPA (bottom panel) and 10 μM forskolin (all panels) in column perifusion studies. Hormone release data (Mean±SE, n=3) are normalized as a percentage of the initial basal secretion rates of the desensitized cell preparations.


sGnRH
Des sGnRH
Gill (% Basal Secretion of Desensitized Cells)
600
500
400
300
200
100
0
0
sGnRH
100
FRSK
200
Time (Min)


cGnRH II
Des cGnRH II
Gill (% Basal Secretion of Desensitized Cells)
600
500
400
300
200
100
0
0
cGnRH II
100
FRSK
200
Time (Min)

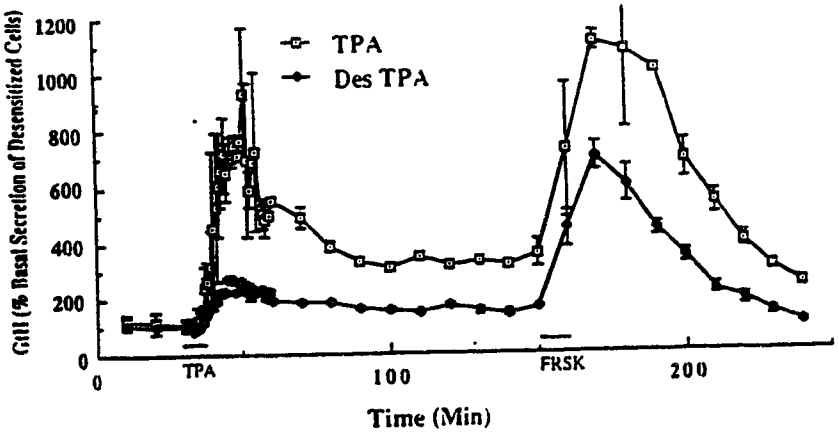
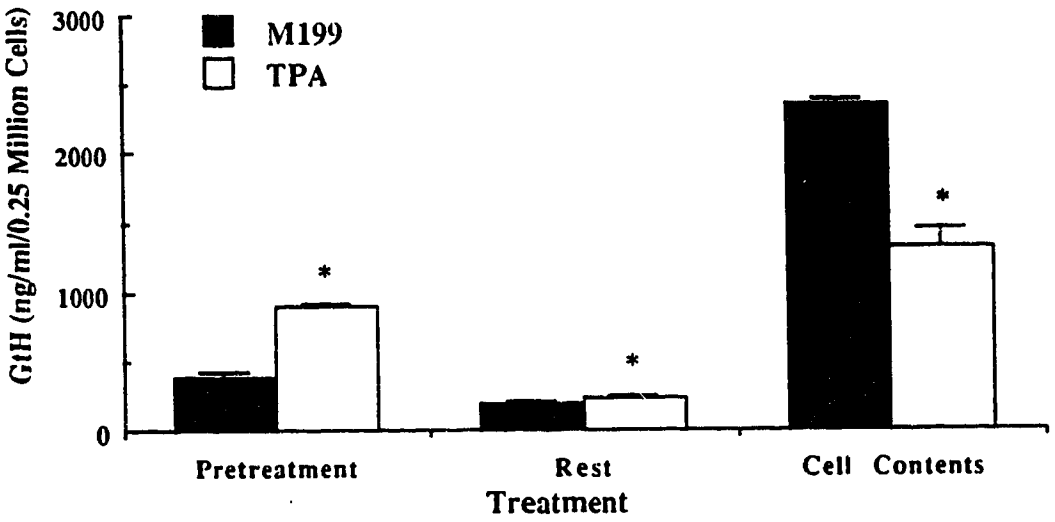

TPA
Des TPA
Gill (% Basal Secretion of Desensitized Cells)
1200
1000
800
600
400
200
0
0
TPA
100
FRSK
200
Time (Min)

Fig. 4.5. Effects of 6-hour TPA pretreatement followed by a 4-hour rest period on cellular contents of GtH. Results presented (Mean±SE) represent pooled data from two separate experiments with treatment groups of 12. * signifies values significantly different from control values ($p<0.05$).


GtH (ng/ml/0.25 Million Cells)
3000
2000
1000
0
M199
TPA
*
*
*
Pretreatment
Rest
Cell Contents
Treatment

Chapter 5

Further Evidence for the Involvement of PKC in the Mediation of GnRH-Stimulated GtH Secretion.

Introduction

In most vertebrates, including the goldfish the release of gonadotropin (GtH) is under the stimulatory influence of gonadotropin releasing hormone (GnRH) [for review see 1]. Two different forms of GnRH, [Trp^7, Leu^8]-GnRH (salmon GnRH, sGnRH) and [His^5, Trp^7, Tyr^8]-GnRH (chicken GnRH II, cGnRH II), are native to the goldfish [2]. Release of both forms can be stimulated from hypothalamic and pituitary preparations; in turn, both GnRH forms are effective in eliciting GtH release from the goldfish *in vitro* and *in vivo* [3,4,5].

Signal transduction mechanisms mediating GnRH-stimulated GtH secretion have been more extensively studied in the rat than in any other animal model. In the rat model system, extracellular Ca^{2+}, intracellular Ca^{2+}, calmodulin, protein kinase C (PKC) and inositol phosphates have been implicated in the mediation of luteinizing hormone (LH) secretion elicited by GnRH [6, 7, 8]. However, there has been some controversy over the involvement of PKC in the LH secretory response to GnRH. This controversy centers over conflicting results obtained with the use of gonadotropes with depleted PKC levels caused by prolonged tetradecanoyl 13 phorbol acetate (TPA) pretreatment [9,10]. However, the preponderance of the evidence implicates the involvement of PKC in GnRH action in the rat. For example, pharmacological stimulators of PKC mimic the LH-releasing and Ca^{2+}-mobilizing abilities of GnRH [9, 11], while inhibitors of PKC have

been shown to antagonize GnRH actions [12].

In the goldfish model system, a substantial amount of evidence indicates that PKC is involved in the mediation of GnRH-stimulated GtH secretion. Activators of PKC, such as TPA and dioctanoyl glycerol (DiC8), mimic the ability of the native GnRHs (sGnRH and cGnRH II) to stimulate release of GtH in an extracellular Ca^{2+} dependent manner [5, 13]. Similar to sGnRH and cGnRH II, activators of PKC can elevate levels of intracellular Ca^{2+} in dispersed goldfish pituitary cells [14]. H7, an inhibitor of PKC, attenuates the GtH response stimulated by TPA, sGnRH and cGnRH II [15]. In addition, another PKC inhibitor, staurosporine, reduces the abilities of TPA and cGnRH II to increase intracellular Ca^{2+} levels in goldfish pituitary cells [14]. Impairment of PKC activity through depletion of PKC levels by prolonged exposure to TPA also led to reductions of TPA-, DiC8-, sGnRH- and cGnRH II-induced GtH release [Chapter 4].

In the present study, further evidence is provided to support the hypothesis that PKC participates in GnRH stimulation of GtH release in the goldfish. The PKC inhibitor staurosporine was used to confirm the attenuation of GtH release known to occur with the inhibition of PKC. Additionally, a new tactic was utilized. Static culture and column perifusion studies were used to determine whether the GtH release responses elicited by sGnRH and activators of PKC (TPA, DiC8) were additive. If PKC did not play a major role in mediating GnRH-induced GtH release, the responses to sGnRH and the PKC activators should be additive, however, if the same pathways are used no significant additivity in the magnitude of the responses should be observed when the secretagogues are presented simultaneously.

Materials and Methods

General. Common goldfish (8-12 cm in length), purchased from Ozark Fisheries Inc., Stoutland, Missouri and Grassyforks Fisheries, Martinsville, Indiana, were transferred to flow-through aquaria (1800 liters) immediately on arrival. The fish were held at 17-20 ^{o}C on a simulated natural (Edmonton, Alberta) photoperiod, and fed to satiation daily with commercial fish food. Fish of both sexes were acclimated to the above conditions for at least 7 days before use. sGnRH and cGnRH II (Peninsula Lab. Inc., Belmont, CA) were dissolved in distilled deionized water. TPA, forskolin (Sigma, St. Louis, MO) and DiC8 (Calbiochem, San Diego, Ca) were dissolved in dimethylsulfoxide. Aliquots of stock solutions were stored at -20 ^{o}C until used.

Dispersed goldfish pituitary cells were used in all experiments in this study. Pituitaries from fish of both sexes were removed and the cells dispersed with a trypsin/DNAse procedure as previously described [16]. Dispersed pituitary cells were cultured overnight prior to all experiments. GtH released was quantified using a radioimmunoassay validated for maturational GtH (GtH II) [17, 18].

Static cell culture and GtH response. Dispersed cells were cultured overnight in 24-well culture plates as previously described [16]. The following day, prior to experiments, the culture medium was replaced with testing medium (medium 199 containing Hank's Salts, (Gibco), 25 mM Hepes, 2.2 g/l sodium bicarbonate, 100 000 units/l penicillin, 100 mg/l streptomycin, 0.1% bovine serum albumin). Test solutions diluted in the proper vehicle were added (1 µl/ml to achieve desired final concentration). All treatments were carried out in quadruplicate. Following the testing period (2 hours), media were removed and stored frozen at -20 ^{o}C until processed for GtH contents by radioimmunoassay [18].

Column perifusion of mixed cell populations. A perifusion system using dispersed cells on Cytodex-I beads as described by Chang et al., [16] was used. After loading onto the columns (2 million cells per column) the cells were perifused for 4 hours

to establish a relatively low and stable rate of unstimulated secretion, at which time the experiment was started. The perifusion began with 10-minute fraction collection time, however during the 10 minute sGnRH pulse and for 20 minutes following the pulse, a 1-minute fraction collection time was used. Three different treatments regimes were applied. These being: 1) continuous application of DiC8 alone, 2) presentation of a 10-minute pulse of sGnRH followed by a 5-minute pulse of forskolin both of which are administered during continuous DiC8 application, 3) a 10-minute pulse of sGnRH followed by a 5-minute pulse of forskolin are presented in the absence of DiC8. Forskolin was used near the end of the perifusion to assess the general GtH releasing ability of the cells. This secretagogue was selected because GnRH does not utilize the cAMP pathway in goldfish gonadotropes [19].

Column perifusion of cells populations enriched with gonadotropes. Dispersed cells were separated using a discontinuous percoll density gradient. The protocol used was a modified version of that used by de Leeuw et al. [20]. Briefly, percoll (Pharmacia, Piscataway, NJ) was diluted with Dulbeco's Ca^{2+}-free phosphate buffered saline with 0.5% BSA so that solutions of 40, 50, 60, 70 and 80% percoll (by volume) were obtained. The percoll gradient was built in a 50 ml centrifuge tube and the dispersed cells were layered at the top of the gradient. The gradient and cells were centrifuged at 1400 x g for 25 minutes at 17 ^{o}C. Following centrifugation, a band of cells was seen at each density interface and one at the bottom of the tube. The 5th fraction, the band of cell found between the 80% and 70% percoll solutions, had approximately 2.5 times the concentration of gonadotropes found in mixed cell populations [21]. These cells were harvested, cultured onto Cytodex-I beads over night and used for perifusion the following day as described by Chang et al., [16] using 0.6 million cells per column. In these experiments a different protocol was used. In the test columns, a 10-minute pulse of sGnRH was given before, during and after DiC8 exposure. In the control columns no sGnRH was administered during DiC8 application. The pulses of sGnRH given before and after DiC8

exposure served as controls for GtH releasing ability. This was done to circumvent the problem of the interaction between PKC and cAMP pathways (Chapter 4).

sGnRH responses were divided into peak and plateau phase in the following manner, the peak phase was the sum of the first 12 fractions of the sGnRH response, whereas the plateau phase consisted of the following 8 fractions. The total GtH response to sGnRH was taken to be the sum of the two phases. The sGnRH-elicited response was calculated by subtracting the basal (unstimulated) level of secretion from the sGnRH-elevated GtH levels observed. Basal GtH levels were calculated as the average of the 5 fractions immediately preceding and following the sGnRH-stimulated response (10 fractions total). However, for the test-pulse GtH values from the control columns (no sGnRH given during DiC8 administration) were used to determine basal GtH secretion.

Results

Staurosporine inhibits GtH release stimulated by activators of PKC. Increasing doses of the PKC inhibitor staurosporine lead to attenuation of GtH release elicited by 10 nM TPA and 100 μM DiC8 in static culture experiments. Basal GtH release was only affected by high (1 μM) levels of staurosporine which caused an elevation in the unstimulated release of GtH (Fig. 5.1). TPA-stimulated GtH release was significantly reduced by dosages of staurosporine higher than 100 pM, while DiC8-stimulated release was significantly attenuated by doses higher than 10 nM. At concentrations of 0.1 and 1 nM, staurosporine was also effective in decreasing 100 nM sGnRH- and 100 nM cGnRH II-stimulated GtH secretion (Fig. 5.2).

Additivity of GtH responses in perifusion. In the experiment which utilized mixed cell populations, no additivity between sGnRH- and DiC8-stimulated GtH release can be

observed (Fig. 5.3). The relatively small size of the sGnRH-stimulated GtH response compared to the DiC8-stimulated response might have contributed to the difficulty in observing the additivity of the two responses. Nevertheless, it is obvious that the forskolin-stimulated response is not only additive, but it is actually potentiated in the presence of DiC8 (Fig. 5.3). This potentiation suggests that the continuous exposure to DiC8 did not impair the cells ability to respond to a stimulator of the cAMP pathway. Therefore, the general GtH-releasing ability of the cells was probably not compromised.

Cell populations enriched with gonadotropes were used to increase the absolute size of the GtH response elicited by sGnRH. The size of the sGnRH response relative to that of the DiC8 response was also increased. To reduce the problem of prolonged DiC8 exposure lowering cellular GtH stores, as was observed in static culture (Chapter 4), the length of DiC8 stimulation was reduced. Pulses of sGnRH were given before (pre-pulse) and after (post-pulse) the DiC8 administration for comparison with the pulse that would be given during the DiC8 exposure (test-pulse) (Fig. 5.4). Upon closer examination it is evident that the test pulse shows a marked reduction in magnitude compared to the other pulses (Fig. 5.4, upper and lower panels). The use of a fraction collection time of one minute allowed for the resolution of the sGnRH responses into peak and plateau phases (Fig. 5.5). The total responses (peak+plateau), of the three pulses were significantly different in size, with the pre-pulse being largest, test-pulse being the smallest and the post-pulse showing substantial recovery. The peak phase of the test-pulse was significantly smaller than those of the pre- and post-pulses, but there was no significant differences seen in the plateau phases of the three responses (Fig. 5.5).

Additivity of GtH responses in static culture. 100 nM sGnRH significantly stimulated GtH release however, The response to sGnRH was not additive to those of 10 nM TPA or 100 μM DiC8 (Fig. 5.6, upper panel). Conversely, the GtH-response elicited by 10 μM forskolin was additive to, if not potentiated by, the GtH-releasing actions of 10

nM TPA and 100 μM DiC8 (Fig. 5.6, lower panel).

Discussion

The ability of the PKC inhibitor staurosporine to reduce GtH responses stimulated by TPA, DiC8, sGnRH and cGnRH II (Figs. 5.1 and 5.2) confirms previous results that show that treatment which impaired PKC activity reduced the GtH-releasing ability of PKC activators and both native GnRH peptides of the goldfish [15, Chapter 4]. Staurosporine is a more specific PKC inhibitor than the previously used H7 [22]. This is reflected in the relatively low (sub nanomolar) dosage which affects GnRH-stimulated GtH release (Fig. 5.2). Although there appears to be a difference between the effectiveness with which staurosporine inhibits TPA-induced, compared to DiC8-stimulated GtH release, this comparison is not appropriate. These experiments were not carried out in tandem, therefore, other factors such as gonadal status of the fish used could account for the differences observed. However, it should be noted that DiC8 has been found to have different GtH-releasing and Ca^{2+}-mobilizing abilities than those possessed by TPA [14]. These differences could also account for DiC8's greater resistance to the inhibitory effects of staurosporine relative to TPA in the present study.

In addition to the confirmation that inhibition of PKC function leads to reduced GnRH-induced GtH release, data indicating that sGnRH-stimulated GtH release was not additive to the GtH response elicited by activators of PKC were also collected. Because of the relatively small GtH response to sGnRH observed in perifusion of mixed populations of pituitary cells, populations of cells with enriched gonadotropes were also used. These enriched gonadotropes preparations yielded sGnRH responses which were larger in both absolute size and size relative to the DiC8-stimulated response (Figs. 5.3 & 5.4). This difference in response, may be due to any number of causes, such as paracrine interactions

due to effects of DiC8 on targets other than gonadotropes, or, isolation of a particular subset of gonadotropes with increased secretory responsiveness to GnRH than those displayed by mixed populations of cells.

Results from perifusion studies using mixed pituitary cell populations and enriched gonadotrope preparations were similar. In experiments using mixed pituitary cells, no additivity between the sGnRH-elicited GtH release and DiC8-induced response was observed. In enriched gonadotrope preparations, the sGnRH GtH response recorded during the application of DiC8 (test-pulse) was less than 25 % of that recorded prior to DiC8 exposure (pre-pulse). Both peak and total responses were diminished. These results suggest that while acute sGnRH-stimulated GtH secretion may utilize some pathways not used during DiC8-elicited GtH release there is still a great deal of overlap in the signal transduction mechanisms used by sGnRH and DiC8.

In the present study, the plateau phase of the sGnRH response was also slightly decreased by the presence of DiC8, but the differences were not statistically significant. this lack of statistical differences in the plateau phase may be an artifact of the technique used to separate the response into its phases. When sGnRH was administed during DiC8 treatment the sGnRH-induced response was so greatly diminished that it is difficult to make out the two phases (Fig. 5.4 bottom panel). The allotment of the phases is done with respect to temporal position of a normal GtH response to the administered sGnRH pulse, therefore, in the case of the test pulse, the plateau phase may be a delayed peak or a combination of peak and plateau phases. In any event, the data do clearly show a great reduction in the peak phase and total amount of GtH released (Fig. 5.5), suggesting that the DiC8 and sGnRH responses are generally not additive.

sGnRH-elicited response following DiC8 exposure (post-pulse) had almost regained the full magnitude of the pre-pulse suggesting that the 90-minute DiC8 administration did not down regulated PKC levels to any significant degree. This

assessment was based on GnRH inhibition that was observed in cells receiving a 6 hour TPA pretreatment (Chapter 4). Despite the slight reduction in size the post-pulse was still significantly larger than the test-pulse, suggesting, that the reduction in size of the test-pulse was largely due to the sharing of activated pathways by sGnRH and DiC8 and not a down regulation of PKC levels by prolonged DiC8 exposure.

In static culture, the GtH response to forskolin, the adenylate cyclase activator, is completely additive to those induced by TPA and DiC8, if not potentiated by these PKC activators (Fig. 5.6, lower pannel). Forskolin also shows a potentiated GtH release response in PKC-depleted cells (see Chapter 4) or when added with DiC8 in perifusion (Fig. 5.3). These results strongly indicate the existence of a synergistic interaction between the PKC and cAMP pathways in goldfish gonadotropes. The chronic GtH response elicited by sGnRH was not additive to the responses stimulated by either TPA or DiC8 in static culture (Fig. 5.6, upper pannel). This data agrees with the results from perifusion experiments obtained in this study (Fig. 5.4).

In summary, the present study has confirmed the effectiveness of inhibition of the PKC pathway for the attenuation of GtH released by PKC activators and the two native GnRH peptides (sGnRH, cGnRH II) of the goldfish. Confirmation of a synergistic interaction between the PKC and cAMP pathways is also provided. Furthermore, lack of additivity of GtH responses elicited by sGnRH and activators of PKC (TPA, DiC8) in both perifusion and static incubation studies add a new type of evidence to the already existing body which supports the hypothesis that PKC is involved in the mediation of acute as well as chronic, GnRH-stimulated GtH secretion [14, 15, Chapter 4].

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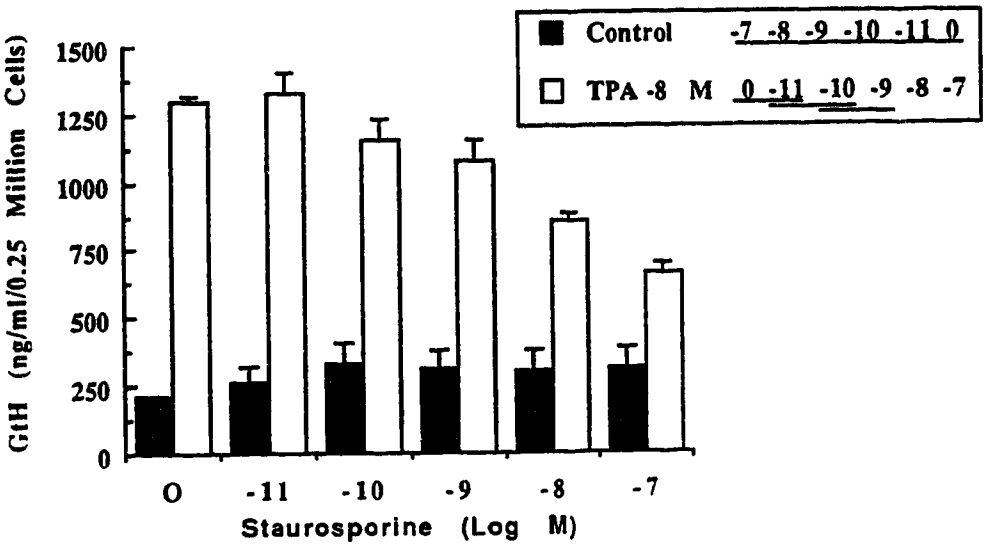
Figure 5.1. Effect of staurosporine on TPA-(upper panel) and DiC8-stimulated (lower panel) GtH release in static culture. Increasing doses of staurosporine were used to inhibit GtH release stimulated by 10 nM TPA and 100 μM DiC8. Representative results from one of three experiments having similar results are presented (mean ± SE, n=4). In controls, TPA- and DiC8-treated groups, concentrations of staurosporine that results in similar GtH values are identified by having the same underscore Values not sharing the same underscore are significantly different as determined by analysis of variance followed by Fisher's least significant difference test ($p<0.05$).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TPA -8 M 0 -11 -10 -9 -8 -7
GtH (ng/ml/0.25 Million Cells)
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1250
1000
750
500
250
0
O -11 -10 -9 -8 -7
Staurosporine (Log M)

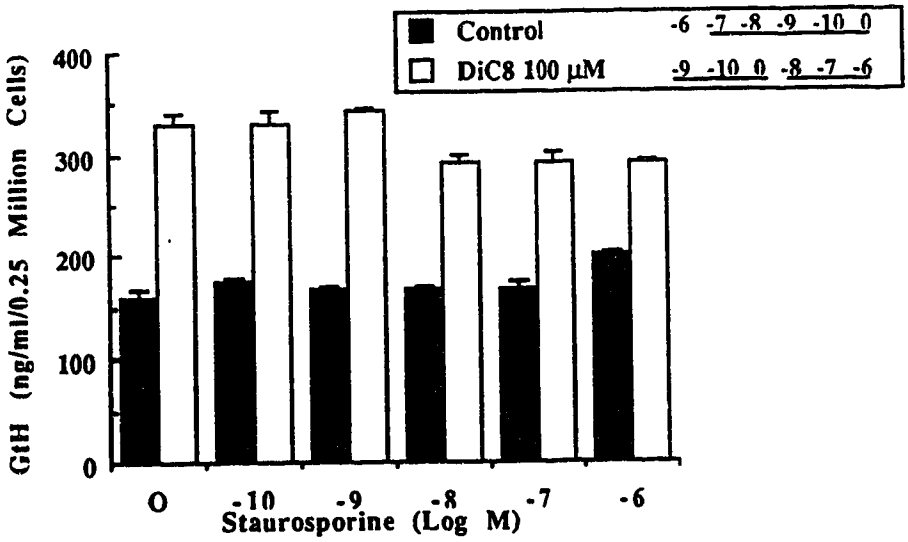

Control -6 -7 -8 -9 -10 0
DiC8 100 µM -9 -10 0 -8 -7 -6
GtH (ng/ml/0.25 Million Cells)
400
300
200
100
0
O -10 -9 -8 -7 -6
Staurosporine (Log M)

Figure 5.2. Effects of staurosporine on GtH secretion elicited by 100 nM sGnRH and cGnRH II. Results presented represent pooled data from two separate experiments (mean ± SE,n=8). Values sharing the same underscore are not significantly different, as determined by an analysis of variance followed by Fisher's least squares difference test ($p<0.05$)

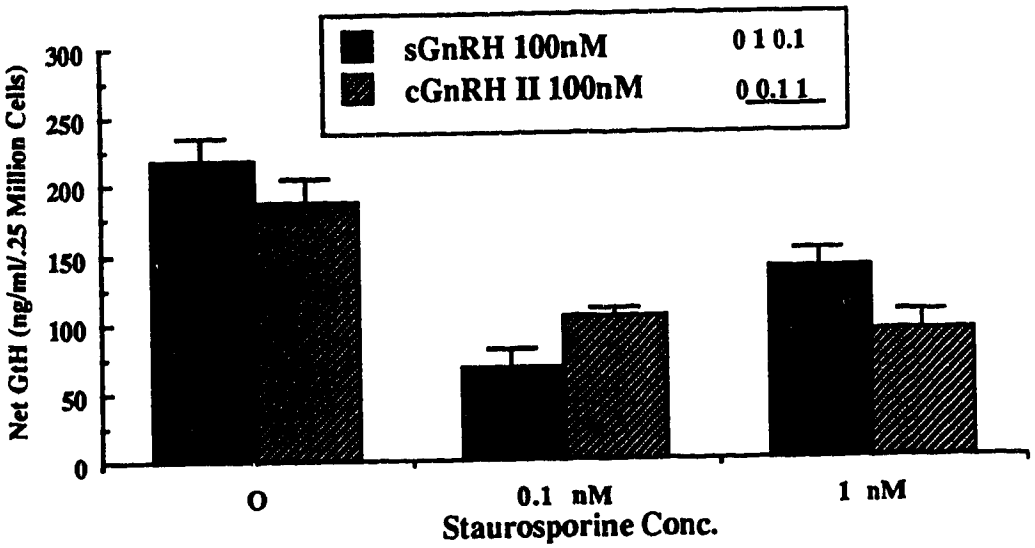
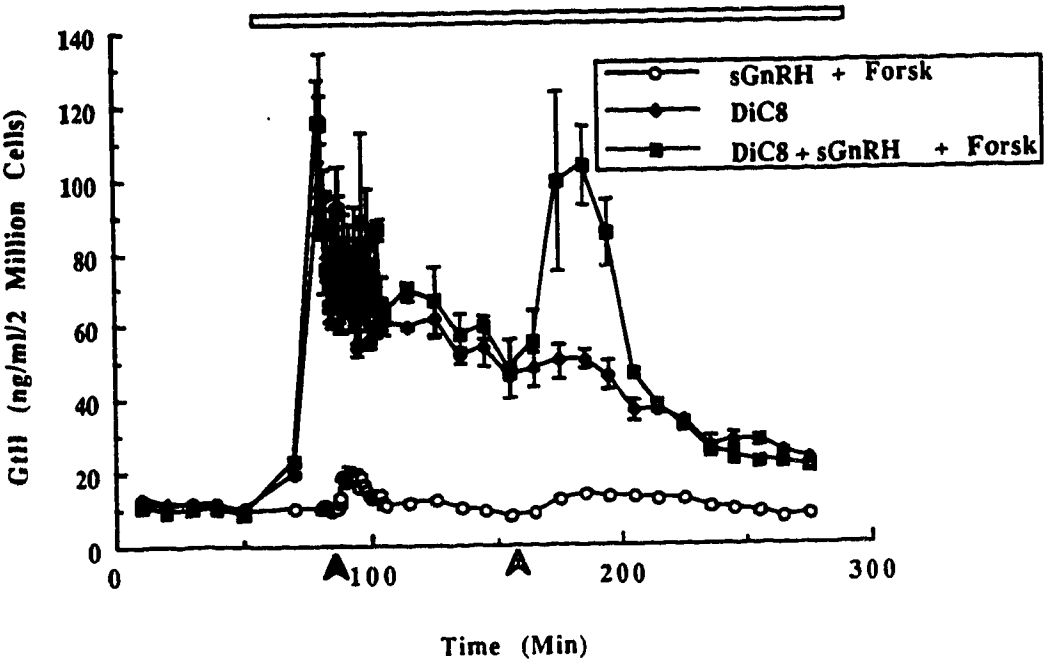

sGnRH 100nM
cGnRH II 100nM
0 1 0.1
0 0.1 1
Net GtH (ng/ml/.25 Million Cells)
300
250
200
150
100
50
0
0
0.1 nM
1 nM
Staurosporine Conc.

