University of Alberta

# A study of the intracellular signalling mechanisms in PACAP-stimulated GH and GTH-II secretion in goldfish pituitary cells.

by

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A thesis submitted to the Faculty of Graduate studies and Research in partial fulfillment of the requirements for the degree of Master of Science

In

Physiology and Cell Biology

Department of Biological Sciences

Edmonton, Alberta, Canada

**Fall 2004**

*Your file Votre référence*
*ISBN: 0-612-95848-5*
*Our file Notre référence*
*ISBN: 0-612-95848-5*

*He may look like an idiot and talk like an idiot but don't let that fool you. He really is an idiot.*

*A child of five could understand this. Fetch me a child of five.*

**Groucho Marx (1890 - 1977)**

*The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' (I found it!) but 'That's funny ...'*

**Isaac Asimov (1920 - 1992)**

*The great tragedy of Science - the slaying of a beautiful hypothesis by an ugly fact.*

**Thomas H. Huxley (1825 - 1895)**

## Acknowledgements

I would like to sincerely thank my supervisor John Chang, who despite having some initial reservations, agreed to take me on as a graduate student. By doing so, you have allowed me to fulfill my dream of one day becoming a medical doctor. I believe that by being in this graduate program, I was able to achieve that little something extra I needed to be accepted into medical school, which had previously been missing. Aside from that, I have also learned more than I had ever thought I would. Not only have I gathered a wealth of theoretical knowledge, but also more importantly, like many graduate students before me, I have gained the ability to think and analyze problems at an entirely different level. No doubt, this skill will be indispensable in my career as a physician. John, thank you for the opportunity and the support you gave me in this program. Your efforts will not be forgotten!

I would also like to thank my committee and examination members. To Dr. Alice Hontela, thank you for taking the time to serve as my external examiner. Considering my fear of flying, the bumpy flight you must have endured from Lethbridge makes your contribution all the more appreciated. To Dr. Declan Ali and Dr. Jeff Goldberg, I thank you both very much for serving on my committee and for all your exceptional suggestions regarding my thesis. To Dr. Bruce Heming, thanks very much for taking the time to act as my committee chair. Your friendly calm demeanor put me at ease throughout my defense.

I also could not have completed this project without the valuable contributions of my colleagues. To Dr. Calvin Wong, I truly appreciate the time you spent showing me the ropes when I first started this program and for all your help when I taught Zoology 344. To Dr. Warren Yunker, thank you for your many, many invaluable suggestions and for your superb advice regarding my medical school interview. To Dr. Aubrey Uretsky, thanks for all your help you provided with my nitric oxide experiments. I would also like to extend my thanks to Shandra Doran who took the time to show me how to use the calcium imaging system. Although I never got around to working with it, your efforts are still very much appreciated.

Finally, I would like to thank my wife Andrea. Without your love and constant support I could not have succeeded in this program or have ever been able to gain entry into medical school. I truly believe that your name should be on this thesis as well since as you did as much, if not more, to help me complete it.

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| | |
|---|---|
| 7-Ni | 7-nitroindazole |
| 8-Br-cADPR | 8-bromo-adenosine 5'-cyclic diphosphate-ribose |
| 1400W | N-(3-aminomethyl) benzylacetamidine dihydrochloride |
| AA | Arachidonic acid |
| AC | Adenylate cyclase |
| ACTH | Adrenocorticotropic hormone |
| AGH | Aminoguanidine hemisulfate |
| ANOVA | Analysis of variance |
| ATPase | Adenosine triphosphatase |
| AVP | Arginine vasopressin |
| BHQ | 2,5-di-(*t*-butyl)-1,4-hydroquinone |
| $Ca^{2+}$ | Calcium |
| cADPR | Adenosine 5'-cyclic diphosphate-ribose |
| Caffeine | 1,3,7-trimethylxanthine |
| CaM | Calmodulin |
| cAMP | Cyclic adenosine monophosphate |
| CCCP | Carbonyl cyanide *m*-chlorophenylhydrazone |
| cGnRH-II | Chicken gonadotropin-releasing hormone-II |
| CNS | Central nervous system |
| CPA | Cyclopiazonic acid |
| D609 | Tricyclodecan-9-yl-xanthogenate |
| DAG | Diacylglycerol |

| | |
|---|---|
| DMSO | Dimethyl sulphoxide |
| EDRF | Endothelial-derived relaxation factor |
| ER | Endoplasmic reticulum |
| ET-18-$OCH_3$ | 1-O-Octadecyl-2-O-methyl-*rac*-glycero-3-phosphorylcholine |
| FSH | Follicle-stimulating hormone |
| GC | Guanylate cyclase |
| GI | Gastrointestinal |
| GH | Growth hormone |
| cGMP | Cyclic guanosine monophosphate |
| GTH-I | Follicle-stimulating hormone-like gonadotropin, gonadotropin I |
| GTH-II | Luteinizing hormone-like maturational gonadotropin, gonadotropin II |
| $IP_3$ | Inositol trisphosphate |
| i.c.v. | Intracerebroventricular |
| i.v. | Intravenous |
| JNK | c-Jun N-terminal kinase |
| LH | Luteinizing hormone |
| LY-83583 | 6-(Phenylamino)-5,8-quinolinedione,6-Anilino-5,8-quinolinedione |
| mRNA | Messenger ribonucleic acid |
| NAD | Nicotinamide adenine dinucleotide |
| NHE | $Na^+/H^+$ exchanger |

| | |
|---|---|
| NO | Nitric oxide |
| NOS | Nitric oxide synthase |
| eNOS | Endothelial NOS |
| iNOS | Inducible NOS |
| nNOS | Neuronal NOS |
| NPY | Neuropeptide Y |
| P chol | Phosphocholine |
| PACAP | Pituitary adenylate cyclase-activating polypeptide |
| PAM | Peptidyl glycine alpha-amidating monooxygenase |
| PC | Prohormone convertases |
| PCh | Phosphatidylcholine |
| PI | Phosphatidylinositol |
| $PIP_2$ | Phosphatidylinositol 4,5 bisphosphate |
| PKA | Protein kinase A |
| PKB | Protein kinase B |
| PKC | Protein kinase C |
| PKD1 | Protein kinase D1 |
| PKG | cGMP-dependent protein kinases |
| $PLA_2$ | Phospholipase $A_2$ |
| PLC | Phospholipase C |
| PLD | Phospholipase D |
| PNS | Peripheral nervous system |
| PRL | Prolactin |

| | |
|---|---|
| PTIO | 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide |
| PVN | Paraventricular nucleus |
| RR | Ruthenium red |
| Ru360 | $\{(u)[(HCO_2)(NH_3)_4Ru]_2Cl_3\}$ |
| Rutin hydrate | Quercetin-3-rutinoside hydrate |
| RyR | Ryanodine receptor |
| SERCA | Sarcoplasmic reticulum Ca-ATPase |
| SCN | Suprachiasmatic nucleus |
| sGC | Soluble guanylate cyclase |
| sGnRH | Salmon gonadotropin-releasing hormone |
| SEM | Standard error of the mean |
| SNP | Sodium nitroprusside |
| SON | Supraoptic nucleus |
| SP | Signal peptide |
| Tg | Thapsigargin |
| TMB-8 | 3,4,5-trimethoxybenzoate |
| TRH | Thyrotropin-releasing hormone |
| TSH | Thyrotropin |
| TTX | Tetrodotoxin |
| U73122 | 1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione} |
| VSCC | Voltage-sensitive $Ca^{2+}$ channels |
| VIP | Vasoactive intestinal polypeptide |