Figure 5.3. Additivity of the GtH responses to sGnRH, DiC8 and forskolin in perifusion. The horizontal bar indicates exposure to 100 μM DiC8. The closed arrow heads indicate the beginning of a 5-minute application of 100 nM sGnRH. The open arrow heads indicate the beginning of a 5-minute application of 10 μM forskolin (F). The lower panel shows the GtH responses to the simultaneous administration of sGnRH and DiC8 in greater detail than the upper panel. Results presented represent one experiment with two columns for each treatment (mean ± SE, n=2)


sGnRH + Forsk
DiC8
DiC8 + sGnRH + Forsk
GtH (ng/ml/2 Million Cells)
140
120
100
80
60
40
20
0
0
100
200
300
Time (Min)

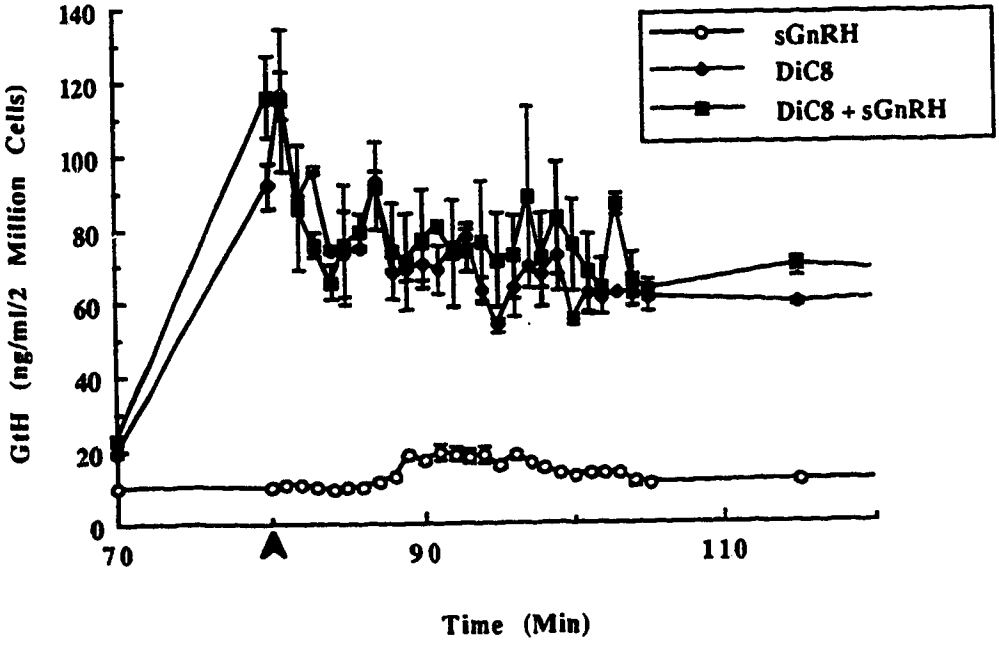
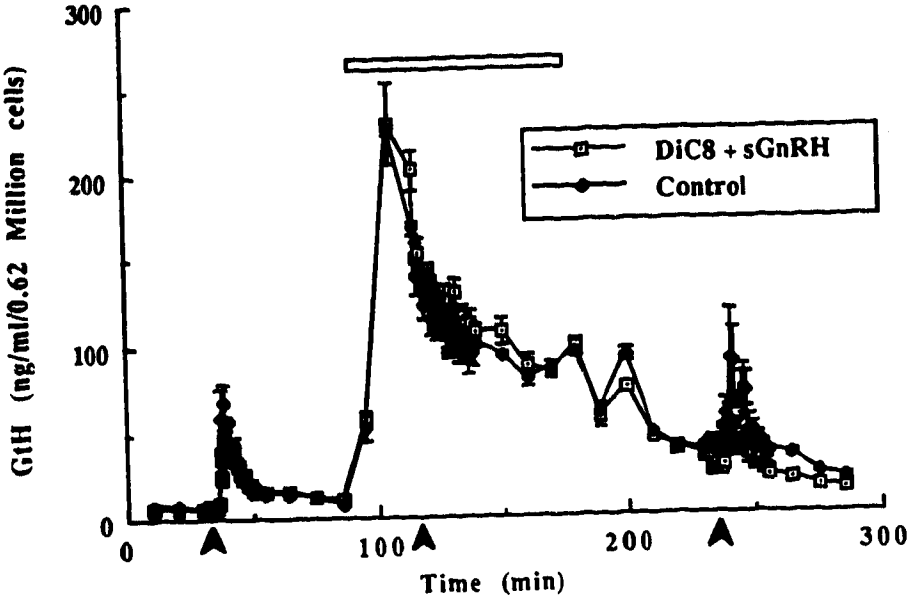

sGnRH
DiC8
DiC8 + sGnRH
GtH (ng/ml/2 Million Cells)
140
120
100
80
60
40
20
0
70
90
110
Time (Min)

Figure 5.4. Additivity of sGnRH- and DiC8-stimulated GtH responses from perifused populations of pituitary cells enriched with gonadotropes. The horizontal bar indicates exposure to 100 μM DiC8. The arrow heads indicate the beginning of a 5-minute application of 100 nM sGnRH. The lower panel shows the GtH response to the simultaneous administration of sGnRH and DiC8 in greater detail than is provided in the upper panel. The data presented (mean ± SE, n=4) represent one of two experiments conducted which yielded similar results.


300
200
100
0
0
100
200
300
GtH (ng/ml/0.62 Million cells)
Time (min)
DiC8 + sGnRH
Control

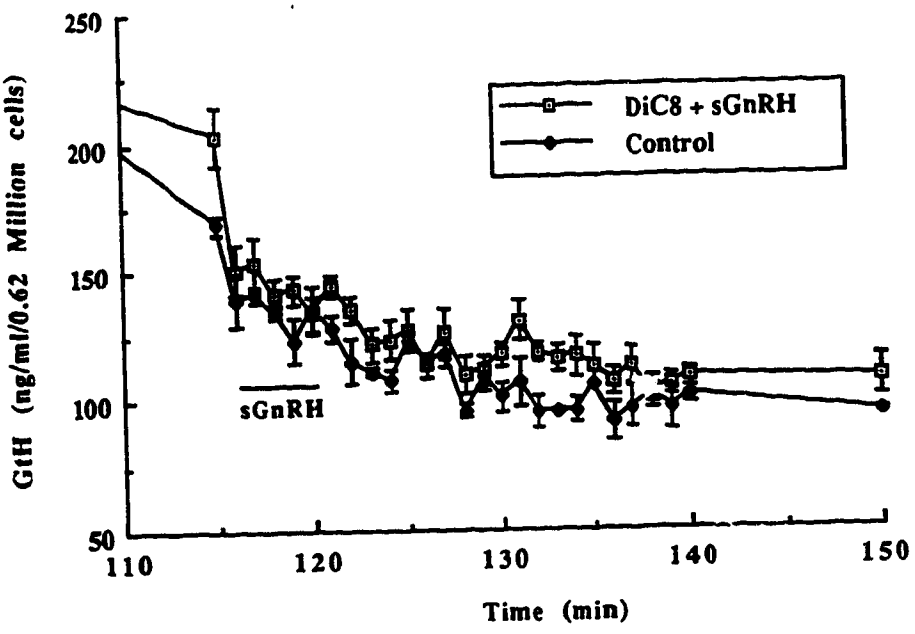

250
200
150
100
50
110
120
130
140
150
GtH (ng/ml/0.62 Million cells)
Time (min)
sGnRH
DiC8 + sGnRH
Control

Figure 5.5. Summarized results of the additivity of sGnRH- and DiC8-stimulated GtH responses from cell populations with enriched gonadotrope contents. The GtH responses to the three 100 nM GnRH pulses, before (pre-pulse), during (test-pulse) and after (post-pulse) 100 μM DiC8 treatment, were divided into peak and plateau phases. The magnitude of these two phases as well as the total GtH response were compared using analysis of variance followed by Fisher's least significant difference test. Values not sharing the same underscore are significantly different from each other ($p<0.05$). Values are presented as mean ± SE (n=4).

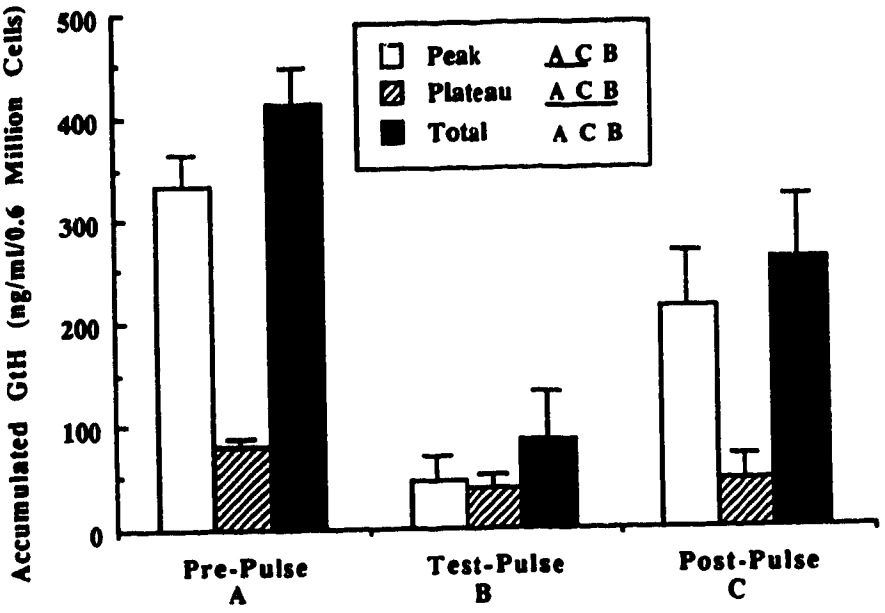
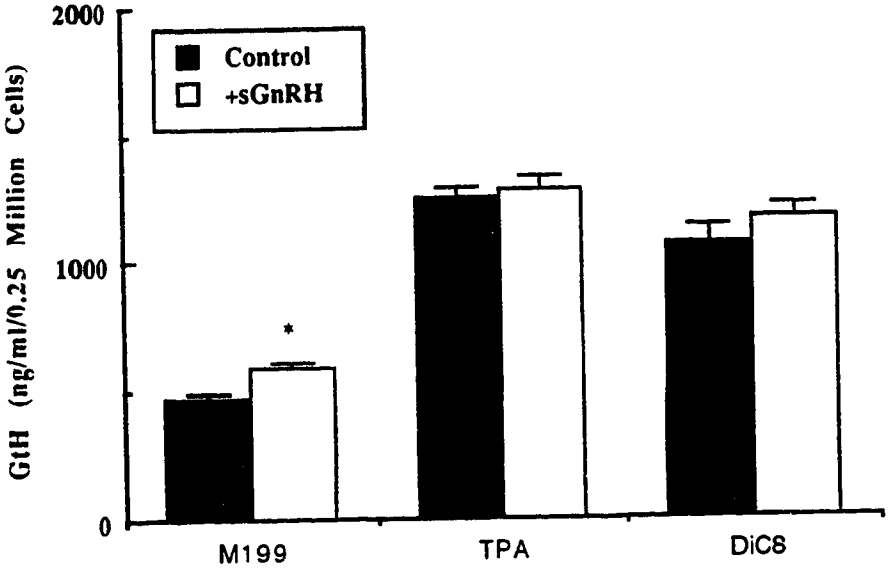
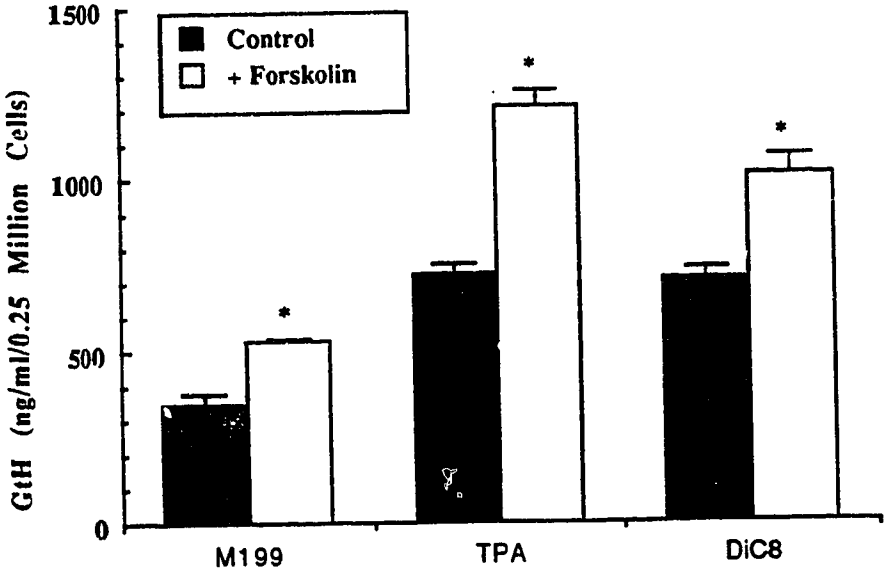

Accumulated GtH (ng/ml/0.6 Million Cells)
500
400
300
200
100
0
Peak A C B
Plateau A C B
Total A C B
Pre-Pulse
A
Test-Pulse
B
Post-Pulse
C

Figure 5.6. Additivity of GtH responses stimulated by PKC activators with those stimulated by 100 nM sGnRH (upper panel) and 10 μM forskolin (lower panel) in static culture. Representative data from one of three experiments with similar results are presented. Results are presented as mean ± SE (n=4). * indicates values that are significantly different from controls as determined by Student's t-test ($p<0.05$).


2000
1000
0
GtH (ng/ml/0.25 Million Cells)
Control
+sGnRH
*
M199
TPA
DiC8


1500
1000
500
0
GtH (ng/ml/0.25 Million Cells)
Control
+ Forskolin
*
*
*
M199
TPA
DiC8

Chapter 6

Actions of Two Native GnRHs and Protein Kinase C Modulators on Goldfish Pituitary Cells. Studies on Intracellular Calcium Levels and Gonadotropin Release.[3]

Introduction

Gonadotropin-releasing hormone (GnRH) has long been known as an important stimulator of gonadotropin (GtH) secretion in vertebrates. The goldfish (*Carassius auratus*) possesses two closely related GnRHs, these being salmon GnRH ([Trp^7, Leu^8]-GnRH, sGnRH) and chicken GnRH II ([His^5,Trp^7, Tyr^8]-GnRH, cGnRH II) [1]. These two forms of GnRH are present in the pituitary and throughout the rest of the brain. Both GnRH forms stimulate the release of GtH *in vivo* and *in vitro* in the goldfish [2-3]. In pituitary membrane preparations sGnRH and cGnRH II compete for the same class of high affinity/low capacity receptors [2, 4-5]. Electronmicroscope studies demonstrate that avidin gold-labelled biotinylated sGnRH analog is displaced from the surfaces of immunohistochemically identified gonadotropes by both unlabeled sGnRH and cGnRH II [6]. These results indicate that both native GnRH peptides bind to the same cell surface receptors to induce GtH secretion in the goldfish. Recent results indicate that sGnRH and cGnRH II stimulation of GtH secretion in the goldfish involves extracellular Ca^{2+} ($[Ca^{2+}]_o$) entry into gonadotropes [7]. In mammals, a similar $[Ca^{2+}]_o$ dependence exists in GnRH-stimulated luteinizing hormone (LH) secretion [8-10]. In goldfish, although the

[3]A version of this chapter has been published. Jobin and Chang 1992. Cell Calcium 13:531-540.

two GnRHs bind to the same class of receptor they appear to stimulate dissimilar signal transduction pathways [7,11]. Specifically, when compared to the GtH secretion elicited by sGnRH, cGnRH II-stimulated GtH release is more sensitive to inhibition by removal of $[Ca^{2+}]_o$ or addition of voltage-sensitive Ca^{2+} channel (VSCC) antagonists [7]. Conversely, sGnRH-, but not cGnRH II-stimulated GtH release, has a component which is blocked by inhibitors of the lipoxygenase enzyme that metabolizes arachidonic acid [11].

Despite the importance of $[Ca^{2+}]_o$ and VSCCs in mediating GnRH action in the goldfish, changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) concentration during GnRH action have not been demonstrated in the goldfish or in any other teleost. To further investigate the involvement of Ca^{2+} in the mediation of sGnRH and cGnRH II action, the influences of both native GnRHs on $[Ca^{2+}]_i$ levels in dispersed goldfish pituitary cells were monitored under normal and Ca^{2+}-deficient incubation conditions. Fluorescence emissions from cells preloaded with the Ca^{2+}-sensitive dye Fura 2 were quantified as indices of free $[Ca^{2+}]_i$ levels.

In mammals, it has been suggested that protein kinase C (PKC) mediates GnRH stimulation of LH release and modulates $[Ca^{2+}]_o$ entry and VSCC function [12]. There is also evidence that suggests that PKC may mediate sGnRH- and cGnRH II-stimulated GtH release in the goldfish [13]. In the present study, the possibility that GnRH-stimulated Ca^{2+} mobilization is mediated through PKC activation in the goldfish was also investigated. Two PKC stimulators, tetradecanoyl phorbol-13-acetate (TPA) and 1,2-dioctanoylglycerol (DiC8), and the PKC inhibitor staurosporine were tested for their effects on $[Ca^{2+}]_i$ concentrations. To further examine the $[Ca^{2+}]_o$ dependence of the actions of these PKC activators, modulators of $[Ca^{2+}]_o$ entry into cells were tested for their effects on TPA- and DiC8-stimulated GtH release.

Materials and Methods

General. Common goldfish (8-12 cm in length), purchased from Ozark Fisheries Inc., Stoutland, Missouri and Grassyforks Fisheries, Martinsville, Indiana, were transferred to flow-through aquaria (1800 liters) immediately on arrival. The fish were held at 17-20 ^{o}C on a simulated natural (Edmonton, Alberta) photoperiod, and fed to satiation daily with commercial fish food. Fish of both sexes were acclimated to the above conditions for at least 7 days before use. Stock solutions of sGnRH, cGnRH II (Peninsula Lab. Inc., Belmont, CA) and KCl were dissolved in distilled deionized water. Verapamil (Sigma, St. Louis, MO) and Bay K8644 (Calbiochem, San Diego, CA) were dissolved in ethanol. TPA (Sigma, St. Louis, MO), DiC8, staurosporine and A23187 (Calbiochem, San Diego, CA) were dissolved in dimethylsulfoxide.

Static Cell Culture and GtH response. Pituitaries from fish of both sexes were removed and enzymatically dispersed [for details see 3]. The dispersed cells were cultured overnight in 24-well culture plates with medium 199 containing Earle's salts (Gibco, Grand Island, NY), 1% horse serum, 25 mM Hepes, 2.2g/l sodium bicarbonate, 100 000 units/l penicillin and 100 mg/l streptomycin, pH 7.2, at a density of 0.25 million cells/ well/ ml. The cells were incubated at 28 ^{o}C, 5% CO_2 and saturated humidity. The following day the medium was replaced with medium 199 containing Hank's Salts (Gibco), 25 mM Hepes, 2.2g/l sodium bicarbonate, 100 000 units/l penicillin, 100 mg/l streptomycin and 0.1% bovine serum albumin at pH 7.2 (M199 with Hank's salts). A special formulation of M199 containing Hank's salts made without $CaCl_2$ (Gibco) was utilized for experiments with altered Ca^{2+} concentrations. Test solutions diluted in the proper vehicle were then added (1 µl/ml to achieve desired final concentration). Inhibitors were usually added 10 minutes prior to application of the secretagogues. The concentration of vehicle was less than 0.1% of the final incubation volume and did not alter GtH release. Following a further 2 hr incubation the media were removed and stored at -20 ^{o}C until their GTH

contents were measured using an established radioimmunoassay [14]. All treatments were carried out in quadruplicate or sextuplicate. Experiments were repeated a minimum of three times. All samples from individual experiments were assayed in duplicate within the same radioimmunoassay. Results from replicate experiments are expressed as a percentage of the net GtH response to maximal TPA (10 nM) or DiC8 (100 μM) stimulation. Data from replicate experiments were pooled prior to statistical analysis by Student's *t* -test.

Measurement of $[Ca^{2+}]_i$ concentrations with Fura 2. Dispersed pituitary cells (prepared as described above) were plated in 35x10 mm culture dishes in 3 ml of medium 199 containing Earle's salts (Gibco), 1% horse serum, 25 mM Hepes, 2.2g/l sodium bicarbonate, 100 000 units/l penicillin and 100 mg/l streptomycin, pH 7.2, at a density of 5 million cells per dish at 28 °C, 5% CO_2 and saturated humidity. The following day the cells were harvested and resuspended in 1.5 ml of M199 with Hank's Salts. Fura 2-AM (Calbiochem, San Diego, CA; 6 μM) was then added and the cells (5 million) were incubated for 60 min at 28°C, 5% CO_2 and saturated humidity to facilitate cellular entry of the Fura 2 AM. Density of cells, concentration of Fura 2 AM, incubation time and temperature have all been varied to determine these optimal loading conditions. Following Fura 2 loading, the cells were spun down (200xg, 10 min) and the unreacted Fura 2 AM remaining in the extracellular medium was removed by washing with Fura medium (M199 with Hank's Salts but without phenol red) or Ca^{2+}-free Fura medium (without addition of $CaCl_2$). The cells were resuspended in Fura medium and transferred to a 3 ml quartz cuvette for assay of $[Ca^{2+}]_i$ concentration by fluorescence analysis at 28°C using a SML8000 spectrofluorometer (SML Instruments, Inc., Urbana, IL) at excitation wavelengths of 350 and 380 nm and an emission wavelength of 510 nm. Test substances were added to the cuvette in 2-5 μl of vehicle. When added alone, none of the vehicles affected $[Ca^{2+}]_i$ levels. Using the equation developed by Grynkiewich et al., [15], $[Ca^{2+}]_i$ concentrations were calculated from the ratio of the emission signals elicited by the

two excitation wavelengths.

$$[Ca^{2+}] = K_d \times (S_{f2}/S_{b2}) \times [(R-R_{min})/(R_{max}-R)]$$

Where: R= the ratio of the two signals at the point you want to measure; R_{min}= the minimum ratio of signals produced (measured in 10 mM EGTA); R_{max}= the maximum ratio of signals produced (measured after addition of 100 μM ionomycin); K_d= the dissociation constant of Fura-2 and Ca^{2+}; and S_{f2}/S_{b2} = free dye proportionality constant/ bound dye proportionality constant.

To determine the Kd and proportionality constants (defined above) calibration curves for free Ca^{2+} concentration were constructed. Different levels of free Ca^{2+} used in calibration curves were achieved by adding $CaCl_2$ in increasing increments to Ca^{2+}-free Fura medium containing 10 mM EGTA. Special attention was given to the maintenance of a constant pH of 7.2. Estimates of free Ca^{2+} concentrations for the calibration curves were determined using a computer program [16]. Increasing levels of free Ca^{2+} cause the fluorescence response to 350 nm excitation to increase while the signal elicited from 380 nm excitation decreases. Ratio of fluorescence at both channels (350 nm/ 380 nm) increased with increasing Ca^{2+} concentration to a maximum at approximately 1 mM free Ca^{2+}. From five separate trials, the Kd of Ca^{2+} and Fura 2 was estimated to be 623 nM and the proportionality constant (S_{f2}/S_{b2}) was estimated to be 6.1. Statistical comparisons were performed with Student's *t* -test. Levels of significance were set at 95%.

Results

Effects of GnRHs, PKC stimulators and a PKC inhibitor on the levels of $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ responses to sGnRH, cGnRH II, TPA, DiC8 and KCl were first examined under normal Ca^{2+} (1.25 mM) incubation conditions. Increases in $[Ca^{2+}]_i$ were measured as the difference between the basal (unstimulated) level and the maximum (peak) value

stimulated by the addition of the test substance to the cell suspension. Addition of both sGnRH and cGnRH II (1 μM doses) increased the concentration of free $[Ca^{2+}]_i$ in the dispersed goldfish pituitary cells (Fig 6.1 A&B). 30 mM KCl also stimulated a rapid elevation in $[Ca^{2+}]_i$ levels (Fig 6.1 C); this same dose of KCl stimulates GtH release from goldfish pituitary cells in perifusion (RM Jobin and JP Chang, unpublished data). The PKC activators, TPA (10 nM) and DiC8 (100 μM), also elicited a marked increase in $[Ca^{2+}]_i$ concentrations (Fig 6.2 A&B). Interestingly, DiC8 produced a biphasic response with two apparent peaks, the first peak (DiC8-1) occurred immediately after stimulation and was short-lived, whereas the second peak (DiC8-2) became apparent only after the first peaked had begun to decay and had a longer duration (Fig 6.2 B). In rat gonadotropes, stimulation of PKC by exposure to TPA has also been reported to have biphasic effects on $[Ca^{2+}]_i$ concentrations [17]. Pre-exposure to either TPA (Fig 6.2 A) or DiC8 (Fig 6.2 B) totally abolished subsequent responses to KCl, suggesting that prior treatment with the PKC stimulators inactivated the VSCCs.

To investigate the relative contributions of $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ pools to the total Ca^{2+} response, experiments were repeated using normal Fura medium and Ca^{2+}-free Fura medium (prepared without $CaCl_2$ but not neccessarily totally devoid of Ca^{2+}) in paired preparations. Incubation with Ca^{2+}-free Fura medium did not significantly alter unstimulated $[Ca^{2+}]_i$ levels in the pituitary cells but abolished increases in $[Ca^{2+}]_i$ levels stimulated by KCl. This result suggests that although a gradient of Ca^{2+} may still exist between the intracellular and extracellular domains when the cells are incubated with the Ca^{2+}-free medium, this gradient is not great enough to facilitate $[Ca^{2+}]_o$ entry when the cells are stimulated, thus making the incubation solution physiologically Ca^{2+}-free. Under these Ca^{2+}-deficient conditions the $[Ca^{2+}]_i$ responses elicited by TPA, cGnRH II and the first phase of DiC8 response (DiC8-1) were also abolished. In contrast the sGnRH and DiC8-2 responses were not totally abolished by incubation with Ca^{2+}-deficient media,

significant elevations still occurred (Fig. 6.3). Compared to the responses observed under normal testing conditions, increases in $[Ca^{2+}]_i$ stimulated by sGnRH in Ca^{2+}-free medium was significantly reduced while the DiC8-2 response was not.

In two separate experiments the addition of 100 nM staurosporine, a PKC inhibitor, significantly suppressed the $[Ca^{2+}]_i$ responses to cGnRH II and TPA. (Increases in $[Ca^{2+}]_i$ - Control: cGnRH II 81.4±18.7 nM n=7, TPA 420.6±83.9 nM n=5; Staurosporine: cGnRH II 36.7±2.3 nM n=2, TPA 223.0±1.48 nM n=2). Staurosporine was added to the cuvette 1 minute prior to treatment with TPA or cGnRH II were added.

Extracellular Ca^{2+} dependence of TPA- and DiC8-stimulated GtH secretion. Since the PKC activators exhibited differences in the Ca^{2+} responses they elicited from populations of mixed cells; the Ca^{2+} dependence of TPA- and DiC8-stimulated GtH release were also compared to determine if the difference in the actions of TPA and DiC8 apply to the gonadotropes. Both DiC8 and TPA stimulated GtH release from static incubations of dispersed pituitary cells in a dose dependent manner (Fig. 6.4-6.7). Incubation with Ca^{2+}-deficient medium inhibited both TPA- and DiC8-stimulated GtH secretion but did not alter unstimulated GtH levels (Fig. 6.4). GtH secretion stimulated by 10 pM, 1 nM and 10 nM TPA as well as 1 and 100 μM DiC8 were significantly inhibited by incubation in Ca^{2+}-deficient medium. GtH responses to maximally effective doses of TPA and DiC8 were reduced by 52% and 33%, respectively.

Treatments with the dihydropyridine VSCC agonist Bay K8644 (100 nM) had no effects on DiC8-stimulated GtH secretion, but significantly potentiated the GtH-releasing properties of submaximal doses of TPA (10 & 1000 pM) (Fig. 6.5). Bay K8644 did not significantly affect basal secretion of GtH.

The phenylalkylamine VSCC antagonist verapamil (1 μM) had been previously shown to reduce the GtH response to increasing concentrations of TPA (inhibition of maximal responses to TPA by verapamil averaged 30 %) [16]. In the present study,

verapamil (1 μM) also reduced the GtH response elicited by 1 mM DiC8 by 20 % (Fig. 6.6). Verapamil had no effect on unstimulated GtH secretion.

The Ca^{2+} ionophore A23187 (10 μM) stimulated GtH release. This stimulated release was not additive to those induced by either TPA or DiC8. However, A23187 significantly attenuated the GtH release response to the maximally effective dose (10 nM) of TPA (Fig.. 6.7).

Discussion

In the goldfish, sGnRH- and cGnRH II-stimulated GtH secretion are inhibited by reduction of $[Ca^{2+}]_o$ levels or treatment with VSCC antagonists, suggesting the involvement of $[Ca^{2+}]_o$ entry into gonadotropes during GnRH action [7,18]. The involvement of $[Ca^{2+}]_o$ in the action on GnRH on GtH release has also been implicated in the African catfish (*Clarias gariepinus*) [19], murrel (*Channa puntatus*) [20] and tilapia (*Tilapia* sp.) [21]. However, there was no direct evidence showing that GnRH can stimulate changes in $[Ca^{2+}]_i$ concentration in pituitary cells of the teleost. The present study provides the first direct evidence that GnRH stimulates increases in $[Ca^{2+}]_i$ in fish pituitary cells. Both sGnRH and cGnRH II stimulate increases in $[Ca^{2+}]_i$ levels of approximately 100 nM; these changes are comparable to those obtained from enriched gonadotrope populations from the rat [9,22-24]. Since both native GnRH peptides stimulate the release of GtH as well as growth hormone (GH) in an $[Ca^{2+}]_o$ dependent manner in the goldfish [7,25] the source of the fluorescent signal in the present study may not be specific to the gonadotropes. Under Ca^{2+}-deficient incubation conditions sGnRH- and cGnRH II-stimulated GH, as well as cGnRH II-stimulated GtH, secretion are abolished but sGnRH still elicits GtH secretion [7]. In the present study, Fura 2 results

obtained under Ca^{2+}-deficient conditions indicate that sGnRH-stimulated elevations in $[Ca^{2+}]_i$ concentrations include a component that is not dependent on $[Ca^{2+}]_o$ entry. This finding parallels the $[Ca^{2+}]_o$ dependency of the GtH response to sGnRH in goldfish [7]. Both results taken together imply that sGnRH but not cGnRH II action has an $[Ca^{2+}]_i$ mobilization component.

In previous studies, TPA-stimulated GtH release was attenuated by the removal of $[Ca^{2+}]_o$ and treatment with the VSCC antagonist verapamil [13]. This study provides further support for the hypothesis that in the goldfish, GtH release stimulated by PKC stimulators is mediated in part by $[Ca^{2+}]_o$ entry through VSCCs. Like TPA, DiC8-stimulated GtH release is reduced by $[Ca^{2+}]_o$ removal and treatment with the VSCC antagonist verapamil (Fig. 6.4 & 6.6). The GtH responses to submaximal doses of TPA are potentiated by the VSCC agonist Bay K8644 (Fig. 6.5). TPA and DiC8 also stimulate increases in $[Ca^{2+}]_i$ concentrations in dispersed goldfish pituitary cells in an $[Ca^{2+}]_o$ dependent manner (Fig. 6.3). Furthermore, KCl-stimulated elevations in $[Ca^{2+}]_i$ concentrations are abolished by pretreatment of cell suspensions with TPA or DiC8 suggesting the involvement of VSCCs in their actions. These data indicate that in goldfish pituitary cells, PKC activators elicit $[Ca^{2+}]_o$ entry at least in part through VSCCs. PKC activators also stimulate $[Ca^{2+}]_o$ entry through VSCCs in other cell types including the gonadotropes of the rat [26, 27].

Several lines of evidence suggest that activation of PKC may mediate GnRH-stimulated GtH secretion in the goldfish. In previous studies, both TPA and the native goldfish GnRHs stimulate the release of GtH. These GtH responses are inhibited by removal of $[Ca^{2+}]_o$, addition of the VSCC antagonist verapamil or use of the PKC inhibitor H7 [7,13]. The present study provides further evidence that TPA- and DiC8-stimulated GtH secretion are partially dependent on $[Ca^{2+}]_o$ entry through VSCCs (see discussion above). Furthermore, increases in $[Ca^{2+}]_i$ levels in response to both TPA and an native GnRH are attenuated by the PKC inhibitor staurosporine. These new data are

consistent with the hypothesis that PKC-activated opening of VSCCs mediate GnRH-stimulated GtH secretion. Although there is controversy over the possible involvement of PKC in GnRH action in the rat model [28], PKC activation has been proposed to mediate GnRH-induced $[Ca^{2+}]_o$ entry and prolonged LH release [9,12].

Although TPA and DiC8 both stimulate PKC, the results of the present study indicate that differences exist in their actions. Both TPA and DiC8 stimulate increases in $[Ca^{2+}]_i$; however, under Ca^{2+}-deficient conditions TPA-induced $[Ca^{2+}]_i$ responses are abolished but the DiC8-2 response persists. This result suggests that the DiC8 response is at least partially due to release from an $[Ca^{2+}]_i$ store, whereas the TPA response is entirely dependent on $[Ca^{2+}]_o$ entry. TPA and DiC8 also exhibit differences in the $[Ca^{2+}]_o$ dependence of their GtH-releasing abilities. Compared to DiC8, the GtH response to TPA was more susceptible to potentiation by the VSCC agonist Bay K8644. TPA-stimulated GtH release was also more easily inhibited by incubation with Ca^{2+}-deficient medium or the addition of the VSCC antagonist verapamil than the corresponding responses to DiC8. TPA and DiC8 also interacted differently with the ionophore A23187. At high doses the non-additivity of the TPA- and DiC8-stimulated GtH increases with that of A23187 is consistent with a general dependence on Ca^{2+} by both PKC activators. However, at maximally stimulatory doses, the presence of A23187 decreased TPA- but not DiC8-induced GtH release. The reason for the reduction of GtH response to TPA during ionophore treatment is at present unknown. In mammals, both phorbol esters and synthetic diacylglycerols compete for the same activation site on PKC, reduce the Ca^{2+} requirement for PKC activation, and share many of the same biological actions [29-30]. When differences between the two stimulators are found, the phorbol esters are usually either more potent or possess actions that are not mimicked by diacylglycerols [31-32]. Recently, synthetic diacylglycerols have been shown to have actions not duplicated by phorbol esters [33], and to have actions independent of PKC [34]. Our results also suggest that synthetic

diacylglycerols can have effects that phorbol esters can not duplicate. Further studies into the cause of the differences between TPA and DiC8 actions in the goldfish pituitary are required.

In this study, TPA, DiC8 and KCl stimulation produced larger Fura 2 signals than did sGnRH or cGnRH II. This difference in the magnitude of $[Ca^{2+}]_i$ response is probably due to the limitation of the effects of GnRHs to only gonadotropes and somatotropes (a small proportion of the entire pituitary cell population). Conversely, TPA, DiC8 and KCl affect all cell types. Although valuable data have been acquired with this system, further studies using single identified cells will be required to distinguish and confirm differences in responses of the different cell types to the different secretagogues.

In summary, the results of the present study are consistent with the involvement of $[Ca^{2+}]_o$ in sGnRH-, cGnRH II-, KCl-, TPA- and DiC8-stimulated GtH secretion, and indicate the involvement of $[Ca^{2+}]_i$ in sGnRH and DiC8 action. The hypothesis that sGnRH and cGnRH II may act through the same receptors to activate non-identical signal transduction pathways is supported by differences in the $[Ca^{2+}]_i$ responses stimulated by the two GnRHs under $[Ca^{2+}]_o$-def[illegible] conditions. This hypothesis may have wide-spread implications as most vertebrate groups (perhaps with the exception of placental mammals) have more than one form of endogenous GnRH peptide [35]. Data from measurements of both GtH release and $[Ca^{2+}]_i$ concentrations also provide further evidence for the involvement of PKC in mediating GnRH-stimulated GtH secretion from goldfish gonadotropes. Evidence for differences in the actions of the PKC activators TPA and DiC8 is also provided.

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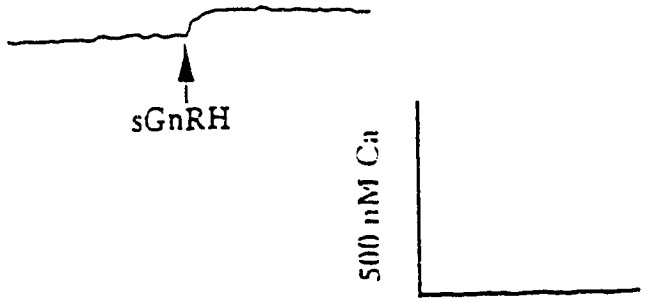
Fig. 6.1. Effects of 1 μM sGnRH (panel A), 1 μM cGnRH II (panel B) and 30 mM KCl (panel C) on levels of $[Ca^{2+}]_i$ as measured by the fluorescent dye Fura 2. Example actual traces from experiments are shown. Points where compounds were added to the cuvette and the nature of the compound are indicated on each panel., Average $[Ca^{2+}]_i$ responses (Δ Ca; Mean±SE) to each compound are also indicated.

A

sGnRH (Δ Ca 110.7 ± 17.0 nM, n=13)



B

cGnRH II (Δ Ca 111.9 ± 37.7 nM, n=7)

C

KCl (Δ Ca 684.6 ± 100.9 nM, n=6)

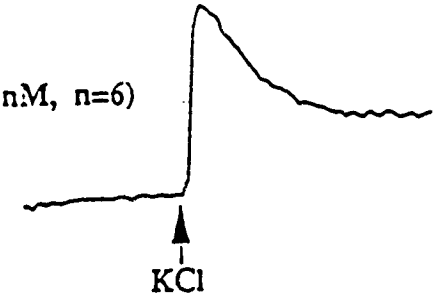
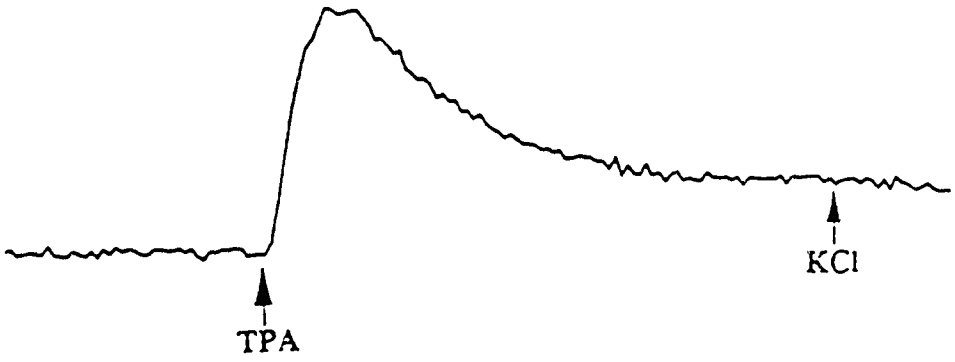
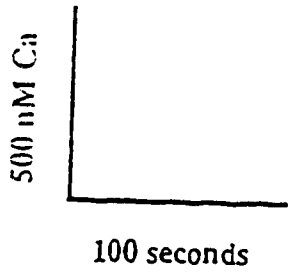


Fig. 6.2. Effects of 10 nM TPA (panel A) and 100 μM DiC8 (panel B) on levels of $[Ca^{2+}]_i$ as measured by the fluorescent dye Fura 2. Examples of actual traces from experiments are shown. Points where compounds were added to the cuvette and the nature of the compound are indicated on each panel. Average $[Ca^{2+}]_i$ responses (Δ Ca; Mean±SE) to TPA and both phases of the DiC8 response are also indicated.

A

TPA (Δ Ca 532.1 ± 75.6 nM, n=8)





B

DiC8-2 (Δ Ca 320.1 ± 40.3 nM, n=5)

DiC8-1 (Δ Ca 209.0 ± 41.1 nM, n=5)

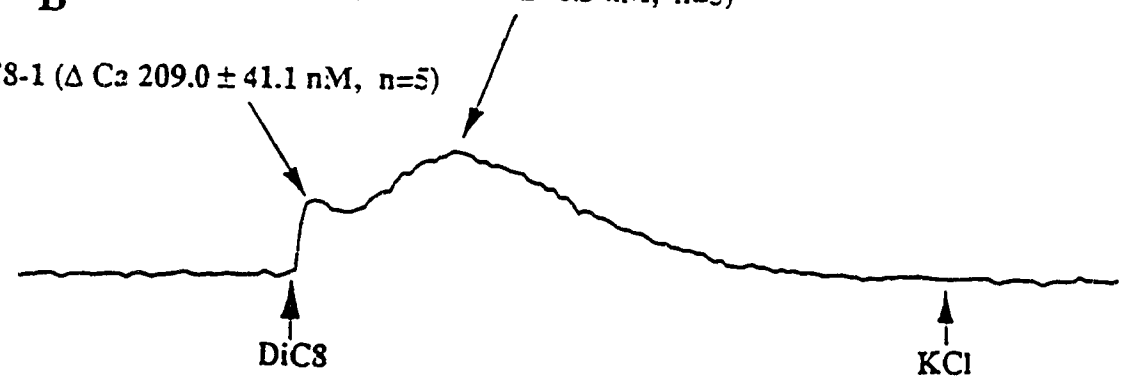


Fig. 6.3. Effects of removal of $[Ca^{2+}]_o$ on changes in $[Ca^{2+}]_i$ concentrations induced by 1 μM sGnRH (n=5), 1 μM cGnRH II (n=4), 100 μM DiC8 (n=4), 10 nM TPA (n=5) and 30 mM KCl (n=4). In six paired experiments unstimulated $[Ca^{2+}]_i$ levels under normal (253.0±36.4 nM) and Ca^{2+}-deficient conditions (203.0±57.9) were not significantly different. *, significantly lower than the value obtained under normal Ca^{2+} conditions and also significantly elevated above unstimulated intracellular Ca^{2+} levels.

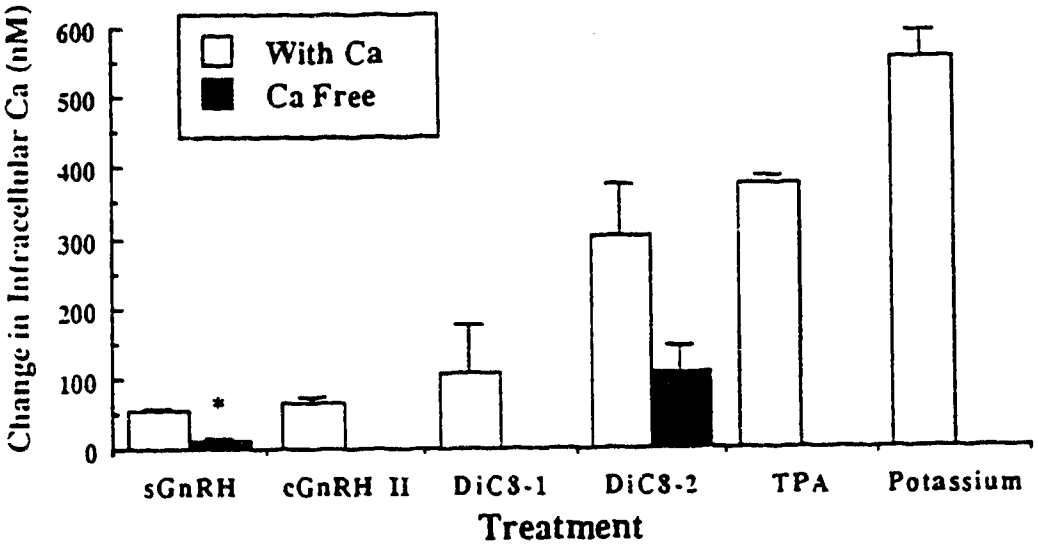
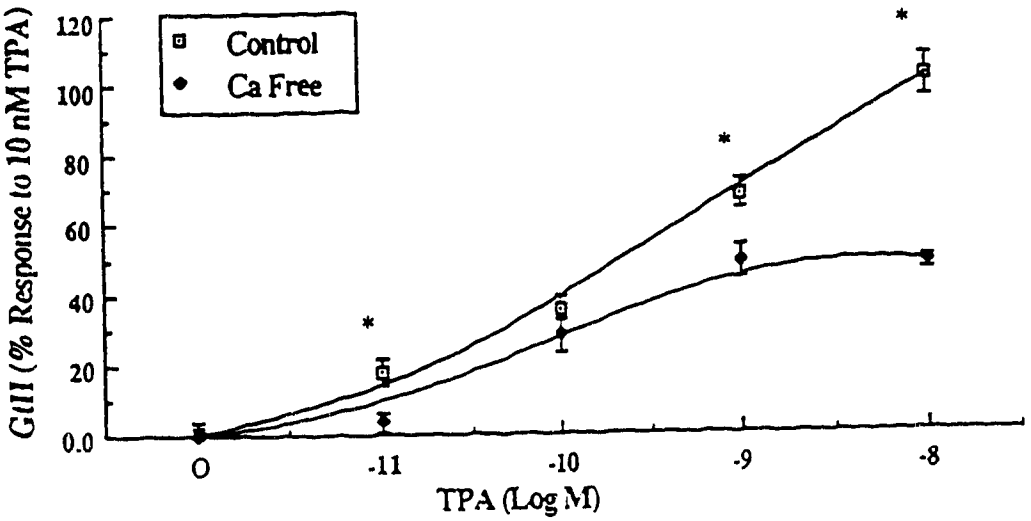

Change in Intracellular Ca (nM)
600
500
400
300
200
100
0
With Ca
Ca Free
*
sGnRH
cGnRH II
DiC8-1
DiC8-2
TPA
Potassium
Treatment

Fig. 6.4. Effects of incubation with Ca^{2+}-deficient medium on TPA (upper panel) and DiC8 (lower panel) stimulation of GtH release from static incubations of dispersed goldfish pituitary cells. Results presented (Mean±SE) are pooled data from three replicate experiments. Average basal GtH release was 217.3±32.5 ng/ml/0.25 million cells. Average net GtH response to 10 nM TPA and 100 μM DiC8 were 426±26 and 464±54 ng/ml/0.25 million cells, respectively. *, significantly ($P<0.05$) different from control values.


GII (% Response to 10 nM TPA)
120
100
80
60
40
20
0.0
Control
Ca Free
*
*
*
0
-11
-10
-9
-8
TPA (Log M)

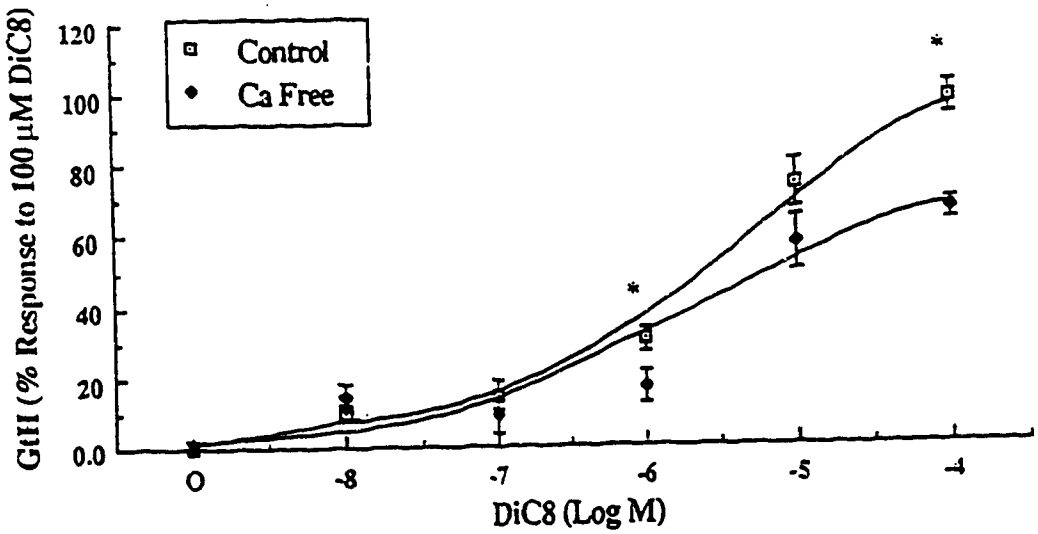
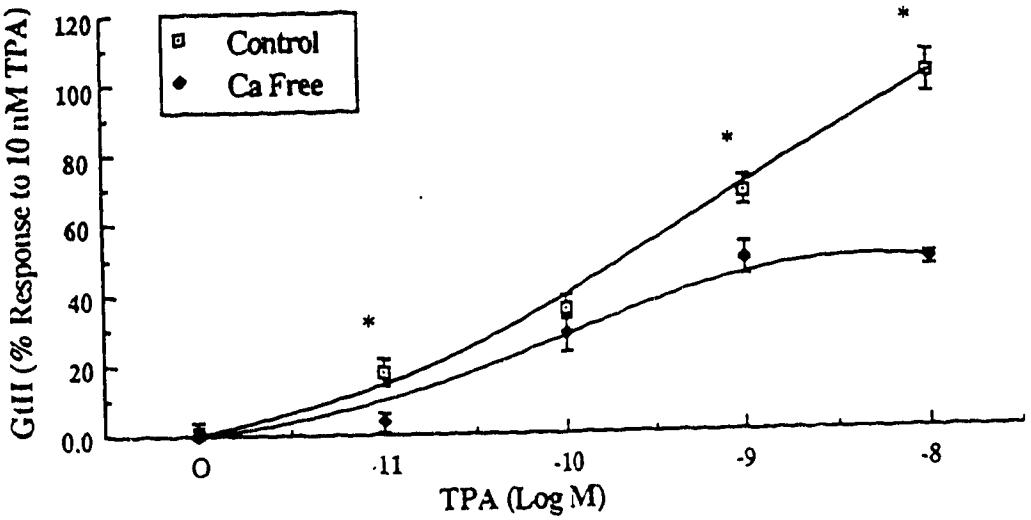

GII (% Response to 100 µM DiC8)
120
100
80
60
40
20
0.0
Control
Ca Free
*
*
0
-8
-7
-6
-5
-4
DiC8 (Log M)

Fig. 6.5. Effects of 100 nM Bay K8644 on TPA (upper panel) and DiC8 (lower panel) stimulation of GtH release from static incubations of dispersed pituitary cells. Results presented (Mean±SE) are pooled data from three replicate experiments. Average basal GtH release was 274.0±41.9 ng/ml/0.25 million cells. Average net GtH response to 10 nM TPA and 100 μM DiC8 were 673±79 and 509±62 ng/ml/0.25 million cells, respectively. *, significantly ($P<0.05$) different from control values.