# 1 Introduction

## 1.1 *Neuroendocrine regulation of growth hormone and gonadotropin release in goldfish*

In goldfish, growth hormone (GH) and gonadotropins (GTH's) are important pituitary factors. Just as in other vertebrates, GH regulates growth (Marchant and Peter, 1986); whereas, the two GTH's, follicle-stimulating hormone (FSH)-like (GTH-I) and luteinizing hormone (LH)-like maturational gonadotropin (GTH-II) affect, reproduction through steroidogenesis and gametogenesis in fish (Van Der Kraak et al., 1990, 1992). In addition, GH affects reproduction because it potentiates the steroidogenic action of GTH-II and regulates the energy requirement for gonadal growth (Trudeau, 1997). Since goldfish are seasonal spawners, the coordinated control of GH and GTH's is important. However, because of the lack of a GTH-I assay, not much is known regarding the regulation of GTH-I secretion. In goldfish, the neuroendocrine control of GH and GTH-II secretion is controlled through the release of hypothalamic factors from neurons that directly innervate the adenohypophysis (Kah et al., 1993; Peter et al., 1990). Some of these factors may only act on one of the cell-types while others can affect hormone release in both cell-types.

The hypothalamic factors stimulating GH release in goldfish include neuropeptide Y (NPY; Peng, et al., 1990), dopamine (Wong, et al., 1992), salmon gonadotropin-releasing hormone (sGnRH) (Marchant et al., 1989; Chang et al., 1990a,b), chicken GnRH-II (cGnRH-II) (Chang et al., 1990a), thyrotropin-releasing hormone (TRH) (Trudeau, et al., 1992), galanin (Rao et al., 1996), and

ghrelin (Unniappan and Peter, 2004). Inhibitors of GH secretion in goldfish are somatostatin (Marchant et al., 1987; Kwong and Chang, 1997), norepinephrine (Lee et al., 2000), and serotonin (Somoza and Peter, 1991). GTH-II is stimulated by sGnRH, (MacKenzie et al., 1984), cGnRH-II (Chang et al., 1990a,b; Khakoo et al., 1994), and NPY (Peng et al., 1990). At this time, dopamine is the only known hypothalamic inhibitor of GTH-II secretion in goldfish (Chang et al., 1990b).

Recently, a newly discovered peptide, pituitary adenylate cyclase-activating polypeptide (PACAP), has been shown to stimulate both GH and GTH-II secretion in goldfish (Wong et al., 1998; Chang et al., 2001). The main goal of this thesis is to elucidate some of the intracellular mechanisms mediating PACAP action on goldfish gonadotropes and somatotropes. In the following sections of this chapter, the known signal transduction mechanisms mediating neuroendocrine regulation of GH and GTH-II release in goldfish, as well as the possible functions and mechanisms of action of PACAP in mammals and in fish will be briefly reviewed.

### *1.1.1 Signal transduction mechanisms mediating GH and GTH-II release in goldfish*

Among the many hypothalamic factors listed above, the signal transduction pathways mediating the GH and GTH-II secretion responses to sGnRH, cGnRH-II, and dopamine are the most well characterized in goldfish.

#### *1.1.1.1 Signal transduction pathways mediating GH release*

Stimulation of GH release by sGnRH and cGnRH-II peptides appears to involve a phospholipase C (PLC)/protein kinase C (PKC)-dependent pathway, as well as that of the signalling molecule, nitric oxide (NO) (Uretsky and Chang 2000; Uretsky et al., 2003; and reviewed in Chang et al., 2000). The dopamine-dependent release of GH is through the adenylate cyclase (AC)/cyclic (c)AMP/protein kinase A (PKA) signalling mechanism (Chang et al., 1994; Wong et al., 1994b). A role for phospholipase $A_2$ ($PLA_2$)/arachidonic acid (AA) in the dopamine signalling pathway leading to GH secretion has also been demonstrated (Chang, et al., 1996a).