Control
Ca Free
GtH (% Response to 10 nM TPA)
120
100
80
60
40
20
0.0
0
-11
-10
-9
-8
TPA (Log M)
*
*
*

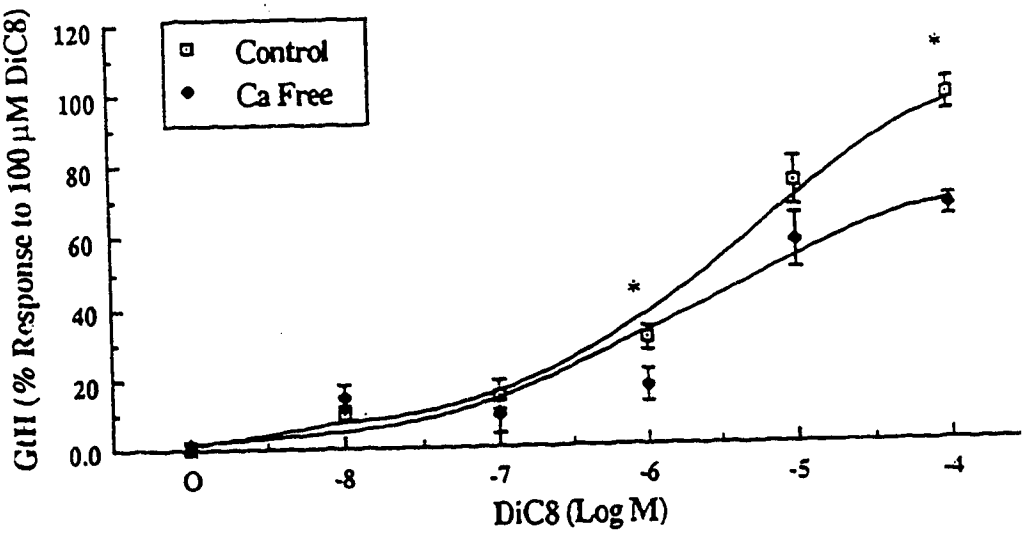

Control
Ca Free
GtH (% Response to 100 µM DiC8)
120
100
80
60
40
20
0.0
0
-8
-7
-6
-5
-4
DiC8 (Log M)
*
*
*

Fig. 6.6. Effects of 1 μM verapamil on DiC8-stimulated GtH release from static incubations of dispersed goldfish pituitary cells. Results presented (Mean±SE) are pooled data from three replicate experiments. Average basal GtH release was 400.0±15.0 ng/ml/0.25 million cells. Average basal GtH release was 274.0±41.9 ng/ml/0.25 million cells. Average net GtH response to 100 μM DiC8 were 647±20 ng/ml/0.25 million cells. *, significantly ($P<0.05$) different from control values.

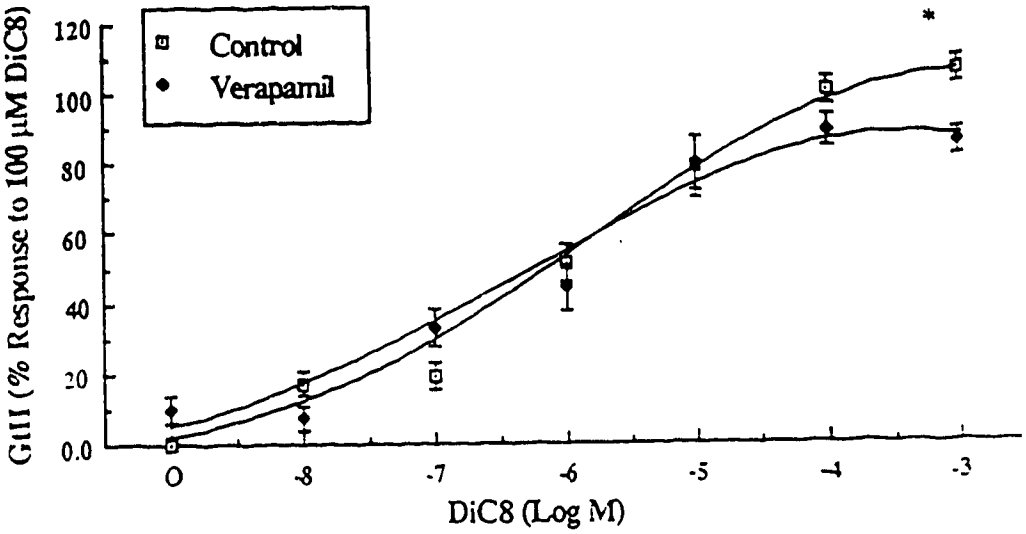
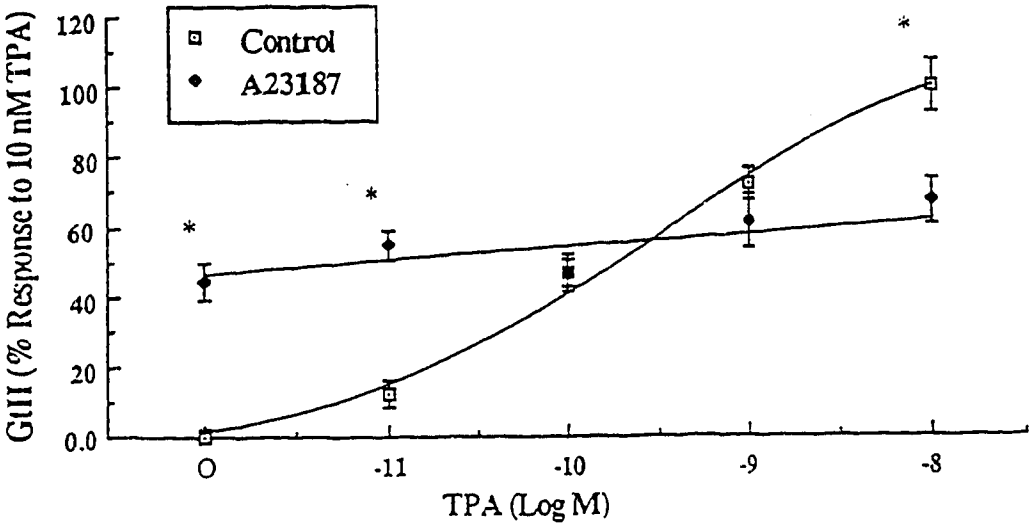
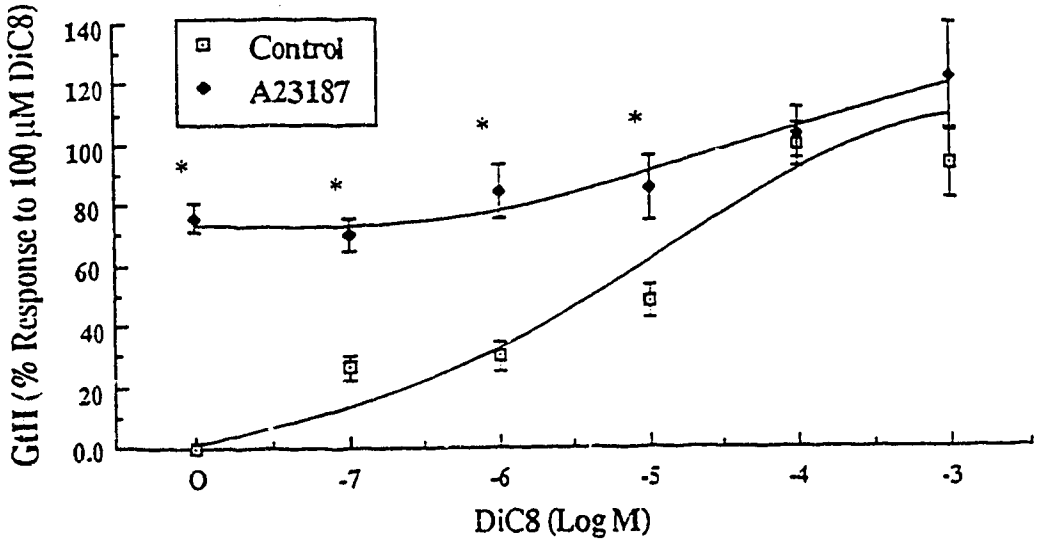

GII (% Response to 100 µM DiC8)
120
100
80
60
40
20
0.0
Control
Verapamil
*
0
-8
-7
-6
-5
-4
-3
DiC8 (Log M)

Fig. 6.7. Effects of 10 μM A23187 on TPA (upper panel) and DiC8 (lower panel) stimulation of GtH release from static incubations of dispersed goldfish pituitary cells. Results presented (Mean±SE) are pooled data from three replicate experiments. Average basal GtH release was 291.6±46.7 ng/ml/0.25 million cells. Average net GtH response to 10 nM TPA and 100 μM DiC8 were 766±90 and 509±62 ng/ml/0.25 million cells, respectively. *, significantly ($P<0.05$) different from control values.


GtII (% Response to 10 nM TPA)
120
100
80
60
40
20
0.0
Control
A23187
*
*
*
*
O
-11
-10
-9
-8
TPA (Log M)


GtII (% Response to 100 µM DiC8)
140
120
100
80
60
40
20
0.0
Control
A23187
*
*
*
*
O
-7
-6
-5
-4
-3
DiC8 (Log M)

Chapter 7

An Assessment of the Roles of Calcium and Protein Kinase C in the Initiation and Amplification of Gonadotropin Secretion from Dispersed Pituitary Cells of the Goldfish.

Introduction

In mammals, calcium (Ca^{2+}) plays a significant role in mediating release of several different pituitary hormones, including, luteinizing hormone (LH), follicle stimulating hormone, thyrotropin and adrenocorticotropin [1]. This is also the case with gonadotropin (GtH) release from dispersed pituitary cells of the goldfish (*Carassius auratus*). GtH secretion elicited by the two native GnRH peptides of the goldfish (sGnRH and cGnRH II), and activators of PKC, tetradecanoyl phorbol 13 acetate (TPA) and dioctanoyl glycerol (DiC8), were found to be at least partially dependent on extracellular Ca^{2+} ($[Ca^{2+}]_e$) entry into cells [2, 3]. Ionophores (A23187, ionomycin) also elevated GtH secretion [3; Chapter 4]. Furthermore, sGnRH, cGnRH II, TPA and DiC8 were all effective in elevating intracellular Ca^{2+} ($[Ca^{2+}]_i$) levels of populations of dispersed pituitary cells [3]. Taken together, the above results indicate that, in goldfish pituitary cells, the GtH-releasing actions of GnRH and several other secretagogues are dependent upon Ca^{2+} and mobilization of Ca^{2+}.

Similar to the goldfish, GnRH and other secretagogues that elicit LH release in the rat also stimulate changes in $[Ca^{2+}]_i$ [for reviews see 4, 5, 6]. The importance of $[Ca^{2+}]_i$ in mediation of LH release is illustrated by the close correlation between both the time course

and dosage dependence of GnRH-stimulated LH release and Ca^{2+} mobilization observed in populations of rat gonadotropes [6]. In the rat, it is believed that GnRH-stimulated LH release is initiated by release of Ca^{2+} from intracellular stores, probably due to inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$). This process elevates $[Ca^{2+}]_i$ and leads to activation of Ca^{2+}-dependent enzymes, such as PKC. PKC serves to amplify the hormone-release signal initiated by Ca^{2+}, leading to continued LH secretion and $[Ca^{2+}]_o$ entry into gonadotropes. $[Ca^{2+}]_o$ entry is required to replenish $[Ca^{2+}]_i$ pools and for sustained LH release [reviewed by 4]. The role of amplifier was assigned to PKC, based mainly on data which showed that in cells with elevated levels of $[Ca^{2+}]_i$ levels, application of TPA elevated LH release while reducing $[Ca^{2+}]_i$ levels [7]. Thus, once the initiator (Ca^{2+}) has started the hormone-release response, the amplifier (PKC) can continue and amplify the LH response while reducing the signal of the initiator, thereby reducing the likelihood of desensitization.

In the present study, the role that $[Ca^{2+}]_i$ and PKC play in the initiation and/or maintenance of GtH release from goldfish pituitary cells is examined. To study the role of $[Ca^{2+}]_i$, the GnRH receptor is bypassed and Ca^{2+} levels are altered by more direct means. The methods used to alter $[Ca^{2+}]_i$ levels include, treatment with medium containing high levels of Ca^{2+}, exposure to Ca^{2+}-deficient medium, use of blockers of $[Ca^{2+}]_o$ entry into cells ($CoCl_2$, nifedipine) and depolarization of cells with KCl. Interactions with PKC were examined through the use of the PKC activator (TPA).

Material and Methods

General. Common goldfish (8-12 cm in length), purchased from Ozark Fisheries Inc., Stoutland, Missouri and Grassyforks Fisheries, Martinsville, Indiana, were

transferred to flow-through aquaria (1800 liters) immediately on arrival. The fish were held at 17-20 °C on a simulated natural (Edmonton, Alberta) photoperiod, and fed to satiation daily with commercial fish food. Fish of both sexes were acclimated to the above conditions for at least 7 days before use. sGnRH (Peninsula Lab. Inc., Belmont, CA) was dissolved in distilled deionized water. TPA (Sigma, St. Louis, MO) was dissolved in dimethylsulfoxide. Nifedipine (Sigma) was dissolved in ethanol. Aliquots of stock solutions were stored at -20 °C until used. Unless otherwise stated, KCl solutions were prepared and osmotically balanced by altering levels of NaCl in the media; the solutions with increased levels of Ca^{2+} were similarly prepared (special M199 preparation from the culture media unit, NIH, Bethesda, MD; courtesy of Dr. Stanko Stojilkovic, ERRB, NICHD, NIH). Ca^{2+}-deficient medium 199 with Hank's salts was obtained from Gib Grand Island, NY (special order).

Dispersed goldfish pituitary cells were used in all experiments in this study. Pituitaries from fish of both sexes were removed and the cells dispersed with a trypsin/DNAse procedure as previously described [8]. Dispersed pituitary cells were cultured overnight prior to all experiments. Amounts of GtH released was quantified using a radioimmunoassay validated for maturational GtH (GtH II) [9, 10].

Static cell culture and GtH response. Dispersed cells were cultured overnight in 24-well culture plates as previously described [8]. The following day, prior to experiments, the culture medium was replaced with testing medium (medium 199 containing Hank's Salts (Gibco), 25 mM Hepes, 2.2 g/l sodium bicarbonate, 100 000 units/l penicillin, 100 mg/l streptomycin, 0.1% bovine serum albumin, pH 7.2). Test solutions diluted in the proper vehicle were added (1 µl/ml to achieve desired final concentration). All treatments were carried out in quadruplicate. Following the testing period (2 hours), media were removed and stored frozen at -20 °C until processed for GtH contents by radioimmunoassay [9].

Column perifusion of mixed cell populations. A perifusion system using dispersed cells cultured on Cytodex-I beads as described by Chang et al., [11] was used. After loading on to the columns the cells were perifused for 4 hours to establish a relatively low and stable rate of unstimulated secretion, at which time the experiment was started. GtH responses to the different treatments were calculated by subtracting basal levels of secretion from those of the stimulated responses. Basal secretion of GtH was calculated by taking the average of the three fractions immediately preceding the stimulated GtH response. Fraction collection time and experimental protocol are described separately for the individual experiments (see figure legends).

Results

Effects of impeding the entry of extracellular Ca^{2+} on GtH release. After prolonged exposure to regular medium 199 with Hank's salts (1.26 mM Ca^{2+}) the dispersed fish pituitary cells were perifused with Ca^{2+}-deficient medium for 1 hour, Ca^{2+}-deficient medium supplemented with 1.26 mM $CoCl_2$ for 1.5 hours or normal medium 199 supplemented with 1 μM nifedipine for 1.5 hours (Fig. 7.1). All of these treatments that were designed to impede $[Ca^{2+}]_o$ entry into cells were effective in eliciting a significant transient increase in the release of GtH.

To further examine the influence of Ca^{2+} concentration on GtH release, an experiment was designed, where, cells were perifused for 90 minutes with perifusion medium containing either normal (1.26 mM) or high (20 mM) Ca^{2+} levels. Upon exposure to medium containing 20 mM Ca^{2+}, there was a significant decrease in basal GtH secretion which returned to control levels by the end of the 90 minute treatment (Fig. 7.2). When compared to controls (cells pretreated with medium containing normal Ca^{2+} levels), GtH release response to exposure to Ca^{2+}-deficient medium was significantly greater in the

cells receiving the high (20 mM) Ca^{2+} pretreatment (Fig. 7.2). No significant difference was observed between experimental and control groups in the sGnRH pulses pulse given at the beginning and end of the experiment (Fig. 7.2). This suggests that GnRH-stimulated GtH release was not compromised by by treatment with medium containing different Ca^{2+} levels.

Effects of KCl on GtH release in perifusion studies. Exposure to a 5-minute pulse of 30 mM KCl stimulated GtH release in perifusion studies (Fig. 7.3). Interestingly, it was apparent that the KCl-stimulated GtH response was delayed by 5 minutes as compared to the known GtH response kinetic to GnRH (Chapter 3, 4 and Appendix 1). Normally the GtH response will appear in the 5 minute fraction collected immediately following the 5-minute test pulse. This suggests that the KCl-stimulated GtH response was only manifested when the KCl was removed from the cells. This effect was clearly observed observed in cells receiving 7- and 20-minute pulses of 30 mM KCl. Note that the GtH response occurs at the end of each of these pulses regardless of pulse duration (Fig. 7.4). The dead volume of the perifusion system is such that an immediate response corresponding to the onset of drug treatment will be seen 6-8 minutes after the commencement of the treatment (Chapters 3 and 4). In perifusion experiments using other doses of KCl, similar delays in the GtH response were also seen (results not shown).

Effects of KCl and TPA, on GtH release in static culture. There was no dose dependent KCl-stimulated increase in GtH secretion in static culture (Fig. 7.5). GtH release was depressed by 10 mM KCl treatment. Conversely, 100 mM KCl significantly stimulated release of GtH (Fig. 7.5). In contrast to the results obtained using KCl alone, a release of GtH that correlated with the dose of KCl, administered was observed when a subthreshold dose of (0.01 nM) TPA was added simultaneously with increasing doses of KCl, (Fig. 7.5).

Discussion

Free $[Ca^{2+}]_i$ levels in cells are highly regulated because of their importance in cellular metabolism. $[Ca^{2+}]_i$ homeostasis can be influenced by $[Ca^{2+}]_o$ entry, Ca^{2+} efflux from the cell, Ca^{2+} sequestration into and release from intracellular stores. In the present study, treatment that were designed to reduce Ca^{2+} entry (incubation in Ca^{2+}-deficient medium, $CoCl_2$, nifedipine) caused a release of GtH. The reason for this release of GtH remains unclear. However, it can be hypothesized that a reduction in the influx of Ca^{2+} may trigger the release of Ca^{2+} from internal stores as a part of the Ca^{2+} homeostatic mechanism. The release of Ca^{2+} from intracellular stores may activate localized Ca^{2+}-sensitive intracellular mechanism which then leads to an increased secretion of GtH (for the importance of localized increases in $[Ca^{2+}]_i$ in GtH release, see discussion below). This hypothesis is supported by results from the experiment in which cells are exposed to medium containing high levels of Ca^{2+} (Fig. 7.2). Under these conditions of high $[Ca^{2+}]_o$, the Ca^{2+} concentration gradient across the plasma membrane would be increased causing an increase in Ca^{2+} influx. This in turn would stimulate the mechanisms regulating homeostasis of Ca^{2+} levels, such as sequestration into intracellular stores and extrusion to the outside of the cell. The ability of cells to restore the change in basal GtH release caused by exposure to high Ca^{2+} provided evidence for the operation of Ca^{2+} homeostatic mechanisms (Fig. 7.2). Following exposure to high $[Ca^{2+}]_o$, the amount of Ca^{2+} sequestered in internal stores was expected to be greater than in cells exposed to normal $[Ca^{2+}]_o$ levels (controls). Subsequently, the GtH responses elicited by exposure to Ca^{2+}-deficient medium was found to be greater from cells that had been pretreated with medium containing high Ca^{2+} than from control cells. These data suggest that the release of Ca^{2+} from intracellular stores participates in the secretion of GtH. Involvement of Ca^{2+} from intracellular Ca^{2+}

stores during GnRH-stimulated GtH release in goldfish pituitary cells has already been implicated [3].

In previous studies, where $[Ca^{2+}]_i$ levels were measured using the Ca^{2+}-sensitive dye Fura 2, KCl (30 mM) administration lead to an immediate and rapid increase of $[Ca^{2+}]_i$ levels in populations of dispersed goldfish pituitary cells [3]. In the present perifusion studies, although an increase in GtH secretion in response to 30 mM KCl was observed (Fig. 7.3), increased GtH release was not observed while the cells were kept depolarized by KCl (Fig. 7.4). A significant increase in GtH release was only observed after removal of KCl (Fig. 7.4). The ineffectiveness of K^+-induced depolarization in elevating GtH release is supported by data from static culture experiments. Continuous incubation (in static culture) with KCl ranging from 10-75 mM failed to increase GtH secretion (Fig. 7.5). These data indicate that, despite elevation of $[Ca^{2+}]_i$ levels during the application of KCl no GtH release was observed during its application (Figs. 7.4 and 7.5).

Similar to the increase in GtH release induced by treatments that impede $[Ca^{2+}]_o$ entry, the GtH-release response to the termination of KCl exposure may be the consequence of a perturbation in the Ca^{2+} homeostatic regulatory mechanisms. During the initial exposure to KCl, Ca^{2+} influx may have increased Ca^{2+} sequestration into internal stores. When KCl is removed, the closure of voltage-sensitive Ca^{2+} channels (VSCC) in the presence of the stimulated homeostatic mechanisms could also result in a transient depression of $[Ca^{2+}]_i$ levels. This decrease may in turn lead to release of Ca^{2+} from intracellular stores and the elevation of GtH release. This hypothesis requires further testing using $[Ca^{2+}]_i$ measurements of single gonadotrophs and populations of cells enriched with gonadotropes before further conclusions can be reached.

A novel finding from the above results is that a general increase in $[Ca^{2+}]_i$ levels alone may not stimulate GtH release from goldfish gonadotropes. In fact, perifusion with medium containing high levels of Ca^{2+} (Fig. 7.2) and static incubation with 10 mM KCl (Fig. 7.5) actually depressed GtH release. These results differ markedly form those

obtained from rat neurons [12], gonadotropes [7] and other pituitary cell types [13], where hormone secretion depends largely on and in correlated with $[Ca^{2+}]_i$ concentration. In rat gonadotropes, KCl stimulated an immediate release of LH as well as an increase in $[Ca^{2+}]_i$ [14]. In melanotropes, elevation of $[Ca^{2+}]_i$ by application of Ca^{2+} through a pipette resulted in an increased rate of exocytosis [13]. Therefore, in these mammalian cells, elevation of $[Ca^{2+}]_i$, independent of its source is sufficient to stimulate hormone release. This does not appear to be the case in goldfish gonadotropes. In addition, the lowering of basal GtH release during exposure to medium containing high Ca^{2+} concentrations in perifusion studies or low concentrations of KCl in static incubation experiments suggest the transient activation of some unknown Ca^{2+}-sensitive inhibitory mechanism on GtH release.

It should be noted that treatments that would cause elevation of overall $[Ca^{2+}]_i$ concentrations to extremely high levels, such as the addition of ionophores (Chapter 4) or 100 mM KCl (Fig. 7.5), do cause secretion of GtH. In rat gonadotropes, it has been observed that GnRH-stimulated Ca^{2+} responses tend to originate at a single intracellular location, and then travel across the cell [15]. This process could result in extremely high localized concentrations of Ca^{2+}. Conversely, the Ca^{2+} response stimulated by the VSCC agonist Bay K8644, which was extracellular in origin, was more dispersed and of lower magnitude. Additionally, Bay K8644 is less effective than GnRH in stimulating release of LH [16]. In the goldfish gonadotrope, Ca^{2+} release from intracellular stores could lead to high localized concentrations of $[Ca^{2+}]_i$ that may act to stimulate GtH release. Treatments which result in extremely high $[Ca^{2+}]_i$ levels may stimulate GtH release because they increase $[Ca^{2+}]_i$ levels to a level which is sufficient to stimulate the localized Ca^{2+} sensitive secretory mechanism. Regardless of whether the mechanism hypothesized above actually exists or not, the question of what triggers GtH release still remains. It is clear from the present study and previous experiments that although elevation of $[Ca^{2+}]_i$ levels

is required, it may not be sufficient to stimulate release of GtH (Figs. 7.4 and 7.5) [2; Chapters 2, 3 and 6].

To determine if activation of another signal transduction pathway is required before release of GtH can be stimulated; a subthreshold dose (one that does not stimulate GtH release) of the PKC activator TPA was added in combination with KCl in static culture experiments. With the addition of TPA, a dose-dependent increase in GtH release was observed with KCl-induced depolarization (Fig. 7.5). In previous studies, the VSCC agonist Bay K8644 also stimulated GtH secretion in the presence of a subthreshold dose of TPA [3, Chapter 6]. Furthermore, additions of PKC inhibitors reduced increases in $[Ca^{2+}]_i$ and GtH release stimulated by GnRH [3, 17 ; Chapter 5]. These data suggest that activation of PKC may initiate release of GtH in the goldfish gonadotrope and Ca^{2+} may act to amplify PKC's signal. The ability of PKC activators (TPA, DiC8) to stimulate elevation of $[Ca^{2+}]_i$ levels in goldfish pituitary cells is consistent with this hypothesis [3]. It is reasonable for the initiator of a signal to also stimulate up regulation of its own amplifier. The hypothesis that PKC is the initiator of the GtH response to GnRH in the goldfish is in direct contrast to the proposed situation in mammals. In rats, PKC has been proposed to amplify the effects of GnRH-induced increases in $[Ca^{2+}]_i$ on LH release [7].