Calcium ($Ca^{2+}$) mobilization is also required for GH secretion. Extracellular $Ca^{2+}$ entry through L-type voltage-sensitive $Ca^{2+}$ channels (VSCC) has been shown to be important to cGnRH-II-, sGnRH-, and dopamine-stimulated GH secretion (Chang et al., 1994, Chang et al. 1996b; Wong et al., 1994a). Experiments with the broad-spectrum intracellular $Ca^{2+}$ release inhibitor 3,4,5-trimethoxybenzoate (TMB-8) indicate that intracellular $Ca^{2+}$ pools are also required for GH secretion in response to the two GnRHs and dopamine (Johnson and Chang, 2000). To date, a number of pharmacologically distinct intracellular $Ca^{2+}$ stores have been identified which participate in the modulation of pituitary hormone secretion in goldfish. These include, 1) ryanodine-sensitive stores, 2) caffeine-sensitive but relatively ryanodine-insensitive stores, 3) xestospongin-C-sensitive (presumably inositol trisphosphate, $IP_3$-sensitive) stores, 4) ruthenium red and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)-sensitive stores

(presumably mitochondria), 5) stores refilled by cyclopiazonic acid (CPA)- and 2,5-di-(*t*-butyl)-1,4-hydroquinone (BHQ)-sensitive sarcoplasmic/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA), and 6) those sensitive to the SERCA inhibitor thapsigargin (Tg). The participation of these pharmacologically distinct stores in GH release is ligand-selective (Table 1.1; Chang et al, 2000; Johnson, 2000). sGnRH and cGnRH-II both utilize caffeine-sensitive stores. In addition, at times of the year when the gonads are regressed, cGnRH-II action appears to be modulated by a ryanodine-sensitive $Ca^{2+}$ store. In contrast, only sGnRH utilizes xestospongin/$IP_3$-sensitive and mitochondrial $Ca^{2+}$ stores to elicit GH secretion (Johnson, 2000). Evidence further suggests that the $Ca^{2+}$ stores mediating GnRH actions are not affected by BHQ, CPA and Tg (Johnson and Chang, 2000). On the other hand dopamine-induced GH secretion involves BHQ-sensitive but not caffeine-sensitive stores (Chang et al., 2003; Wong et al., 2001); Tg-sensitive SERCA also enhances the GH response to dopamine suggesting that this SERCA normally negatively modulates dopamine action (Chang et al., 2003). Downstream of $Ca^{2+}$, calmodulin (CaM) kinase II participates in the signalling cascades leading to GH secretion induced by sGnRH, cGnRH-II and dopamine (Chang et al., 2000; 2003).

#### *1.1.1.2 Signal transduction mediating GTH-II release*

PLC/PKC mechanisms are important in mediating sGnRH and cGnRH-II actions on GTH-II release (reviewed in Chang et al., 2000). sGnRH action also involves an additional $PLA_2$/ AA pathway (Chang et al., 1991).

The modulation of cytosolic free $Ca^{2+}$ ($[Ca^{2+}]_c$) levels is also important for the control of GTH-II secretion by GnRH. $Ca^{2+}$ entry through VSCC has been shown to be important for GTH-II release by both sGnRH and cGnRH-II (Chang et al., 1996b; Chang et al., 1997). Results with TMB-8 indicate that intracellular $Ca^{2+}$ stores are important in the control of GTH-II release by the two GnRHs (Johnson et al., 2000). The specific $Ca^{2+}$ stores used by these two peptides has been elucidated (Table 1.2). Stores involved in sGnRH and cGnRH-II appears to be refilled by CPA- and BHQ-sensitive SERCA (Johnson et al., 2000; Johnson, 2000). Differences between the two peptide's requirement in intracellular $Ca^{2+}$ usage are also evident. Caffeine-sensitive $Ca^{2+}$ pools mediate sGnRH, but not cGnRH-II, stimulation of GTH-II release (Johnson et al., 2000). Conversely, ryanodine-sensitive $Ca^{2+}$ stores are used by sGnRH to evoke GTH-II secretion in sexually regressed goldfish whereas these pools are involved in cGnRH-II-dependent GTH-II release of this hormone at all stages of gonadal development (Johnson and Chang, 2002). In addition, $IP_3$-sensitive $Ca^{2+}$ stores seem to be used only by sGnRH to stimulate GTH-II release (Johnson, 2000; Johnson and Chang, 2002).