If PKC activation is an important initiator of the intracellular signaling cascade which leads to GtH release, then the GtH release response to secretagogues that stimulate high levels of $[Ca^{2+}]_i$ should be sensitive to PKC inhibition. In support of this supposition, it was found that in cells with depleted PKC levels, ionomycin-stimulated GtH release was totally abolished (Chapter 4). Furthermore, the ionophore A23187 was not able to cause an increase in GtH release sumulated by maximally effective doses of either TPA or DiC8 [3]. Finally, the amounts of GtH released by TPA or DiC8 were greater than those released by either ionomycin or A23187 alone [3; Chapter 4]. These results are in agreement with the hypothesis that GtH release stimulated by elevations of $[Ca^{2+}]_i$ are

mediated through the PKC pathway. Perhaps the GtH response elicited by release of Ca^{2+} from intracellular stores is due to activation of localized PKC-dependent mechanisms.

In summary. GtH release in the goldfish appears to be sensitive to the release of Ca^{2+} from intracellular stores which may activate localized release mechanisms. In contrast, elevations of $[Ca^{2+}]_i$ over the entire cell is generally ineffective or may even be inhibitory to GtH release. However, very large increases in overall $[Ca^{2+}]_i$ levels lead to stimulation of GtH release, but this appears to be achieved through the activation of PKC. The novel hypothesis that GnRH-stimulated GtH release in the goldfish is initiated by the activation of PKC and then amplified by elevated $[Ca^{2+}]_i$ levels is proposed.

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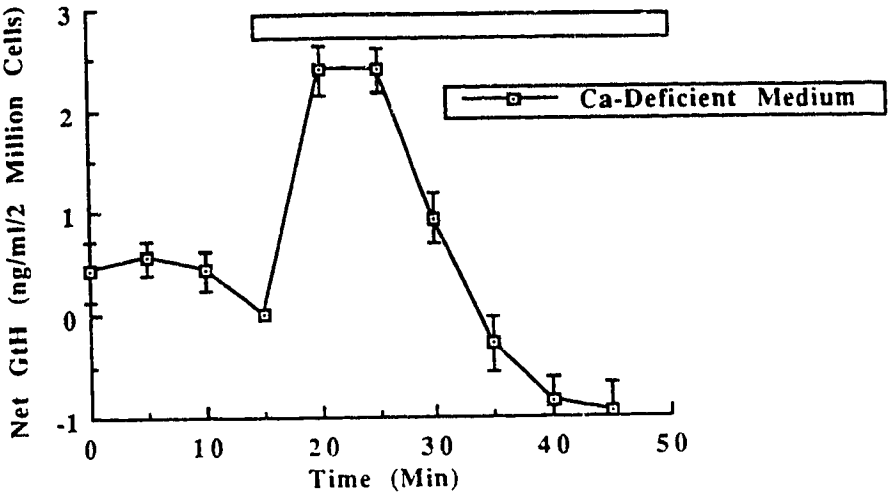
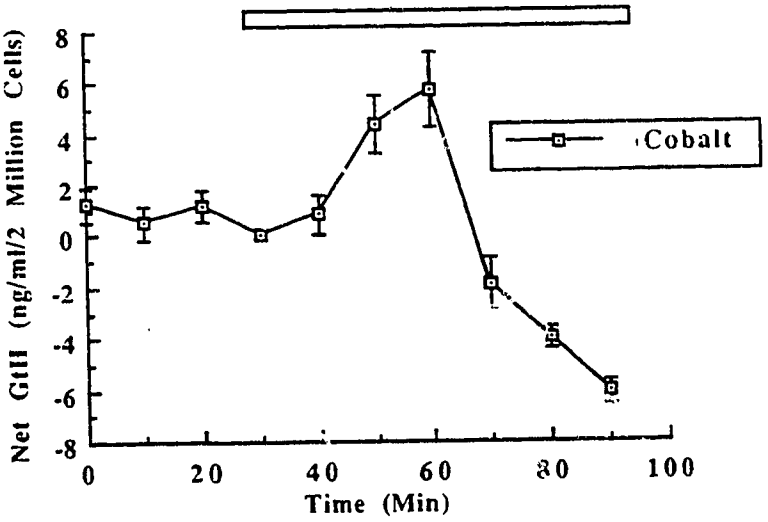
Figure 7.1. Effects of impeding extracellular calcium entry into cells on GtH release in perifusion. Horizontal bar indicates treatments with Ca^{2+}-deficient medium (upper panel), 1.26 mM $CoCl_2$ (center panel) and 1 μM nifedipine (lower panel). In experiments where nifedipine or $CoCl_2$ were applied, a fraction collection time of 10 minutes was used. In the experiments where Ca^{2+}-deficient medium was applied, a 5-minute fraction collection time was used. Results are presented as mean ± SE (n=6).


Net GtH (ng/ml/2 Million Cells)
3
2
1
0
-1
0
10
20
30
40
50
Time (Min)
Ca-Deficient Medium


Net GtH (ng/ml/2 Million Cells)
8
6
4
2
0
-2
-4
-6
-8
0
20
40
60
80
100
Time (Min)
Cobalt

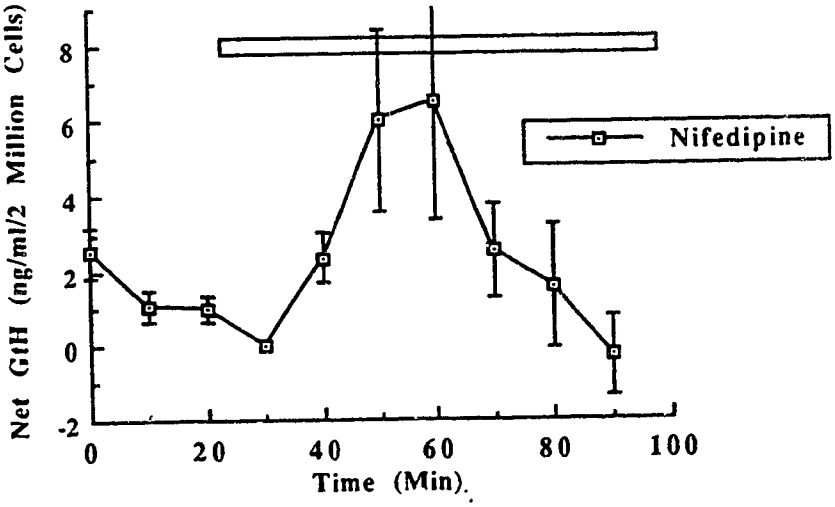
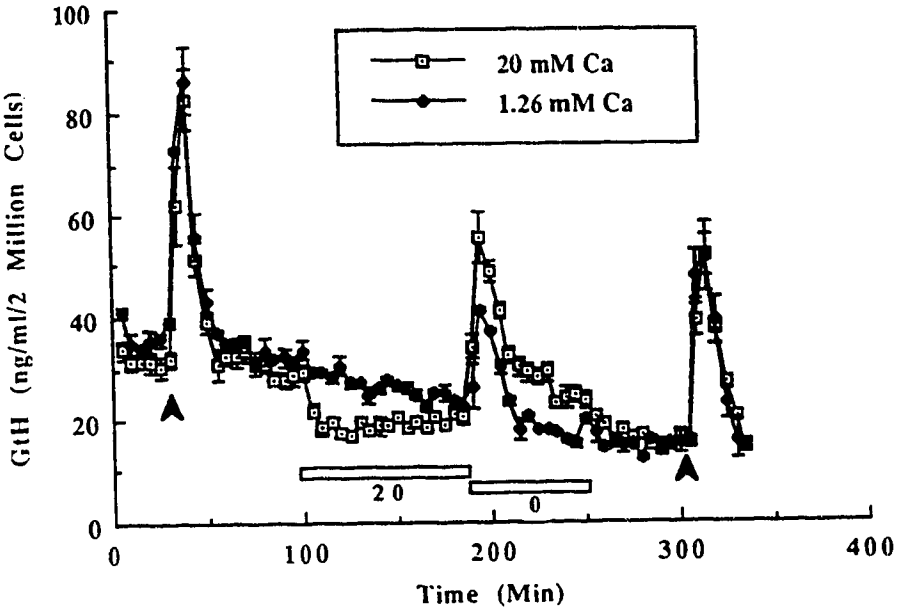

Net GtH (ng/ml/2 Million Cells)
8
6
4
2
0
-2
0
20
40
60
80
100
Time (Min)
Nifedipine

Figure 7.2. Effects of perifusion with medium containing high (20 mM) and low (no added $CaCl_2$) calcium have on GtH release. In the top panel, horizontal bars indicate exposure to medium with 20 mM (20) or low $CaCl_2$ (0). Arrow heads indicate the beginning of a 5-minute application of 100 nM sGnRH. A 5-minute fraction collection time was used. Results are presented as mean ± SE (n=4). In the bottom panel, total GtH responses to the various treatments are compared. Total GtH response was calculated by subtracting the non-stimulated GtH levels from those of the stimulated responses. Non-stimulated levels of GtH were calculated by taking the average of the three fractions immediately preceding the stimulated GtH response. In addition to the GtH responses stimulated by sGnRH and exposure to Ca^{2+}-deficient medium (0 calcium), the effects of perifusion with medium containing 20 mM Ca^{2+} on basal GtH release (average of 5 fractions from xx to xx minutes) are also compared to controls (1.26 mM Ca^{2+}). Results are presented as mean ± SE (n=4). * indicates values that are significantly different from controls as determined by Student's t-test ($p<0.05$).


100
80
60
40
20
0
GtH (ng/ml/2 Million Cells)
20 mM Ca
1.26 mM Ca
20
0
0
100
200
300
400
Time (Min)

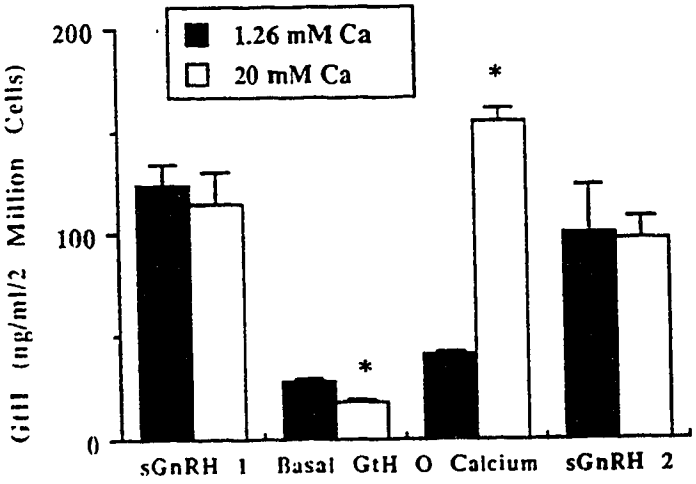

200
100
0
GtH (ng/ml/2 Million Cells)
1.26 mM Ca
20 mM Ca
*
*
sGnRH 1
Basal GtH O
Calcium
sGnRH 2

Figure 7.3. Effects of a 5-minute pulse of 30 mM KCl on GtH release in perifusion. Pooled results (mean ± SE, n=6) from 3 experiments are presented. GtH values are normalized as the net change from values collected at the time of KCl application. 5-Minute fractions were collected in these studies. KCl application is indicated by the horizontal bar and the position of the expected GtH response is indicated by the arrow head.

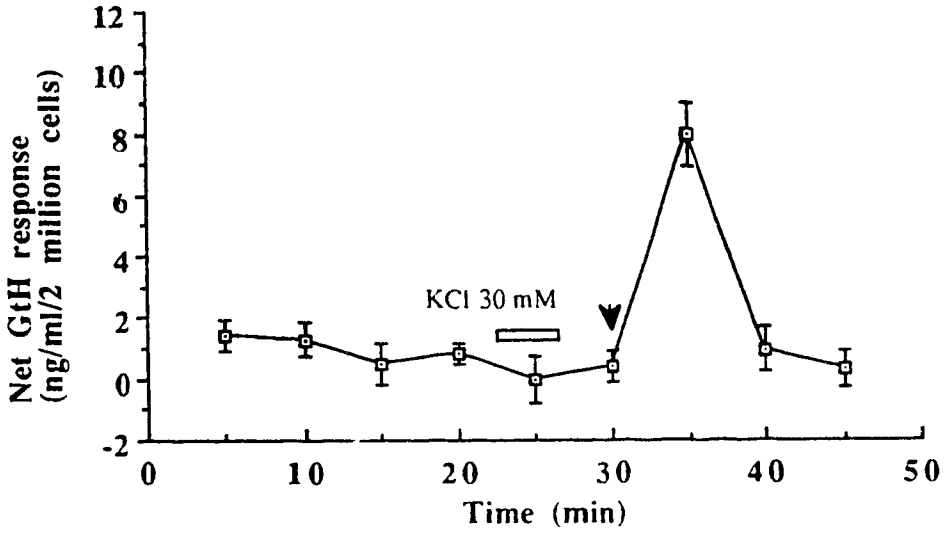
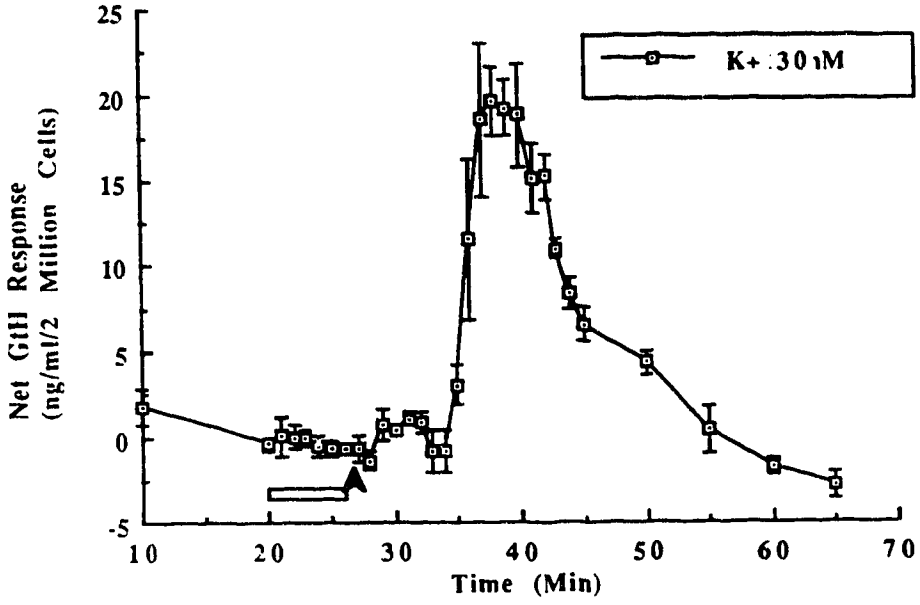

Net GtH response
(ng/ml/2 million cells)
12
10
8
6
4
2
0
-2
KCl 30 mM
0
10
20
30
40
50
Time (min)

Figure 7.4. Effects of 7- (top panel) and 20-minute exposures to 30 mM KCl (lower panel) on GtH release in perifusion. GtH results are expressed as a net change from values collected at the beginning of the KCl application. The horizontal bar indicates the duration of the 30 mM KCl administration. The arrow indicates when the GtH response was expected. In the upper panel, a 1-minute fraction collection time was used. Results are presented as mean ± SE (n=3). In the lower panel, a 5-minute fraction collection time was used. Results are presented as mean ± SE (n=2).


K+ 30nM
Net GtH Response
(ng/ml/2 Million Cells)
25
20
15
10
5
0
-5
10
20
30
40
50
60
70
Time (Min)

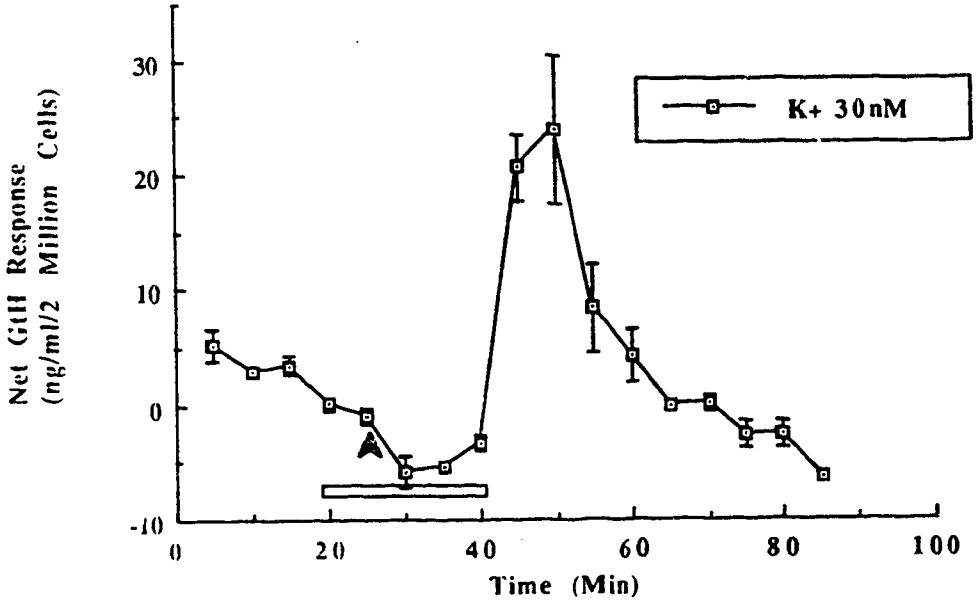
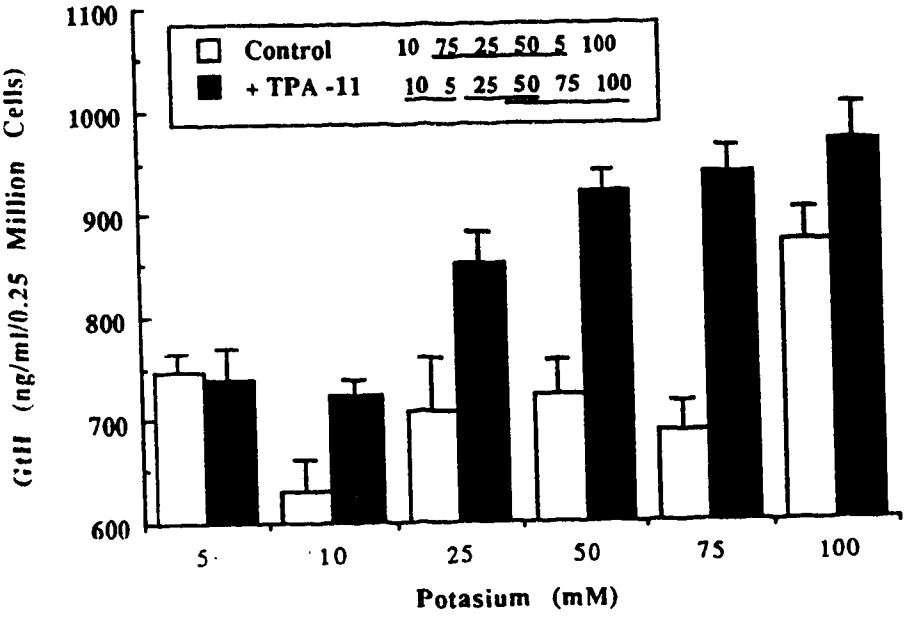

K+ 30nM
Net GtH Response
(ng/ml/2 Million Cells)
30
20
10
0
-10
0
20
40
60
80
100
Time (Min)

Figure 7.5. Effects of KCl and TPA on GtH release in static culture. Results are pooled from two experiments and presented as mean ± SE (n=8). Values were compared using an analysis of variance followed by Fisher's least significant difference test. In controls and 10 pM TPA-treated cells, concentrations of KCl that result in similar GtH responses are identified by sharing the same underscore ($p<0.05$).


Control 10 75 25 50 5 100
+ TPA -11 10 5 25 50 75 100
GtH (ng/ml/0.25 Million Cells)
1100
1000
900
800
700
600
5
10
25
50
75
100
Potasium (mM)

Chapter 8

Possible Involvement of Calmodulin in the Mediation of Gonadotropin Releasing Hormone-Stimulated Gonadotropin Secretion.

Introduction

In the goldfish, as in many other teleosts, the release of gonadotropin (GtH) is stimulated by gonadotropin releasing-hormone (GnRH) [1]. Unlike eutherian mammals which possess only one GnRH peptide, the goldfish has two native GnRH peptides. These two GnRH forms, salmon GnRH (sGnRH) and chicken GnRH II (cGnRH II), have been found in the pituitary and throughout the rest of the goldfish brain [2]. Both forms are released from the pituitary and hypothalamus [3, 4]. sGnRH and cGnRH II are both able to stimulate secretion of GtH *in vivo* and *in vitro* in the goldfish [4, 5].

Signal transduction of GnRH-stimulated GtH release in the goldfish has been shown to involve extracellular Ca^{2+}, intracellular Ca^{2+}, protein kinase C, and arachidonic acid metabolism [6, 7, 8, 9]. However, there is a paucity of information concerning the involvement of calmodulin in mediating GnRH action in both the teleost and the more extensively studied rat model systems. No known data has been collected implicating the involvement of calmodulin in GnRH-induced GtH secretion in any teleost model system. In the rat, micromolar doses of the calmodulin inhibitor pimozide and penfluridol were effective in attenuating GnRH-stimulated luteinizing hormone (LH) release [10]. Exposure to GnRH stimulates redistribution of calmodulin from the cytosolic to the membrane fractions [11]. Calmodulin has also been found to regulate the Ca^{2+}-dependent vesicle

binding protein, caldesmon, which may act to regulate interactions with secretory granules [12].

Given the importance of the roles that Ca^{2+} and Ca^{2+}-dependent enzymes play in GnRH action, calmodulin is a likely candidate as a mediator of GnRH-stimulated GtH secretion in the goldfish. In the present study, inhibitors of calmodulin activity were used to determine involvement of this protein in prolonged and acute sGnRH- and cGnRH II-stimulated GtH release from dispersed pituitary cells of the goldfish.

Materials and Methods

General. Common goldfish (8-12 cm in length), purchased from Ozark Fisheries Inc., Stoutland, Missouri and Grassyforks Fisheries, Martinsville, Indiana, were transferred to flow-through aquaria (1800 liters) immediately on arrival. The fish were held at 17-20 ^{o}C on a simulated natural (Edmonton, Alberta) photoperiod, and fed to satiation daily with commercial fish food. Fish of both sexes were acclimated to the above conditions for at least 7 days before use. sGnRH and cGnRH II (Peninsula Lab. Inc., Belmont, CA) stock solutions were made up in distilled deionized water. Calmidazolium (Research Biochem. Inc., Natick, MA) was dissolved in ethanol. Napthalene sulfonamides (W5-N-(aminohexyl)-1-napthalene sulfonomide HCl, W7-N-(aminohexyl)-5-chloro-1-napthalene sulfonomide HCl, W12-N-(4 aminobutyl)-2-napthalene sulfonomide HCl) (Sigma, St. Louis, MO) were dissolved in dimethyl sulfoxide. Aliquots of stock solutions were stored at -20 ^{o}C until used.

Static cell culture and hormone response. Pituitaries from fish of both sexes were removed and enzymatically dispersed as described by Chang et al., [13]. The dispersed cells were cultured overnight in 24-well culture plates with medium 199 containing Earle's

salts (Gibco, Grand Island, NY), 1% horse serum, 25 mM Hepes, 2.2 g/l sodium bicarbonate, 100 000 units/l penicillin, and 100 mg/l streptomycin, pH 7.2, at a density of 0.25 million cells/well/ml. The cells were incubated at 27 ^{o}C, 5% CO_2 and saturated humidity. The following day, the culture medium was replaced with testing medium (medium 199 containing Hank's Salts, (Gibco), 25 mM Hepes, 2.2 g/l sodium bicarbonate, 100 000 units/l penicillin, 100 mg/l streptomycin, 0.1% bovine serum albumin, pH 7.2). Test solutions diluted in the proper vehicle were then added (1 µl/ml to achieve desired final concentration). Inhibitors were usually added 10 minutes prior to application of sGnRH or cGnRH II. The concentration of vehicle was less than 0.1% of the final incubation volume and did not alter GtH release. Following a further 2 hr incubation, the media were removed and stored at -20 ^{o}C until their GtH contents were measured using an established radioimmunoassay [14]. All treatments were carried out in quadruplicate. Experiments were repeated two times and the GtH contents were measured in both replicate experiments. Results from replicate experiments were similar and the data were pooled prior to statistical analysis by analysis of variance followed by Fisher's least significant difference test. GtH values from replicate experiments were normalized by expressing the data as a percentage of the net hormone response to a maximal or near maximal (10 nM) stimulation by sGnRH or cGnRH II; net hormone response being hormone level for the treatment minus the hormone level in unstimulated controls.

Column perifusion of dispersed pituitary cells A perifusion system using dispersed cells cultured on Cytodex-I beads as described by Chang et al., [15] was used. The mixture of cells and beads were loaded onto the columns and perifused with testing medium. After 4 hours of perifusion with testing medium, a relatively low basal secretion rate was achieved and the experiment commenced with the collection of perifusate in 10 minute fractions. 30 minutes after commencement of the experiment, a 10-minute pulse of 100 nM of sGnRH or cGnRH II was applied (pre-pulse). During the GnRH application and for 20 minutes following GnRH treatment, 1-minute fractions of perifusate were

collected, following which the coliection interval was returned to 10 minutes. 1 hour after administration of the GnRH pulse the cells were perifused with medium containing 100 nM calmidazolium. 1 hour after commencement of the calmidazolium treatment another 10-minute GnRH pulse was given (test-pulse) in the presence of calmidazolium during which time, fractions were again collected once per minute for the next 30 minutes. The fraction collection time was then returned to 10 minutes and the calmidazolium treatment continued for an additional 30 minutes. Thus, the entire duration of the calmidazolium treatment was 2 hours. The cells were then perifused with normal testing medium for one hour, following which a final 10-minute pulse of GnRH was given (post-pulse) while one-minute fractions were collected for the next 30 minutes. For the remainder of the experiment, the perifusion continued with normal testing medium and fractions were collected every 10 minutes.

GtH response to sGnRH and cGnRH II treatment was divided into two phases, peak and plateau. The peak response was calculated as being the sum of the first 12 fractions of the GtH response to the GnRHs, while the plateau response was the sum of the subsequent 12 fractions. Total GtH response to GnRH was taken as the sum of the peak and plateau responses. The size of the responses was measured as the amount of GtH release above basal levels. Responses in replicate columns were normalized by expressing the data as a percentage of the total pre-pulse response elicited by either sGnRH or cGnRH II. Basal release was calculated as the average release during the 5 fractions preceding and 5 fractions following the GtH response elicited by GnRH.

Results

Use of calmodulin inhibitors in static culture. Napthalene sulfonomides (W5, W7,

W12) were reported to inhibit calmodulin action an micro molar levels [16]. At these concentrations preliminary and repeated experiments yielded inconclusive results (not shown) primarily due to extreme elevation of basal GtH release by the napthalene sulfonomides (e.g., 50 μM W7 elevated basal GtH release 6 fold).

In contrast to the napthalene sulfonomides, calmidazolium produced a dosage dependent decrease in GnRH-stimulated GtH release in static culture. Generally, doses of calmidazolium greater than 1 nM resulted in a significant inhibition of sGnRH- and cGnRH II-induced GtH release (Fig. 8.1, top and center panel). A 1 μM dose of calmidazolium resulted in a large (50%) increase in basal GtH release, a 10 nM dose also elevated basal but not to as great an extent as the napthalene sulfonomides. All other doses had no significant effects on basal GtH release in static culture (Fig. 8.1, bottom panel). Similar inhibitory actions of 1 μM calmidazolium on sGnRH- and cGnRH II-stimulated GtH release were observed in repeated single dose experiments (results not shown).

Effects of calmidazolium in column perifusion of cells. Perifusion experiments allow for the examination of acute hormone release response as well as changes of hormone secretion with respect to time. In the rat, LH secretion from pituitary cells in perifusion is biphasic. The LH response to GnRH displays a distinct peak and a plateau phase; these two phases appear to be mediated differently by signal transduction pathways [for a review see 17]. In addition, the secretion of GnRH is pulsatile in mammals. Although pulsatile GnRH release has not been clearly established in the goldfish, treatments with a transient pulse of GnRH, as permitted in perifusion studies, are probably a better approximation of the natural condition than a chronic administration of GnRH, as is the case in static culture experiments. Furthermore, with the high resolution afforded by rapid fraction collection, a biphasic GtH release response to GnRH challenge can be observed as previously reported in the goldfish (Appendix 1). Thus, second messenger systems responsible for mediating the peak and plateau phases of the GtH response to

GnRH in the goldfish can also be examined using perifusion studies. A dosage of 100 nM calmidazolium was selected in these perifusion experiments because it was the highest concentration of the compound that did not greatly affect basal secretion of GtH but still inhibited GnRH-stimulated GtH release in static culture (Fig. 8.1). Treatment with calmidazolium exerted no significant effects on basal, sGnRH- or cGnRH II-stimulated GtH release in perifusion (Figs. 8.2 and 8.3). In the GnRH-elicited responses, neither the peak, plateau nor the total GtH responses was altered due to exposure to calmidazolium (Fig. 8.3).

Discussion

Previous work in the rat have utilized penfluridol and pimozide to inhibit calmodulin action [10]. However, at doses of these drugs that inhibit calmodulin activity they also interact with dopaminergic and serotonergic receptors [18]. Dopamine and serotonin have been shown to alter GtH release in the goldfish by actions at the level of the pituitary [19]. These compounds are therefore not appropriate for use as calmodulin inhibitors in the present studies on fish pituitary cells On the other hand, the calmodulin inhibitor calmidazolium is not only 10-100 times more potent than penfluridol but it also does not interact with dopamine, serotonin, histamine or several other receptor types [20]. The use of calmidazolium as a calmodulin inhibitor is thus more appropriate in the present experiments.

In the present study, calmidazolium attenuated sGnRH- and cGnRH II-stimulated GtH secretion in static incubation, but not in column perifusion experiments. This suggests that calmodulin is involved in the mediation of prolonged but not acute GnRH-stimulated GtH secretion. This marks the first time that this signal transduction pathway has been implicated in the involvement of GnRH-stimulated GtH secretion in a teleost.

In the previous chapter (Chapter 7) it has been proposed that GnRH-stimulated GtH secretion is initiated by the activation of PKC. This PKC signal is then amplified by subsequent elevation of intracellular Ca^{2+} levels. Presently, the results implicating the involvement of calmodulin in the GnRH-induced GtH response suggest that this pathway is only involved in chronic GtH release (that which takes place after at least 10 minutes of GnRH exposure). Calmodulin, which is Ca^{2+}-sensitive, may be activated by the elevated Ca^{2+} levels that occur subsequent to PKC activation and may play a role in the sustained GtH release response stimulated by both native GnRH peptides.

In summary, this study provides the first evidence in teleosts that calmodulin may be involved in the mediation of GnRH-stimulated GtH secretion. If this is the case, it would appear that calmodulin is only involved in the mediation of chronic GnRH-induced GtH release, while PKC and Ca^{2+} are responsible for the initial stimulation and amplification of the GtH response.

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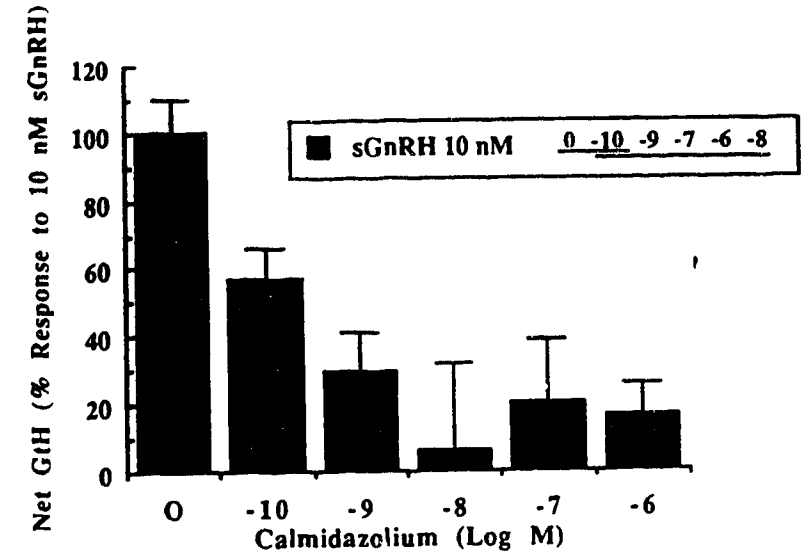
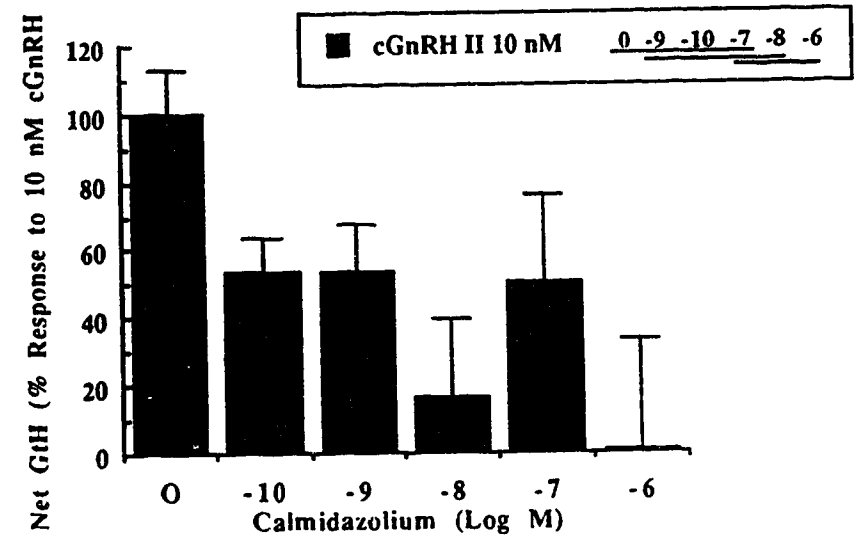
Figure 8.1. Effects of calmidazolium on 100 nM sGnRH-(top panel), 10 nM cGnRH II-stimulated (center panel), and basal (bottom panel) GtH release in static culture. Data presented are pooled results from two experiments (mean ± SE, n=8). Values are compared using analysis of variance followed by Fisher's least significant difference test. In each panel concentrations of colmidazolim that resulted in similar GtH values are identified by sharing the same underscore ($p<0.05$). GtH responses to 100 nM sGnRH, cGnRH II and unstimulated release are 151.2 ± 11.1, 187.2 ± 29.2 and 395.0 ± 8.9 respectively.


Net GtH (% Response to 10 nM sGnRH)
120
100
80
60
40
20
0
sGnRH 10 nM
0 -10 -9 -7 -6 -8
O -10 -9 -8 -7 -6
Calmidazolium (Log M)


Net GtH (% Response to 10 nM cGnRH II)
120
100
80
60
40
20
0
cGnRH II 10 nM
0 -9 -10 -7 -8 -6
O -10 -9 -8 -7 -6
Calmidazolium (Log M)

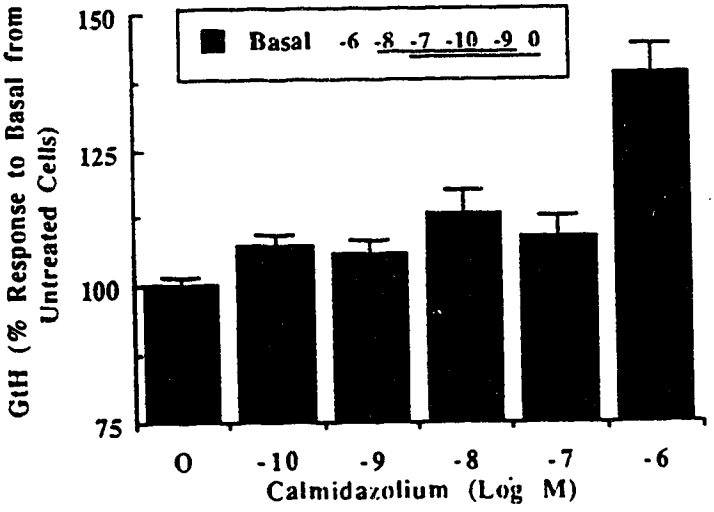
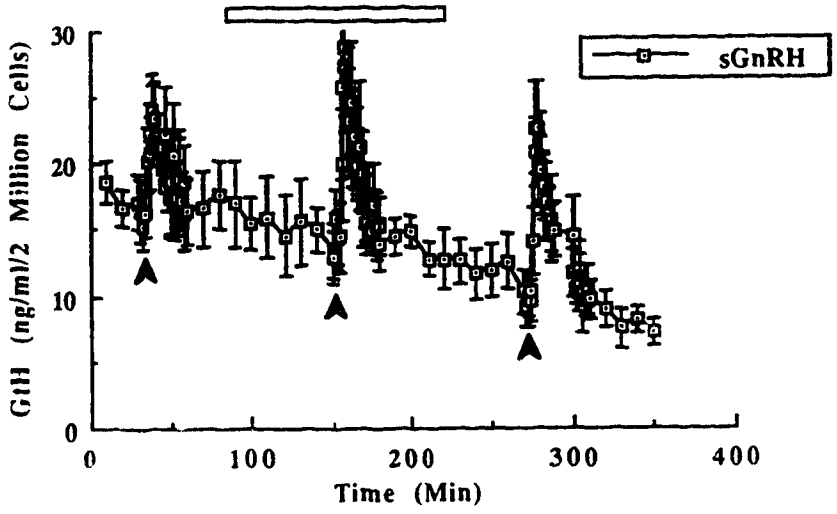

GtH (% Response to Basal from Untreated Cells)
150
125
100
75
Basal -6 -8 -7 -10 -9 0
O -10 -9 -8 -7 -6
Calmidazolium (Log M)

Figure 8.2. Effects of calmidazolium on 100 nM sGnRH- and 100 nM cGnRH II-stimulated GtH release in perifusion. The horizontal bars indicate administration of 10 nM calmidazolium. Arrow heads indicate the beginning of a 10 minute application of sGnRH (upper panel) or cGnRH II (lower panel). Representative data from one of three experiments with similar results are shown. All data are expressed as mean ± SE (n=2). 10 nM calmidazolium had no significant effect on unstimulated GtH release in perifusion.


sGnRH
GtH (ng/ml/2 Million Cells)
30
20
10
0
0
100
200
300
400
Time (Min)

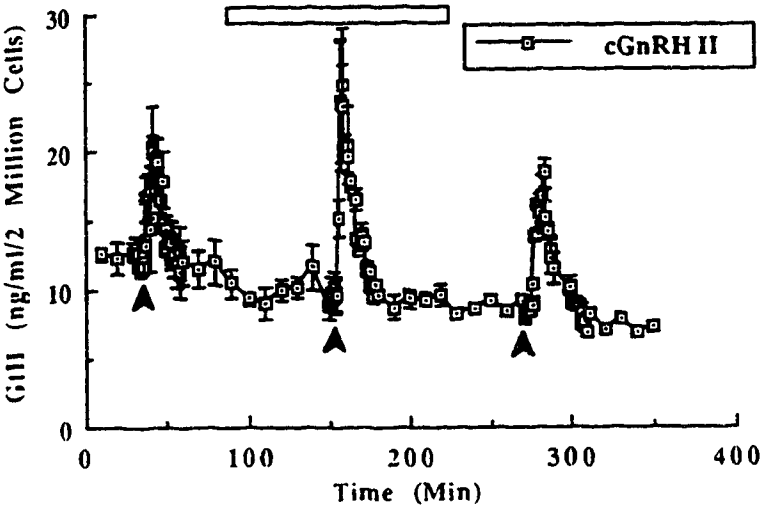
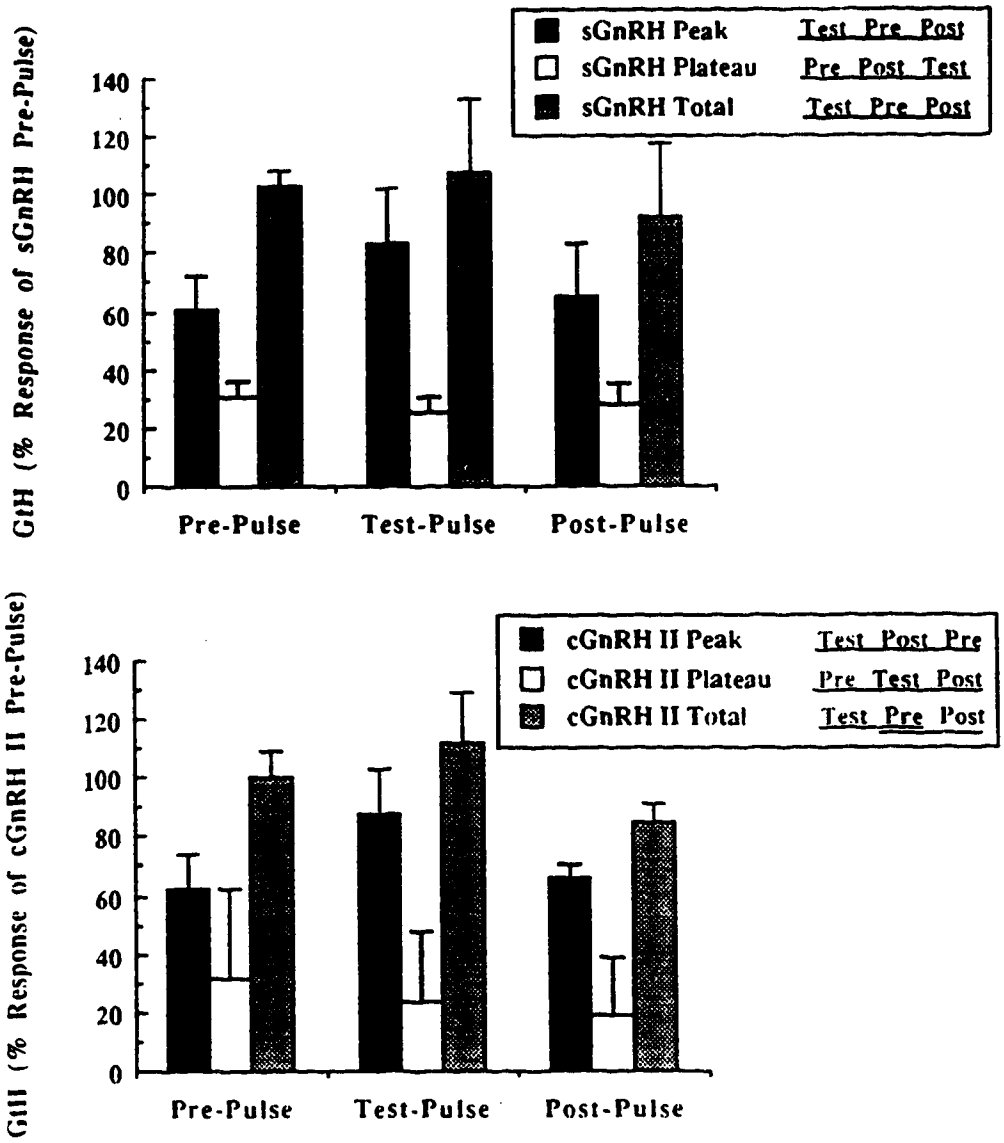

cGnRH II
GtH (ng/ml/2 Million Cells)
30
20
10
0
0
100
200
300
400
Time (Min)

Figure 8.3. Summarized results on the effects of calmidazolium on 100 nM sGnRH- and 100 nM cGnRH II-stimulated GtH release in perifusion. The GtH responses to the three GnRH pulses, before (pre-pulse), during (test-pulse) and after (post-pulse) 10 nM calmidazolium treatment, were divided into peak and plateau phases. The magnitude of these two phases as well as the total GtH response were compared using analysis of variance followed by Fisher's least significant difference test. Values not sharing the same underscore are significantly different from each other ($p<0.05$). GtH values were expressed as a percentage of the total GtH response to pre-pulse GnRH (185.0 ± 34.3) in the control columns. Data presented represent pooled results of 3 separate experiments (mean $\pm$ SE, n=6).


GtH (% Response of sGnRH Pre-Pulse)
sGnRH Peak Test Pre Post
sGnRH Plateau Pre Post Test
sGnRH Total Test Pre Post
140
120
100
80
60
40
20
0
Pre-Pulse
Test-Pulse
Post-Pulse
GtII (% Response of cGnRH II Pre-Pulse)
cGnRH II Peak Test Post Pre
cGnRH II Plateau Pre Test Post
cGnRH II Total Test Pre Post
140
120
100
80
60
40
20
0
Pre-Pulse
Test-Pulse
Post-Pulse

Chapter 9

General Conclusions

Ca^{2+} and Ca^{2+}-related signal transduction pathways play the major roles in GnRH-induced LH release from rat gonadotropes [1, 2]. In the present thesis the involvement of these pathways in the mediation of GnRH-stimulated GtH secretion in the goldfish pituitary was studied. The goldfish model is an interesting system for the study of signal transduction mediating hormone release because one can compare mechanisms underlying secretion of two different hormones (GH and GtH) stimulated by two closely related releasing peptides (sGnRH and cGnRH II). The goldfish model has the added feature of a direct inhibitory influence on GtH release exerted by dopamine via dopamine D_2 receptors [3]. These qualities make this system ideal for study of integration of stimulatory and inhibitory stimuli regulating hormone secretion.

The present studies using the goldfish model has at least two advantages over other current teleost studies. In the goldfish, native GnRH peptides have been identified whereas the identity of GnRH forms in other teleost species have only recently been investigated. In this goldfish study, the native GnRH peptides (sGnRH and cGnRH II) are used as stimulators of GtH release, while other investigator using teleost models tend to use GnRH analogues such as Buserelin or other LHRH analogs [4, 5]. Given the differences that have been found between signal transduction stimulated by sGnRH and cGnRH II, which are the natural GnRHs of goldfish [6; Chapters 2&6], doubt is cast on the physiological relevance of signal transduction pathways stimulated by GnRH analogs. Several studies examining the second messenger pathways mediating GnRH-induced GtH release in other teleost fishes were carried out on pituitary fragments and not dispersed pituitary cells [4, 5]. Teleosts pituitaries are directly innervated by hypothalamic neurons [7]. Therefore,

pituitary fragments possess neuronal terminals that may contain physiological modulators of GtH release such as dopamine or GnRH. The presence of nerve terminals in fragment preparations is critical because, treatments and drugs used to investigate second messenger pathways are not specific to cell type. For example, Yu [8] found that GnRH release from hypothalamus and pituitary fragments is stimulate by exposure to depolarizing doses of KCl, which acts to elevate $[Ca^{2+}]_i$ in all excitable cells. The use of dispersed pituitary cells in this study reduces these confounding effects by removing the neuronal terminals [9].

Results obtained from the presented work represent substantial contributions to the understanding of the signal transduction mechanisms mediating pituitary hormone release in teleosts. In particular, the roles of Ca^{2+} and Ca^{2+}-related signal transduction pathways that mediated GnRH-stimulated GtH secretion from the goldfish pituitary were examined in fair detail. In addition, preliminary studies examining the mechanisms that mediate the actions of the endogenous GtH-release inhibitor, dopamine were also carried out. Results from the present study also contribute to the knowledge of GnRH signal transduction mechanisms mediating GH release in the goldfish and in teleosts in general.

Calcium in GtH Release

Previously, it has been shown that ionophores and sGnRH stimulate GtH release from dispersed goldfish pituitary cells in static culture, in an $[Ca^{2+}]_o$ dependent manner [10]. In my studies, a comparison of GtH secretion induced by sGnRH and cGnRH II in static culture revealed several interesting results (Chapter 2). Using several different methods to impede $[Ca^{2+}]_o$ entry into the cells, both sGnRH and cGnRH II-stimulated GtH release was found to be heavily dependent on $[Ca^{2+}]_o$. Use of blockers of VSCC action indicated that these channels are at least partially responsible for mediating the

GnRH-induced $[Ca^{2+}]_o$ entry into gonadotropes. Interestingly, results from studies using different techniques to impede $[Ca^{2+}]_o$ entry (ie., use of Ca^{2+}-deficient medium, $CoCl_2$, blockers of VSCCs) all indicated that chronic cGnRH II-stimulated GtH release was more sensitive to manipulations of $[Ca^{2+}]_o$ entry and was more dependent on $[Ca^{2+}]_o$ than that which was stimulated by sGnRH. These results lead to the introduction of a novel hypothesis that two closely related peptide hormones could bind to the same class of receptors and show differences in the manner in which they activate signal transduction mechanisms mediating hormone release (see discussion in Chapter 2 and summary model below).

In perifusion experiments, like static incubation studies, both sGnRH and cGnRH II-stimulated GtH release were found to be dependent on $[Ca^{2+}]_o$ entry into cells, and in particular, the Ca^{2+} entry occurred partially through VSCCs (Chapter 3). Both the peak and plateau phases of the native GnRH-stimulated GtH were reduced by inhibitors of $[Ca^{2+}]_o$ entry through channels and the reduction of $[Ca^{2+}]_o$ concentrations (See Chapters, 3, 4, 5, 8). In perifusion studies which examined the acute GtH response, the inhibitory effects of different doses of blockers of VSCCs on the GtH-release responses to sGnRH and cGnRH II were not tested. However, no difference in the effectiveness of reduction of sGnRH- and cGnRH II-stimulated GtH release by a high dose (1 μM) of nifedipine was observed. This indicates that the difference in $[Ca^{2+}]_o$ involvement between the two native GnRHs occurs only in the sustained and not the acute release of GtH. The ability of KCl to induce GtH release in perifusion also indicates an involvement of $[Ca^{2+}]_o$ entry in mediation of GtH release (Chapter 7). Treatment with KCl also stimulates an increase in $[Ca^{2+}]_i$ levels in populations of dispersed goldfish pituitary cells which is completely dependent on the presence of $[Ca^{2+}]_o$ (Chapter 6). These data indicate that entry of $[Ca^{2+}]_o$ into gonadotropes plays a role in mediating GnRH-induced acute as well as prolonged (2 hour) GtH release.

To further examine whether GnRH-induced GtH release is accompanied by changes in $[Ca^{2+}]_i$ levels, $[Ca^{2+}]_i$ concentrations were estimated using the Ca^{2+}-sensitive fluorescent dye Fura-2. Both sGnRH and cGnRH II stimulate increases in $[Ca^{2+}]_i$ levels in populations of dispersed goldfish pituitary cells. However, sGnRH stimulated increases in $[Ca^{2+}]_i$ levels possess a component that is independent of $[Ca^{2+}]_o$, this component appears to be lacking in cGnRH II-stimulated Ca^{2+} mobilization (Chapter 6). These results added a new line of evidence emphasizing the importance of the differences in the $[Ca^{2+}]_o$ dependence of sGnRH and cGnRH II-induced GtH release. Furthermore these results also suggest that the $[Ca^{2+}]_o$ independent portion of the sGnRH-stimulated GtH response may be mediated via release of Ca^{2+} from intracellular stores (Chapter 6).

Involvement of $[Ca^{2+}]_i$ stores is also implicated in the GtH release stimulated by treatments which impede $[Ca^{2+}]_o$ entry in perifusion studies. Cells pretreated with medium containing high levels of Ca^{2+} subsequently displayed larger GtH responses when stimulated by exposure to Ca^{2+}-deficient medium than cells receiving no such pretreatment. These results suggest that cells given an opportunity to fill their intracellular stores to capacity can then produce larger GtH responses (Chapter 7). Taken together with results from studies using blockers of VSCCs and Fura-2 preloaded cells, these results further substantiate the suggestion that both entry of $[Ca^{2+}]_o$ and release of Ca^{2+} from intracellular stores play roles in the mediation of GnRH-stimulated GtH secretion in the goldfish.