Dopamine-dependent inhibition of basal GTH-II levels is reported to be through the reduction of cyclic adenosine monophosphate (cAMP) levels (Chang et al., 1992). Inhibition of GnRH-stimulated GTH-II release by dopamine appears to operate through the inhibition of PKC and PLC activity in gonadotropes (Chang et al., 1993, Chang et al., 1997). The inhibition of basal and stimulated GTH-II release by dopamine is also thought to involve modulation of VSCC (Van

Goor et al. 1998); however, the intracellular $Ca^{2+}$ stores affected during dopamine-induced suppression of GTH-II release has not been investigated.

*1.2 PACAP in mammals*

*1.2.1 Discovery and synthesis*

PACAP is a member of the vasoactive intestinal polypeptide (VIP) family of peptides. PACAP is a 38 amino acid peptide (PACAP38) first identified in ovine hypothalamus and is shown to activate adenylate cyclase (AC) and GH release in rat pituitaries (Miyata et al., 1989). Since that time, another PACAP isoform has also been identified from rat pituitaries; this corresponds to the N-terminal 27 amino acid sequence of PACAP38 and is called, PACAP27 (Miyata et al., 1990).

The two functional neuropeptide isoforms are derived from a common prohormone consisting of 176 amino acids termed, proPACAP (Li et al., 1999). In rat pituitary GH4C1 cells, posttranslational proteolysis by the prohormone convertases, PC1 and PC2, convert proPACAP to the mature forms, PACAP38 and PACAP27 (Li et al., 1999). Another convertase isoform, PC4, performs this function in rat testis (Li et al., 1998). PACAP38 has been shown to contain three discrete domains located at the 1-8, 9-26, and 27-38 amino acid positions with the first 27 amino acids postulated to be responsible for biological activity and receptor binding (Wray et al., 1993). While PACAP38 contains all of these regions PACAP27 only has the first two (Figure 1.1). Although there are two PACAP forms identified, PACAP38 is by far the most common, representing 70 – 90% of the detected form depending on the tissue (reviewed in Vaudry et al.,

2000). Because of the predominance in tissue levels of PACAP38 over PACAP27, this review will focus only on the characteristics of the 38 amino acid isoform unless otherwise noted.

*1.2.2 Tissue distribution*

Both isoforms of PACAP have a wide tissue distribution in mammals. They are found throughout the central nervous system (CNS) with the highest concentrations in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus in pigs (Koves et al., 1990), primates (Vigh et al., 1991) and rats (Masuo et al 1993). Although PACAP has not been found within the anterior pituitary, results indicate that it is likely produced in the neurons of the hypothalamus and released into the portal circulation, which carries it to the anterior pituitary where a response is initiated (Tamada et al., 1994; Dow et al., 1994). In rats, immunohistochemical and *in situ* hybridization experiments have shown that PACAP is present in cells of the retinal ganglion layer, suprachiasmatic nucleus (SCN) nerve fibers within the retinohypothalamic tract, vestibulo nucleus, cochlear nucleus, cerebellum, paraventricular thalamic nucleus, posterior pituitary, choroid plexus, the dorsal and ventral horn of the spinal cord, hippocampus, and the dentate gyrus (Arimura and Shioda,1995; Hannibal et al., 1997; Koves et al., 1991; Hannibal, 2002;). In humans, immunoreactive PACAP has been shown to be present in the spinal cord (Dun et al., 1996). Within the peripheral nervous system (PNS), PACAP immunoreactivity is seen in the small nerve cell bodies of the spinal ganglia and trigeminal ganglia, in nerve fibers in the skin, nose, tongue, larynx-trachea, and

around the ducts of the submandibular gland in rats (Moller et al., 1993; Mulder et al., 1994), and in the dorsal root ganglia in humans (Dun et al., 1996).