The unstimulated (basal) GtH release does not appear to be dependent on $[Ca^{2+}]_o$ in earlier experiments using static incubations of dispersed pituitary cells (Chapter 2). However, there is some indication that basal GtH release in perifusion may be partially dependent on the presence of $[Ca^{2+}]_o$ (Chapters 3&7). Both of these findings may be correct. The transient GtH-releasing ability of treatments that impede $[Ca^{2+}]_o$ entry to cells may offset the subsequent attenuation of unstimulated GtH release, resulting in no overall change in basal GtH release. Interestingly, treatments which lead to an elevation of

$[Ca^{2+}]_i$ levels such as low doses of KCl or medium containing high Ca^{2+} levels lead to a decrease in unstimulated GtH release (Chapter 7). Taken together, the above results suggest that there is an optimal level of $[Ca^{2+}]_i$ above or below which basal GtH release is inhibited.

Protein Kinase C (PKC) in GtH Release

Part of my initial work on PKC showed that β-phorbol esters (including TPA) which are known to activate PKC also stimulated release of GtH from static cultures of goldfish pituitary cells. However, 4α-phorbol 12,13 didecanoate, which does not activate PKC also has no stimulatory effect on GtH release in goldfish pituitary cells [11]. Like GtH release stimulated by GnRH, TPA-stimulated GtH release is partially dependent on $[Ca^{2+}]_o$ entry through VSCCs. Additionally, the PKC inhibitor H7 attenuated GtH release stimulated by TPA, sGnRH and cGnRH II [11]. These results suggest that PKC plays a role in mediating GtH release in the goldfish.

In the present work, involvement of PKC in GnRH action was further investigated; in addition, the interactions between the PKC and Ca^{2+} pathways were examined. Impairment of PKC activity through the use of the PKC inhibitor staurosporine or PKC-depleted cells attenuated GtH release stimulated by sGnRH, cGnRH II and activators of PKC in static incubation experiments (Chapters 4, 5 and Appendix 1). In perifusion studies, cells with reduced PKC levels showed attenuation of both the peak and plateau phases of sGnRH- and cGnRH II-stimulated GtH release (Chapter 4). These finding provide important evidence supporting the participation of PKC in mediating sGnRH and cGnRH II stimulation of GtH release in the goldfish.

In other studies, activators of PKC (TPA, DiC8) stimulated increases in $[Ca^{2+}]_i$ in

dispersed goldfish pituitary cells as measured by the Ca^{2+}-sensitive fluorescent dye Fura 2 (Chapter 6). These increases in Ca^{2+} are dependent on $[Ca^{2+}]_o$ entry but the DiC8-stimulated response possesses a component that is apparently mediated by release from intracellular stores (Chapter 6). The ability of these PKC activators to mimic the Ca^{2+} mobilization abilities of the GnRHs provides another line of evidence that PKC is involved in the mediation of GnRH-stimulated GtH release in the goldfish. These data also demonstrate the ability of the PKC pathway to interact with the Ca^{2+} pathway by stimulation of $[Ca^{2+}]_o$ entry and possible release of Ca^{2+} from intracellular stores.

The lack of a potent GtH-releasing response to treatments with KCl and Bay K8644 in static culture, suggest that Ca^{2+} by itself may not be an adequate signal to stimulate GtH release in goldfish gonadotropes (Chapters 6 and 7). Subthreshold doses of TPA added simultaneously with KCl or Bay K8644 lead to a substantial secretion of GtH. In fact, when KCl is added in combination with a subthreshold dose of TPA a dose-dependent GtH to KCl is observed. These data indicate that minimal activation of PKC is required for the generation of a GtH release-response to physiological ranges of increases in $[Ca^{2+}]_i$ concentration. Although treatments that lead to extremely high levels of $[Ca^{2+}]_i$ do stimulate release of GtH, this release appears to be mediated through activation of PKC. In PKC-depleted cell, the GtH response to ionomycin was completely abolished (Chapter 4). Furthermore, the GtH-releasing effects of ionophores and activators of PKC are not additive (Chapter 6). When combined, these result suggest that PKC activation initiates the signal for GtH response and elevation of $[Ca^{2+}]_i$ levels acts to amplify this response (Chapter 7).

The data presented in this thesis not only confirm the involvement of PKC in GnRH action on GtH release, but also describe PKC's actions on the temporally identified phases of GtH release in the goldfish. Furthermore, the current data are also the first to define the interactions that exist between the PKC and Ca^{2+} pathways mediating GtH

release in teleosts.

Calmodulin in GtH Release

In many systems, the biological response to increases in $[Ca^{2+}]_i$ concentration is mediated through the actions of the intracellular protein, calmodulin. In this study, the specific calmodulin inhibitor, calmidazolium, attenuates sGnRH- and cGnRH II-stimulated GtH release in static culture. However, GtH responses to 10-minute pulses of sGnRH and cGnRH II are not affected by treatments with calmidazolium in perifusion experiments (Chapter 8). These data indicate that involvement of calmodulin in GnRH-stimulated GtH release from goldfish gonadotropes occurs only during sustained GtH release and the activation of calmodulin may be result of prolonged elevations of $[Ca^{2+}]_i$ levels.

Proposed Model of GnRH Stimulation of GtH Release in the Goldfish

Based on results from this study as well as those from the literature, a model for the signal transduction mechanisms mediating sGnRH and cGnRH II stimulation of GtH release in the goldfish can be proposed (Fig. 9.1). sGnRH and cGnRH II both occupy the same class of cell surface receptors on gonadotropes [12, 13, 14]. Both GnRHs initiate GtH release by activating PKC, probably via the production of diacylglycerol (DG). DG is most commonly believed to be generated by phospholipase C (PLC) activation; but DG can also be produced via phospholipase D (PLD) action on phospholipids. Actions of PLC and PLD have been shown to stimulate GtH release from goldfish pituitary cells [15]. In the case of sGnRH, activation of PLC probably also leads to production of inositol

phosphates (such as $InsP_3$) which stimulates the release of Ca^{2+} from intracellular stores. The ability of $InsP_3$ to induce release of $[Ca^{2+}]_i$ is well known [reviewed by 16]. sGnRH also stimulated mobilization of arachidonic acid through phospholipase A2 action and is subsequently metabolized via the lipoxygenase pathway, thus contributing to the GtH response [15, 6]. Additions of PLA_2 enhanced GtH release and the GtH response to sGnRH can be reduced by coincubation with an inhibitor of PLA_2 [17] or an inhibitor of the lipoxygenase enzyme [15].

On the other hand, cGnRH II does not stimulate release of Ca^{2+} from intracellular stores or arachidonic acid metabolism but is more dependent on the actions of $[Ca^{2+}]_o$ entry into gonadotropes.

The reason for the differences in the $[Ca^{2+}]_o$ dependence and the difference in the involvement of $[Ca^{2+}]_i$ mobilization between sGnRH and cGnRH II action in goldfish gonadotropes is not entirely understood. However, it may be that cGnRH II generates DG from the plasma membrane of gonadotropes through PLD- rather than PLC-induced hydrolysis. This would result in a lack of production of inositol phosphates and a loss in intracellular Ca^{2+} mobilizing ability in cGnRH II action. Since arachidonic acid stimulation of GtH release in the goldfish is independent of the presence of $[Ca^{2+}]_o$ [15, 6], the lack of involvement of an arachidonic acid component in cGnRH II action, as compared to sGnRH, may also account for the increased $[Ca^{2+}]_o$ dependence of the GtH response to prolonged stimulation by cGnRH II. Once activated by DG, PKC stimulates entry of $[Ca^{2+}]_o$ partially through VSCCs and, in the case of sGnRH, this may also stimulate release of Ca^{2+} from intracellular stores. Elevation of $[Ca^{2+}]_i$ levels either by the mobilization of Ca^{2+} from extracellular and intracellular sources serves to amplify the GtH response to PKC. After prolonged elevation of $[Ca^{2+}]_i$ levels activation of calmodulin aids in the maintenance the sustained sGnRH- and cGnRH II-induced GtH responses.

Signal Transduction Mechanisms Mediating Dopamine Inhibition of GtH Secretion

Previously, it has been shown that dopamine serves as a GtH release inhibitory factor in the goldfish [18, 3]. In studies using static incubations of dispersed goldfish pituitary cells, the dopamine agonist, apomorphine inhibits sGnRH- and cGnRH II-stimulated GtH release [10]. The selective dopamine D_2 agonist LY171555 also has been shown to inhibit sGnRH-stimulated GtH release in static incubation and perifusion studies on goldfish pituitary cells [19].

In our preliminary studies [17], I have shown that LY171555 reduces the increases in $[Ca^{2+}]_i$ levels stimulated by cGnRH II. Furthermore, addition of the Ca^{2+} ionophore A23187 reversed the inhibitory effects of LY171555 on sGnRH- and cGnRH II-stimulated GtH release in static incubation experiments. These results suggest that the inhibitory effects of dopamine on GtH release may be mediated via alteration of $[Ca^{2+}]_o$ entry into gonadotropes. Although no equivalent data for the action of dopamine D_2 mechanisms were available from pituitary cells of other teleosts, D_2 stimulation can reduce $[Ca^{2+}]_o$ entry in mammalian pituitary cells [20].

In this thesis, LY171555 was shown to have an inhibitory effect on the GtH release induced by activators of PKC (TPA and DiC8) (Appendix 1). This result suggests that one possible site for the inhibitory action of dopamine is the PKC pathway. As indicated in the earlier section of the conclusions, PKC has also been shown to be an important pathway in the stimulatory effects of sGnRH and cGnRH II on GtH release and $[Ca^{2+}]_o$ entry [11; Chapters 4, 5, 6 and 7]. Therefore dopamine could act strictly on PKC which would in turn affect Ca^{2+} mobilization.

Signal transduction of GnRH-Stimulated GH Secretion in the Goldfish

Despite the fact that most of the emphasis has been placed on the signal transduction mechanisms mediating GnRH action on GtH secretion, some studies on intracellular signaling systems involved in GnRH stimulation of GH release have also been performed for this thesis.

Previously, it has been suggested that GnRH-stimulated GH release from dispersed cells of the goldfish pituitary, is dependent on $[Ca^{2+}]_o$ but not the mobilization of arachidonic acid [21]. In our initial studies, the GH-releasing abilities of phorbol esters and the attenuation of GnRH-stimulated GH release by the PKC inhibitor H7 in static incubation experiments implicated the involvement of PKC in GnRH-stimulated GH release [11]. In the current studies, involvement of PKC in GnRH-induced GH release is further supported by the inability of sGnRH to stimulate GH release from PKC-depleted cells (Appendix 1). Interestingly, unlike results from gonadotropes, the $[Ca^{2+}]_o$ dependence of sGnRH and cGnRH II were found to be similar in the mediation of GH release from somatotropes (Chapter 2). Also contrary to results obtained from GtH release, unstimulated GH release in static culture was found to be dependent on $[Ca^{2+}]_o$ entry (Chapter 2). The above results indicate that despite sharing some common signal transduction pathways, the GnRH-induced GH and GtH release in the goldfish are mediated quite differently at the second messenger level. Not only may some components be absent in GnRH action on one pituitary cell type (eg. arachidonic acid), but the details of the participation or relative importance of common components (eg. $[Ca^{2+}]_o$) may also be different between cell types.

Summary and Further Directions

The studies presented, elucidated the importance of Ca^{2+} and Ca^{2+}-related signal transduction mechanisms in GnRH-induced GtH and GH release in the goldfish, as well as the mediation of dopamine inhibition of GtH release. Furthermore, these findings indicate that unlike the situation in mammals [reviewed by 1], PKC probably acts as an initiator, rather than an amplifier, of the GtH release response to GnRH in the goldfish. Besides investigating other signal transduction pathways in the GtH and GH responses, studies on the temporal interactions of different second messenger pathways mediating the hormone responses will be required if we are to further understand the intracellular actions of GnRH and other neuroendocrine regulators in goldfish pituitary cells.

More significantly, results from the current series of studies clearly indicate that two closely related GnRH peptides act by different signal transduction mechanisms not only on two different cell types (GtH, GH) but also on the same cell type (GtH). The presence of distinct GnRH receptor subtypes on the same cell type or the existence of subpopulations of gonadotropes responsive to only one of the two forms of GnRH are other possible explanations to the collected results that still require further investigation. Nevertheless, the novel hypothesis that two peptide hormone from the same peptide family bind to the same set of receptors on the same cell type to elicit the same hormone response through activation of different second messenger pathways remains a significant and testable contribution of the present study.

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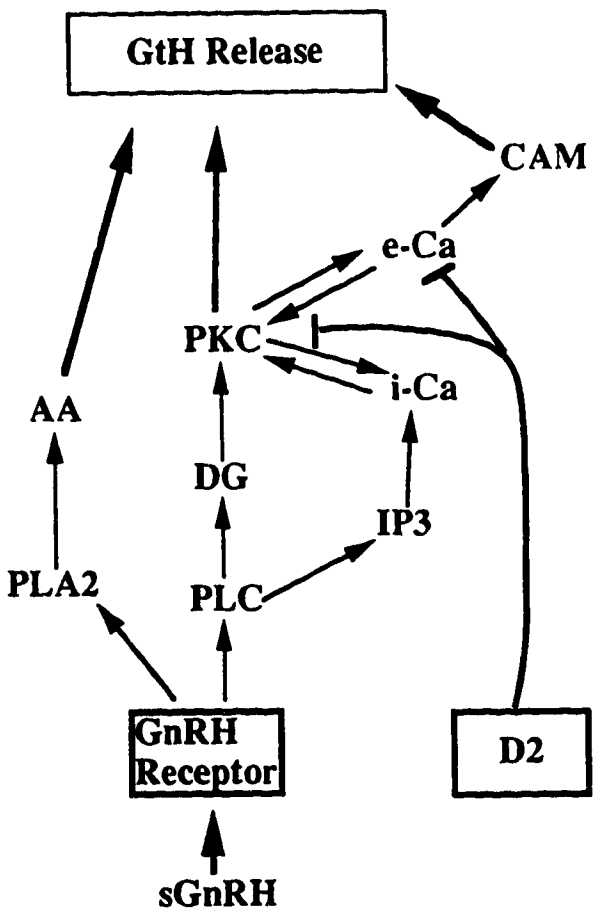
Figure 9.1. Schematic model of the signal transduction pathways mediating GtH release in the goldfish. (Abbreviations: AA, arachidonic acid; CAM, calmodulin; e-Ca, extracellular calcium; i-Ca, intracellular calcium; DG, diacylglycerol; DA, dopamine, D2, Dopamine D2 receptor; InsP3, inositol-1,4,5-trisphosphate; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; PLA2, phospholipase A2; other abbreviations are defined in the text).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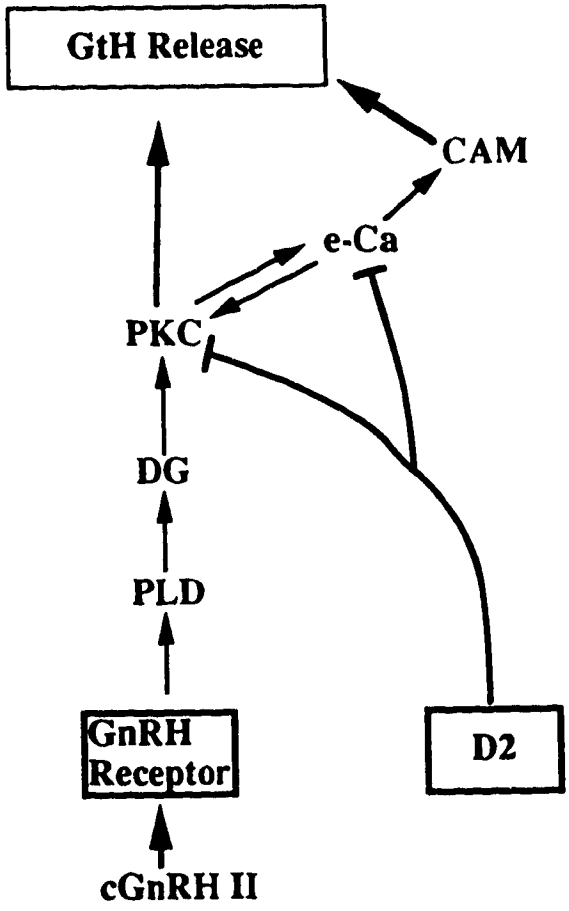
e-Ca
PKC
i-Ca
AA
DG
IP3
PLA2
PLC
GnRH Receptor
D2
sGnRH


GtH Release
CAM
e-Ca
PKC
DG
PLD
GnRH Receptor
D2
cGnRH II

Appendix 1

Involvement of Protein Kinase C in the Modulation of Gonadotropin and Growth Hormone Secretion from Dispersed Goldfish Pituitary Cells.[4]

Introduction

Gonadotropin (GtH) release in many teleosts, including the goldfish, is directly under the stimulatory influence of GtH-releasing hormone (GnRH) and the direct inhibitory effects of dopamine (DA) D2 action [1]. In addition to eliciting GtH release, GnRH directly stimulates growth hormone (GH) release and enhances the rate of increase in body length in goldfish [2, 1].

In mammals, the intracellular second messengers mediating luteinizing hormone (LH) and GH release have been extensively studied. GnRH-stimulated LH release appears to be mediated by several different signal transduction pathways, including extracellular Ca^{2+}, intracellular Ca^{2+}, arachidonic acid, and protein kinase C [3]. However, the participation of PKC in GnRH action in rat gonadotropes remains controversial. Depletion of cellular PKC activity has been reported to have no influence on GnRH action in one study [4], but to reduce the LH response to GnRH in another study [5]. In general, GH release in mammals is mediated by cAMP as well as by Ca^{2+}-dependent mechanisms [6].

In the goldfish, GnRH action on GtH and GH release also appears to be mediated by multiple intracellular signalling components [for a review see 7] including PKC [8, 9].

[4]A version of this chapter has been accepted for publication. Jobin and Chang (in press). Fish Physiology and Biochemistry.

In the present study, the possible role of PKC as a mediator of GtH and GH secretion responses to GnRH in the goldfish is further investigated using dispersed goldfish pituitary cells pretreated with TPA. Prolonged preincubation with TPA depletes PKC levels in goldfish pituitary cells [10], and is used to impair PKC activity in pituitary cells. Subsequent challenges with salmon (s)GnRH and a sGnRH superagonist ([D-Ala^6, Pro^9-Net]-sGnRH; sGnRHa) are used to determine if PKC is a component of the signal transduction mechanisms invoked by GnRH. Challenges with TPA are used to assess the extent of PKC depletion. To determine if DA inhibition of GtH release involves PKC, the DA D2 agonist LY171555 is used to inhibit hormone release elicited by the PKC stimulators, TPA and DiC8. GH responses were also measured to determine if the D_2 effects were specific to the secretion of GtH.

MATERIALS AND METHODS

General. Common goldfish (8-12 cm in length), purchased from Ozark Fisheries Inc., Stoutland, Missouri and Grassyforks Fisheries, Martinsville, Indiana, were transferred to flow-through aquaria (1800 liters) immediately on arrival and maintained as previously described [11]. Stock solutions of sGnRH (Peninsula Lab. Inc., Belmont, CA) and sGnRHa (Syndel lab., Vancouver, B.C.) were dissolved in distilled deionized water. TPA (Sigma, St. Louis, MO), DiC8 (Calbiochem, San Diego, CA) and LY171555 (Research Biochemicals Inc., Natick, MA) were dissolved in dimethylsulfoxide. Aliquots of stock solutions were stored at -20 ^{o}C until used.

Static cell culture and hormone response. Pituitaries from fish of both sexes were removed and the enzymatically dispersed cells were cultured overnight in 24-well culture plates as previously described [11]. The following day, prior to experiments, the culture medium was replaced with testing medium (medium 199 containing Hank's Salts, (Gibco), 25 mM Hepes, 2.2 g/l sodium bicarbonate, 100 000 units/l penicillin, 100 mg/l streptomycin, 0.1% bovine serum albumin). Test solutions diluted in the proper vehicle were added (1 µl/ml to

achieve desired final concentration). All treatments were carried out in quadruplicate o sextuplicate. Following the testing period (2 hours), media were removed and stored frozen a -20 ^{o}C until processed for GtH and GH contents by radioimmunoassay [12, 13].

Experiments with D_2 agonist. LY171555 was added 10 minutes prior to applicatio of TPA or DiC8. Unless otherwise indicated, experiments were repeated a minimum of thre times. Hormone levels from replicate experiments were normalized by expressing the data as a percentage of the net hormone response to a maximal or near maximal stimulation by TPA o DiC8; net hormone response being hormone level for the treatment subtracting the hormon level in unstimulated controls. Results from replicate experiments were similar and the dat were usually pooled prior to statistical analysis by analysis of variance followed by Fisher' least significant difference test.

TPA-desensitization in static culture. The experimental protocol for this section i summarized in Fig. A1.1. Cells were incubated for 4 hours during the pretreatment perio with testing medium in the absence (control) or presence of (TPA-pretreated group) 10 nM TPA. The medium from the pretreatment period was then collected and replaced with fres testing medium for 1 hour (rest period). Medium from the rest period was collected an replaced with fresh testing medium for a 2 hour test period. During the testing period the cell were exposed to 10 nM sGnRH, 10 nM TPA or testing medium alone. Following the tes period, medium was again collected. The cells were then lysed with distilled water, freezin and thawing, after which the hormone content (cell contents) were assessed. For eac individual treatment, total hormone measurable was calculated by adding the amount o hormone released during the pretreatment, rest and test periods as well as the cell conten remaining. Data from two individual experiments were pooled and statistical analysis wa performed by analysis of variance followed by Fisher's least significant difference test.

TPA-desensitization in cell column perifusion. A perifusion system using dispers cells cultured on Cytodex-I beads as described by Chang et al., [14] was used. Prior t

loading into columns, half of the preparations of pituitary cells were exposed to 10 nM TPA for 6 hours (TPA-pretreated groups); the remaining control cells received no such treatment. After 4 hours of perifusion with testing medium a relatively low basal secretion rate was achieved and the experiment commenced with the collection of perifusate in 10 minute fractions. 30 minutes after commencement of the experiment, a 10-minute pulse of 100 nM of sGnRHa was applied. During the GnRH application and for 20 minutes following GnRH treatment, 1 minute fractions of perifusate were collected, following which the collection interval was returned to 10 minutes. To evaluate the GtH-releasing ability of the pituitary cells, a 5-minute pulse of 10 μM forskolin was then applied 80 minutes after the termination of the sGnRHa pulse. Activation of adenylate cyclase by forskolin has previously been shown to increase GtH release independent of GnRH in the goldfish [15]. Perifusate from the columns was frozen at -20 °C until their GtH contents could be determined by radioimmunoassay [13]. Results from each individual column were expressed as a percentage of the average rate of hormone secretion obtained from the five fractions prior to the first sGnRHa response in TPA-pretreated (desensitized) preparations. Data presented are pooled results from three control and three TPA-pretreated columns. GH measurements were not obtained in this part of the study due to the limited supply of GH tracer.

Results

TPA-desensitization in static culture. Exposure to TPA resulted in a significant elevation in GtH release during the pretreatment period (Fig. A1.2A). During the rest period there was no difference between the amounts of GtH secreted by the TPA-pretreated and controls groups (Fig. A1.2A). During the test period, TPA-pretreatment did not alter unstimulated GtH secretion; conversely, TPA and sGnRH-stimulated GtH release were greatly

reduced and totally abolished, respectively (Fig. A1.2B). Cellular GtH contents and total amount of GtH measurable during the experiment were both significantly reduced by either TPA-pretreatment or TPA applied during the testing period (Fig. A1.2C).

TPA-pretreatment elevated GH release during the pretreatment period but had no effect during the resting period (Fig. A1.3A). During the test period TPA-pretreatment reduced basal release of GH and totally abolished GH secretion elicited by TPA and sGnRH (Fig. A1.3B). Cellular GH contents and the total amount of GH measurable during the experiment were both significantly reduced by TPA-pretreatment and TPA applied at the testing period (Fig. A1.3C).

TPA desensitization in perifusion. The effects of TPA-pretreatment on GtH responses were further investigated in a cell column perifusion system. The use of superactive sGnRHa in combination with the rapid fraction collection of perifusate, allowed for greater resolution of the GtH release response. As in other studies with sGnRH [10], GtH release stimulated by sGnRHa had two distinct phases. The first phase consisted of a rapid increase in hormone release and was of high magnitude (peak phase); this was followed by a more sustained GtH release of lower magnitude (plateau phase) (Fig. A1.4 inset). The existence of a biphasic LH response to GnRH has been well characterized in studies using rats [3]. Compared to control columns, the GtH response in both of these release phases as well as the total amount of GtH released by sGnRHa (cumulative total of hormone release during entire response) were significantly attenuated in the TPA-pretreated cells (Table A1.1). In contrast, the forskolin-induced GtH release was potentiated in the TPA-pretreated, PKC depleted cells (Fig. A1.4, Table A1.1). Basal secretion of GtH was lower from the TPA-pretreated cells than from control cells (Fig. A1.4).

Inhibition of PKC stimulators by LY171555. In a preliminary experiment high doses (0.1 μM, 1 μM) of the DA D_2 agonist LY171555 appeared to inhibit GtH, but not GH release stimulated by 10 nM TPA (Fig. A1.5 A,B). In three subsequent experiments, 1 μM LY171555 also significantly reduced GtH release elicited by 10 nM TPA and 100 μM DiC8 (Fig. A1.5C). This inhibition occurred despite the unexpected slight stimulatory effect of

LY171555 on basal GtH release. In previous studies on goldfish DA is generally inhibitory to basal GtH release, *in vivo* [1].

Discussion

Using TPA-pretreatment to down-regulate PKC levels in goldfish pituitary cells, the present study confirms the hypothesis that PKC is involved in GnRH-stimulated GtH and GH secretion from goldfish pituitary cells. In previous studies TPA pretreatment of goldfish pituitary cells has been shown to decrease PKC levels [10]. Currently, TPA-pretreatment attenuated GtH and GH responses to TPA. These results demonstrate that PKC-dependent hormone release processes are effectively reduced by TPA pretreatment. Under these conditions of impaired PKC function, both the GtH and GH release responses to sGnRH are abolished in static incubation studies. This indicates that PKC mediates prolonged GnRH stimulation of GtH and GH release. Similar reduction of sGnRHa stimulation of GtH release in -pretreated cells in perifusion studies suggests that rapid GnRH actions on GtH secretion in goldfish are also mediated by PKC-dependent mechanisms.