Outside of the nervous system, immunocytochemistry studies have demonstrated that PACAP is present in significant concentrations in the lung, gastrointestinal (GI) tract, adrenal gland, testes, ovaries, bladder, urethra, and retina in rats (Arimura et al., 1991; Arimura and Shioda 1995; Seki et al., 1997; Mohammed et al., 2002) and the brain, GI tract, kidney, and adrenal glands of mice (Miyata et al., 2000). In humans, PACAP mRNA has been localized in the prostate gland (Garcia-Fernandez et al., 2002) and the female genital tract (Steenstrup et al., 1995). In addition, immunoreactive PACAP is present in the lower oesophageal sphincter (Ny et al., 1995) and in the adrenal medulla (Mazzocchi et al., 2002).

### *1.2.3 Functions of PACAP*

The broad tissue distribution of PACAP in mammals suggests that it may have numerous functions. This assertion has been supported by a number of studies, most of which focused on PACAP38.

#### *1.2.3.1 PACAP actions within the CNS*

Masuo et al. (1993) and Kozicz et al. (1997) postulated that PACAP acts as a neurotransmitter and a neuromodulator within the CNS of rats, and Chiba et al. (1996) showed that it may act as a neurotransmitter in preganglionic sympathetic neurons in rat spinal cord. A role as a neuromodulator in rat

hippocampus is evident since PACAP could depress synaptic transmission in this part of the brain (Kondo et al., 1997).

PACAP also has many other functions within the CNS. When given by intracerebroventricular injection (i.c.v.), PACAP increases the level of the dopamine metabolite, DOPAC, in sheep hypothalamus, indicating that it plays a role in the control of dopamine turnover within the CNS (Anderson and Curlewis, 1998). Localization of PACAP immunoreactivity in corticotrophin-releasing hormone and TRH neurons in rats suggests that PACAP may be involved in the regulation of the hypothalamic-pituitary-adrenal axis and may function as a cofactor for TRH neurons (Legradi et al., 1997,1998). In addition, PACAP has been implicated in the regulation of the inflammatory response within the CNS (Delgado, 2002). PACAP serves a neuroprotective function as PACAP treatment decreases neuronal death in the CA1 region of the hippocampus following forebrain ischemia (Uchida et al., 1996), reduces infarct size after middle cerebral artery occlusion (Reglodi et al., 2000), and reduces rat neuronal death due to exposure to human immunodeficiency virus envelope protein gp120 (Brenneman et al., 2002).

PACAP also modulates behavior. It may influence the circadian rhythm as SCN neuronal activity was significantly changed upon its application in rats (Hannibal et al., 1997; Fukuhara et al., 1997) and hamsters (Harrington et al., 1999). When given by i.c.v. injection, PACAP produces an anorectic effect in several species (Morley et al., 1992; Chance et al., 1995; Tachibana et al., 2003). In PACAP-knockout mice, altered psychomotor behaviors were observed

(Hashimoto et al., 2001). A role in learning and memory has also been postulated for PACAP; its application caused an increase in the consolidation of the passive avoidance response in rats (Telegdy and Kokavszky, 2000; Sacchetti et al., 2001).

Neurotrophic properties have also been attributed to this peptide. PACAP promotes the production of interleukin 6 in cultured astrocytes (Tatsuno et al., 1996), stimulates neurite outgrowth in rat cerebellar neuroblasts (Gonzalez et al., 1997), increases the number of mesencephalic dopaminergic neurons in primary embryonic rat cultures (Takei et al., 1998), increases the volume of the cerebellar cortex (Vaudry et al., 1999), and is reported to have general neurotropic activity throughout the CNS and PNS (Lindholm et al., 1998). It also promotes olfactory neurogenesis and olfactory receptor neuronal survival in rats (Hansel et al., 2001) and mediates adult neural stem cell proliferation in mice (Mercer et al., 2004).

*1.2.3.2 PACAP's hypophysiotropic actions*

One of the best-characterized actions of PACAP is its hypophysiotrophic properties. PACAP can act directly at the level of the pituitary or indirectly through modulation of other neuroendocrine regulators. I.c.v. administration of PACAP in rats (Murase et al., 1993) and peripheral infusion of this peptide in humans (Chiodera et al., 1995) stimulate a rise in plasma arginine vasopressin (AVP) levels. PACAP-containing nerve fibers also innervates AVP-containing cells in the rat SON (Shioda et al., 1997). Together these results indicate that PACAP is involved in the modulation of water balance.

PACAP is also involved in the regulation of LH secretion. PACAP infusion into the arcuate nucleus reduces LH levels in ovariectomized ewes (Anderson et al., 1996). In contrast, in rats, LH secretion is stimulated by PACAP, both *in vitro* (Tsujii et al., 1994) and *in vivo* (Osuga et al., 1992; Szabo et al., 2002), and PACAP acts synergistically with LH-releasing hormone to increase LH levels in rat pituitary cultures (Culler and Pasachall, 1991). These results suggest a possible role for PACAP in reproduction.

PACAP has been shown to affect prolactin (PRL) secretion. Depending on the study, it acts either in an inhibitory or stimulatory role. Infusion of PACAP into the arcuate nucleus reduces plasma PRL levels in ovariectomized ewes (Anderson et al., 1996). When given by intravenous (i.v). injection or i.c.v. infusion, PACAP increases plasma PRL in rats (Jarry et al., 1992; Yamauchi et al., 1995). In pituitary cell culture experiments, PACAP has been reported to have either stimulatory (Nagy et al., 1993) or inhibitory (Jarry et al., 1992) effects on PRL production, as well as to increase PRL mRNA (Coleman and Bancroft, 1993; Velkeniers et al., 1994). In humans, i.v. injection of PACAP increases PRL secretion (Chiodera et al., 1996).

PACAP is also involved in the control of adrenocorticotropic hormone (ACTH) secretion. Intravenous administration of PACAP increases ACTH levels in rats (Leonhardt et al., 1992) and humans (Chiodera et al., 1996). Similarly, PACAP increases ACTH production in cultured rat pituitary cells (Hart et al., 1992; Propato-Mussafiri et al., 1992).

The role of PACAP in regulating thyrotrope activities appears controversial. On one hand, application of PACAP produces no change in the levels of thyrotropin (TSH) production *in vivo* (Hart et al., 1992; Chiodera et al., 1996) or *in vitro* (Culler and Paschall, 1991). On the other hand, PACAP has been reported to act synergistically with LH-releasing hormone to increase TSH secretion (Culler and Paschall, 1991). Thus, PACAP may behave as a modulator, rather than a primary regulator of TSH secretion. Available evidence consistently demonstrates a stimulatory role for PACAP in the regulation of GH secretion. PACAP increases GH release from rat (Goth et al., 1992; Hart et al., 1992; Nagy et al., 1993; Velkeniers et al., 1994) and from sheep pituitary cell cultures (Sawangjaroen et al., 1997). Similarly, i.v. injections of PACAP elevate circulating GH levels in rats (Jarry et al., 1992) and cattle (Radcliff et al., 2001).

*1.2.3.3 Peripheral PACAP actions*

Outside the CNS, PACAP has numerous functions. In the respiratory system, PACAP relaxes tracheal smooth muscles and causes bronchodilation in guinea pig (Foda et al., 1995; Linden et al., 1998). It modulates small bronchi tone in humans (Kinhult et al., 2000) and increases airway mucus secretion in rats and ferrets (Wagner et al., 1998; Liu et al., 1999).

PACAP action on vasomuscular tone is mixed. It has been shown to be a potent vasorelaxant in guinea pig lung tissue (Cardell et al., 1991), rat-tail artery (Absood et al., 1992), human pulmonary artery (Cardell et al., 1997), and rabbit cardiovascular tissue (Dalsgaard et al., 2003); however, other researchers have

suggested it has vasoconstrictive properties as well (Minkes et al., 1992; Santiago and Kadowitz, 1993).