Incubation with TPA lowered cellular GtH and GH contents, as well as the total measurable GtH and GH. These results suggest that PKC affects synthesis and/or degradation of hormones in gonadotropes as well as somatotropes. It can be argued that the impaired hormone responses to GnRH following TPA pretreatment are due to the reduction of the releasable hormone pool and not the result of a specific inhibition of PKC-dependent mechanisms. However, evidence from the perifusion studies presented does not support this hypothesis. In perifusion studies, stimulation of cAMP-dependent pathways by forskolin, resulted in elevation of GtH release in both TPA-pretreated and control cells.

The effectiveness of forskolin was actually enhanced in TPA-pretreated cells, conversely, the GtH response to sGnRHa was severely impaired. This clearly demonstrates that following TPA pretreatment, the releasable hormone pool is not depleted and only a specific hormone release pathway has been affected. These results support the hypothesis that GnRH stimulation of pituitary hormone release in the goldfish involves PKC.

The enhancement of forskolin-induced GtH release in TPA-pretreated cells suggests that PKC exerts an inhibitory influence on the cAMP-dependent mechanisms in goldfish gonadotropes. Since TPA-pretreatment severely reduces cellular GtH content, these data further indicate that the releasable GtH pool is not directly proportional to cell contents. This observation may have strong implications in the controversy surrounding the involvement of PKC in GnRH action in rats, where TPA pretreatment also causes reduction in the cellular contents of LH [5]. Some of the arguments against the participation of PKC in GnRH stimulation of LH release in rats, are based on the ineffectiveness of TPA-pretreatment to reduce the LH response to GnRH. This is only the case when GnRH-stimulated LH release is expressed as a percentage of cellular contents of LH [4]. This transformation of the data makes an inherent assumption that cellular LH contents are a direct measure of the releasable hormone pool, which at least in the case of the goldfish, is untrue.

In the present study, TPA pretreatment appears to have greater effects on GH as compared to GtH cells. Cellular GH contents and the TPA-induced GH release were more severely affected by prior exposure to TPA than the corresponding GtH responses. TPA pretreatment also reduced GH, but not GtH, basal release rates in static incubation studies. Perhaps GH cells in the goldfish are more sensitive to manipulations of the PKC pathways than GtH cells.

DA D2 receptor activation in the goldfish, inhibits stimulated GtH release [1]. In the present study, the D2 agonist LY171555 reduces the GtH response to two PKC

activators, TPA and DiC8. These findings suggest that DA negatively modulates GtH secretion by inhibition of the PKC pathway. In rat pituitary cells, it has previously been shown that DA D2 action reduces the phospholipase C activity that generate diacylglycerol which activates PKC [16].

In summary, PKC pathways are important in mediating the effects of native regulators of GtH and GH release in the goldfish. PKC is involved in the GnRH-induced stimulation of GtH and GH secretion, and is also a component of the signalling cascade affected by DA, a physiological inhibitor of GtH release.

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Table. A1.1. Effects of PKC desensitization, induced by TPA-pretreatment, on GtH responses to 100 nM sGnRHa-stimulated GtH release in perifusion studies. Total response is calculated as the net increase (ng GtH) in hormone contents for the entire response. Average values of the GtH level (ng/ml) in the fraction containing the peak response (Peak, 1st phase) and the plateau phase (20 min following the sGnRHa pulse) are also presented (Mean ± SE; n=3).

	GTH response	
	Control	Desensitized
sGnRHa-Total	174.27 ± 7.54	70.33 ± 15.17*
sGnRHa-Peak	22.83 ± 13.30	5.30 ± 0.17*
sGnRHa-Plateau	4.18 ± 0.38	2.08 ± 0.22*
Forskolin-Total	393.05 ± 63.75	1010.54 ± 182.51*

*, Significantly different from control, $P<0.05$, t-test.

Fig. A1.1. Experimental protocol for TPA-induced desensitization experiments in static incubation studies.

Experimental protocol for desensitization experiments with phorbol ester TPA. PKC is downregulated by 4-h pretreatment with TPA.

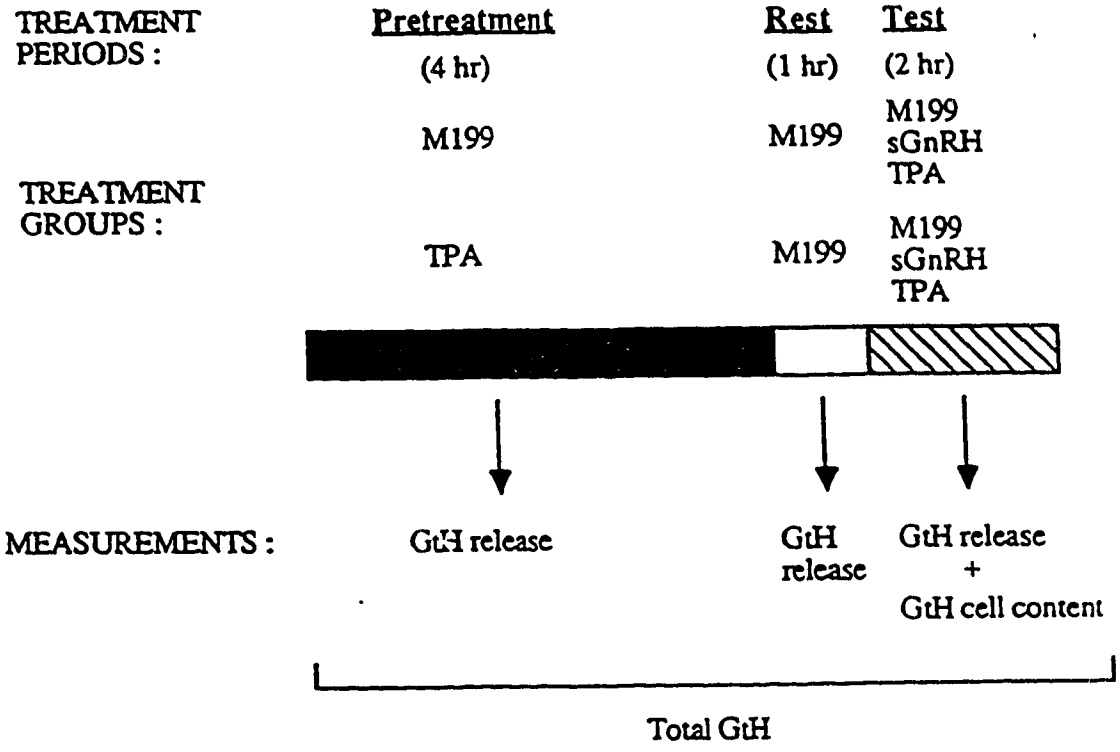
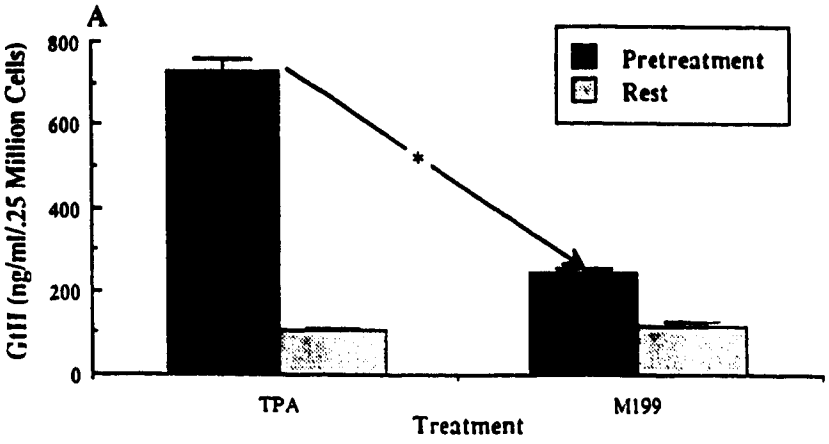


Fig. A1.2. Effects of TPA pretreatment on control (M199), TPA- (10 nM), and sGnRH-induced (10 nM) GTH responses during pretreatment, rest (A) and test stages (B), and the cell content (C) of GtH and the total hormone measurable. Treatment groups are identified by numbers and those with similar GtH values (Mean ± SE, n=8) are identified by having the same underscore.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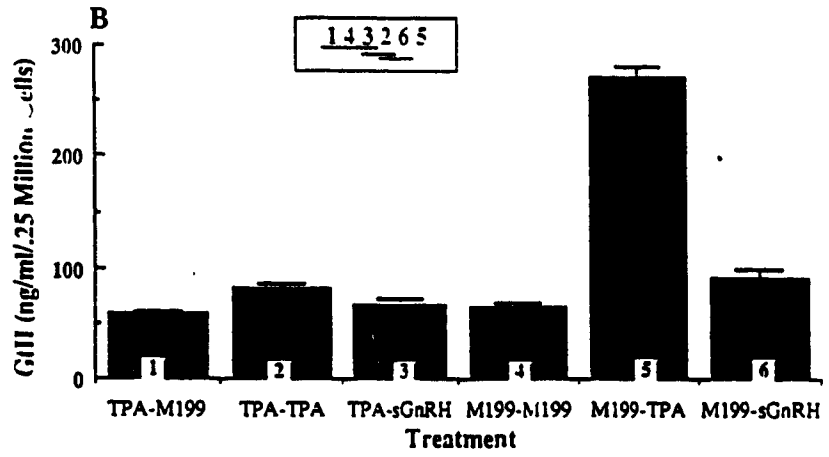
200
0
GtH (ng/ml/.25 Million Cells)
Pretreatment
Rest
TPA
M199
Treatment


B
143265
300
200
100
0
TPA-M199
TPA-TPA
TPA-sGnRH
M199-M199
M199-TPA
M199-sGnRH
Treatment

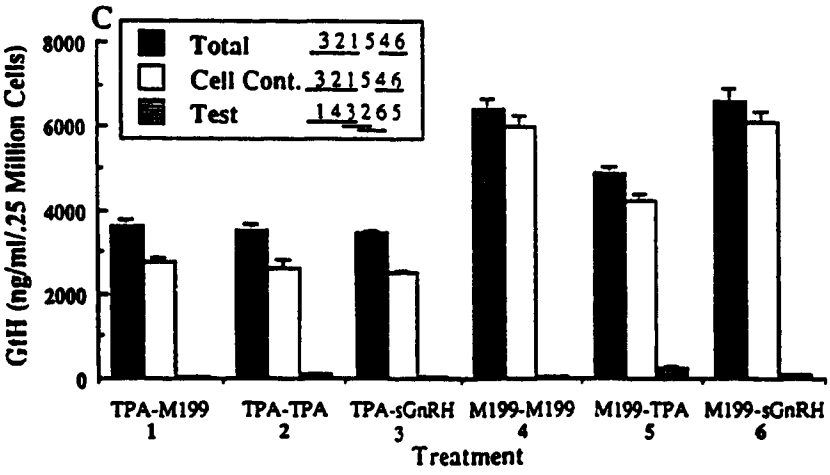
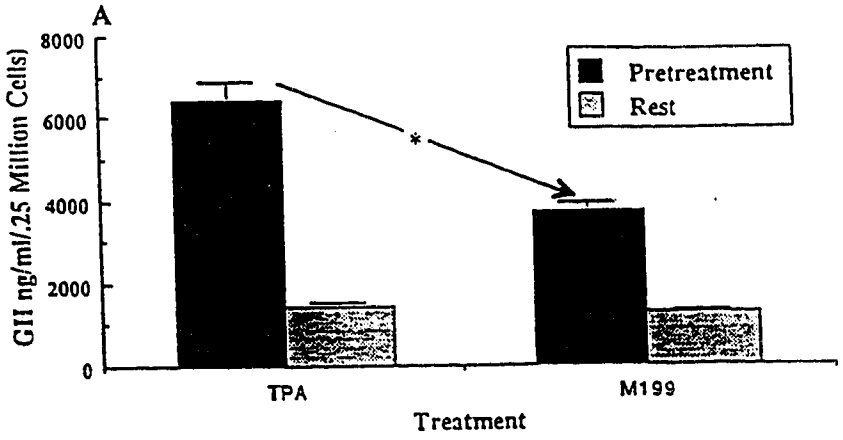
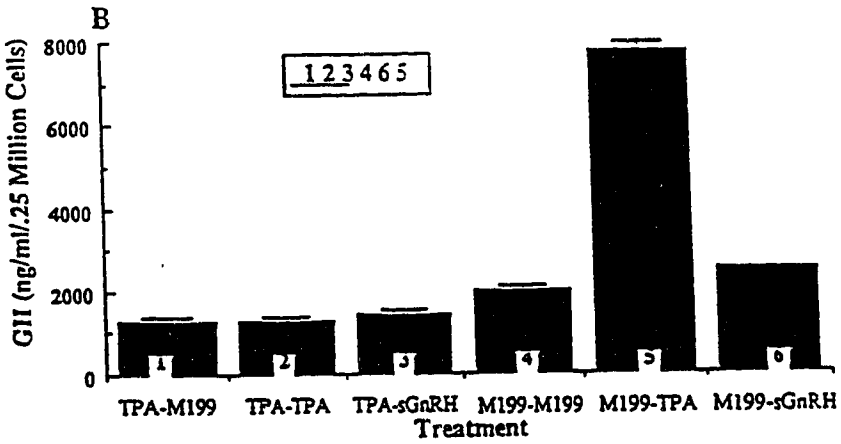

C
Total 321546
Cell Cont. 321546
Test 143265
8000
6000
4000
2000
0
GtH (ng/ml/.25 Million Cells)
TPA-M199 1
TPA-TPA 2
TPA-sGnRH 3
M199-M199 4
M199-TPA 5
M199-sGnRH 6
Treatment

Fig. A1.3. Effects of TPA pretreatment on control (M199), TPA-(10 nM), and sGnRH-induced (10 nM) GH responses during pretreatment, rest (A) and test stages (B), and the cell content (C) of GH and the total hormone measurable. Treatment groups are identified by numbers and those with similar GH values (Mean ± SE, n=8) are identified by having the same underscore.


A
8000
6000
4000
2000
0
GII ng/ml/.25 Million Cells)
Pretreatment
Rest
*
TPA
M199
Treatment


B
123465
8000
6000
4000
2000
0
GII (ng/ml/.25 Million Cells)
1
2
3
4
5
6
TPA-M199
TPA-TPA
TPA-sGnRH
M199-M199
M199-TPA
M199-sGnRH
Treatment

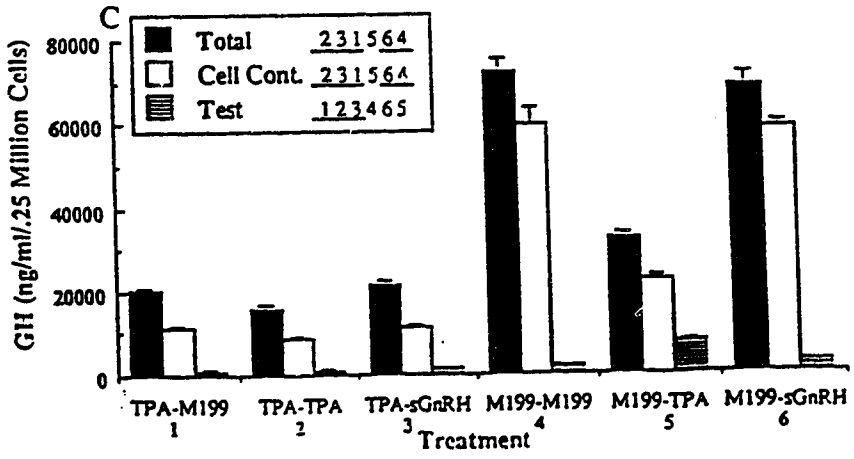

C
Total 231564
Cell Cont. 231564
Test 123465
80000
60000
40000
20000
0
GII (ng/ml/.25 Million Cells)
TPA-M199
1
TPA-TPA
2
TPA-sGnRH
3
M199-M199
4
M199-TPA
5
M199-sGnRH
6
Treatment

Fig. A1.4. Effects of TPA pretreatment on the GtH responses to sGnRHa and forskolin in column perifusion studies. Results (Mean ± SE, n=3) are normalized as a percentage of the initial basal secretion rates of the desensitized cell preparations. The upper inset is an enlargement of the sGnRHa-induced GtH response.

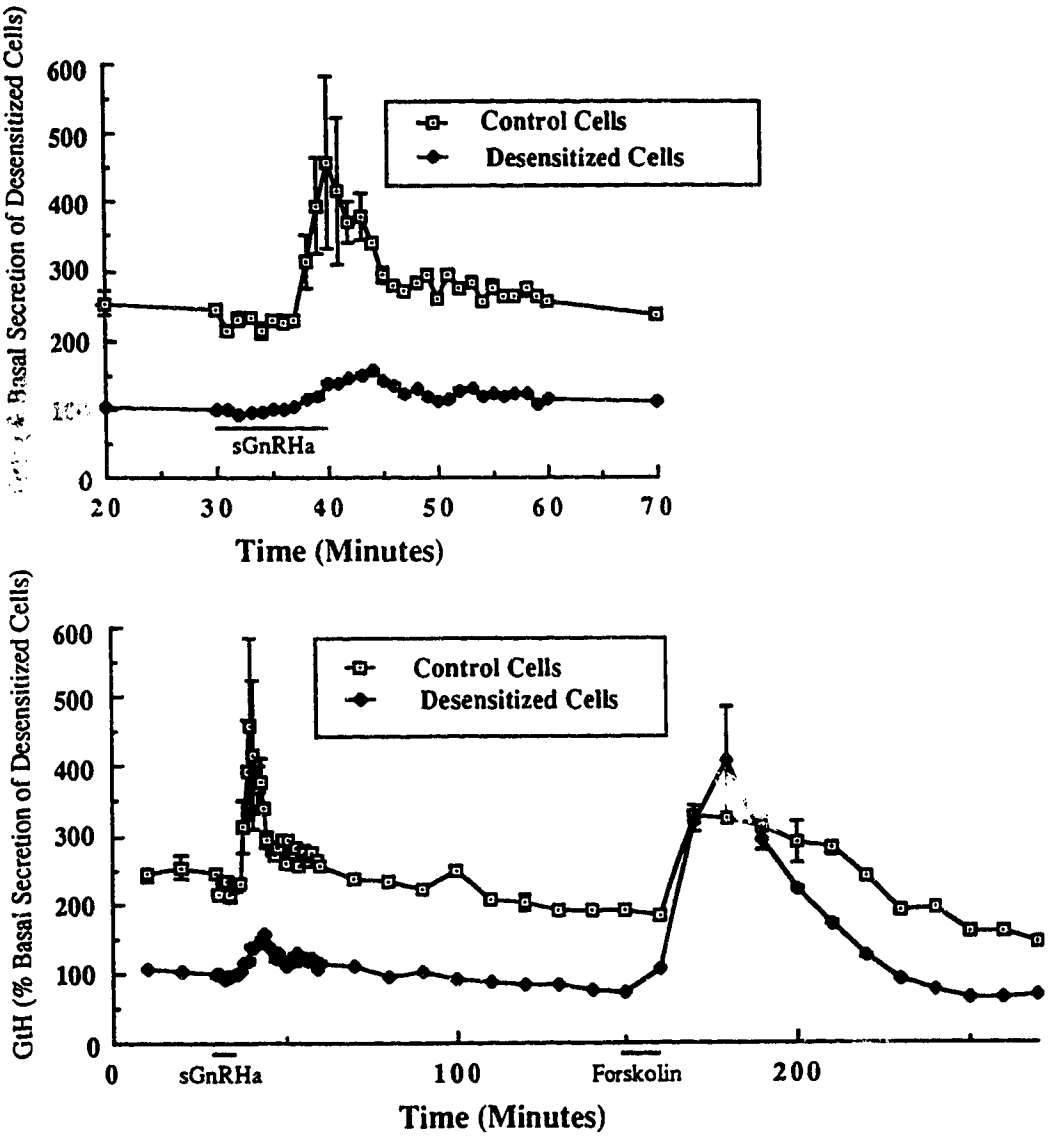
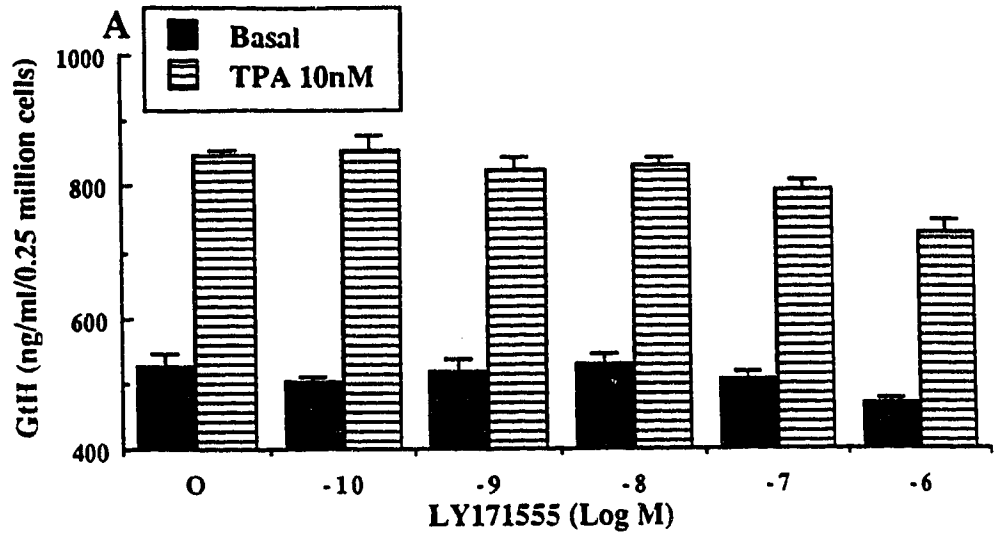
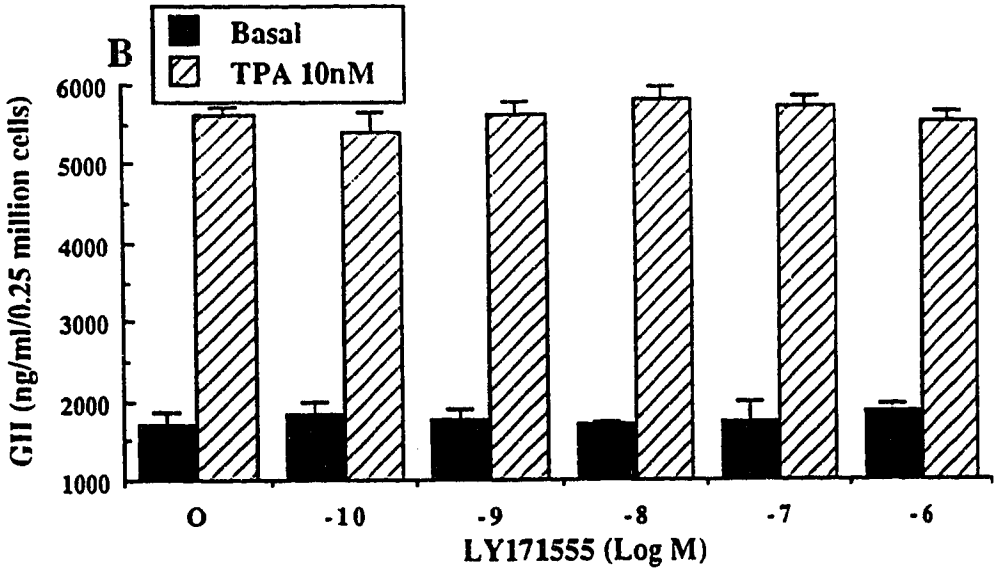
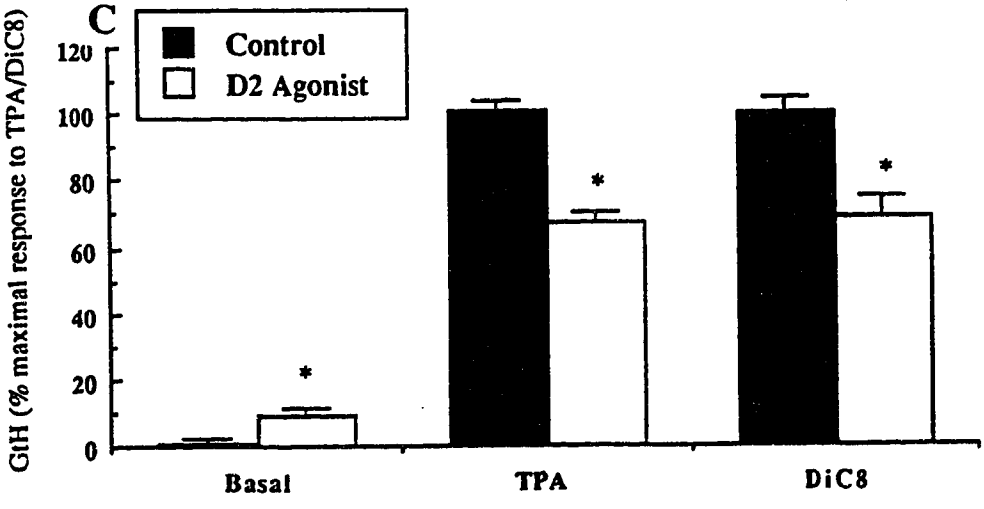

GtH (% Basal Secretion of Desensitized Cells)
600
500
400
300
200
100
0
Control Cells
Desensitized Cells
sGnRHa
20
30
40
50
60
70
Time (Minutes)
GtH (% Basal Secretion of Desensitized Cells)
600
500
400
300
200
100
0
Control Cells
Desensitized Cells
0
sGnRHa
100
Forskolin
200
Time (Minutes)

Fig. A1.5. Effects of increasing concentrations of a D2 agonist LY171555 on TPA-induced GtH (A) and GH response (B), and (C) the inhibitory action of 1 μM LY171555 on 10 nM TPA- and 100 μM DiC8-stimulated GH release. *, significantly different from control $P<0.05$, t-test. Figures A and A represent data from single experiments (4 replicates/treatment), while figure C represents pooled data from three experiments (Mean ± SE, n=12).


A
Basal
TPA 10nM
GtH (ng/ml/0.25 million cells)
1000
800
600
400
O
-10
-9
-8
-7
-6
LY171555 (Log M)


B
Basal
TPA 10nM
GH (ng/ml/0.25 million cells)
6000
5000
4000
3000
2000
1000
O
-10
-9
-8
-7
-6
LY171555 (Log M)


C
Control
D2 Agonist
GtH (% maximal response to TPA/DiC8)
120
100
80
60
40
20
0
*
*
*
Basal
TPA
DiC8