The action of PACAP on the GI tract is also well documented. PACAP modulates gut motility in guinea pig (Katsoulis et al., 1996), rat (Katsoulis et al., 1993; Ozawa et al., 1999) and humans (Schworer et al., 1993). It increases the secretion of saliva in rats (Mirfendereski et al., 1997), stimulates the release of pepsinogen from the cultured guinea pig chief cells (Felley et al., 1992), enhances bicarbonate release from rat duodenum (Takeuchi et al., 1997), and increases chloride secretion in rat colon (Kuwahara et al., 1993) and in human colonic T84 cells (Leung et al., 2001). It has also been shown to regulate gastric acid secretion in rats (Mungan et al., 1992,1995) and mice (Piqueras et al., 2004) and to stimulate intestinal ion transport in humans (Fuchs et al., 1996).

PACAP is also an important controller of peripheral hormone release. PACAP increases catecholamine secretion from adrenal chromaffin cells in rats (Watanabe et al., 1992) and pigs (Isobe et al., 1993). In rats, it stimulates testosterone release from cultured Leydig cells (Romanelli et al., 1997; Rossato et al., 1997), estradiol secretion from cultured rat sertoli cells (Heindell et al., 1992) and progesterone production in granulosa cells (Zhong and Kasson, 1994; Gras et al., 1999). In addition, PACAP enhances glucagon and insulin secretion in mice (Fridolf et al., 1992; Filipsson et al., 1997) and humans (Filipsson et al., 1998). It also has been demonstrated to stimulate atrial natriuretic peptide secretion in cultured rat myocardiocytes (Basler et al., 1995).

PACAP also affects bone, liver, pancreas, synaptic plasticity in the PNS, and immune system function. The peptide has been found to modulate bone metabolism (Kovacs et al., 1996; Winding et al., 1997), stimulate glucose release from the liver (Yokota et al., 1995; Sekiguchi et al., 1994), induce pancreatic amylase and bicarbonate secretion (Mungan et al., 1991, Raufmann et al., 1991), increase N-methyl-D-aspartate receptor-mediated responses in the rat sympathetic preganglionic neurons (Wu et al., 1997), and stimulates histamine release from mast cells (Odum et al., 1998).

*1.2.4 Tissue receptors for PACAP*

Three receptors for the PACAP peptide have been identified: $PAC_1$, $VPAC_1$, and $VPAC_2$ (Harmar et al., 1998). They are members of the G-protein coupled secretin/glucagon receptor subfamily (Spengler et al., 1993; Pisegna and Wank, 1993; Journot et al., 1995; Hezareh et al., 1996; Seebeck et al., 1998; Liu et al., 2000). $PAC_1$ has been cloned in several species including humans (Ogi et al., 1993), bovine (Miyamoto et al., 1994), mice (Susuki et al., 2003), and rats (Hashimoto et al., 1993). The $VPAC_1$ and $VPAC_2$ receptors have been cloned in rat (Ishihara et al., 1992; Lutz et al., 1993) and in humans (Sreedharan et al., 1993; Adamou et al., 1995). The messenger ribonucleic acid (mRNA) for all three receptors have been found in most areas of the CNS, including the anterior pituitary gland, and with $PAC_1$ being the most dominant (Vaudry et al., 2000). In peripheral tissues, $PAC_1$ mRNA is strongly expressed in the PNS, pancreas, testis, lung, liver, placenta and adrenal medulla; $PAC_1$ is also expressed in a lesser degree in many other tissues (Vaudry et al., 2000; Sherwood et al., 2000).

The pattern of $VPAC_1$ and $VPAC_2$ mRNA expression in tissues is similar to that of $PAC_1$ but these were generally expressed at lower levels (Vaudry et al., 2000). The binding affinity of the $PAC_1$ receptor has been determined to be about 1000 times higher for PACAP38 and PACAP27 than for VIP (Cauvin et al., 1990; Gottschall et al., 1990; Rawlings and Hezareh, 1996); whereas, $VPAC_1$ and $VPAC_2$ have similar affinities for PACAP and VIP (Gottschall et al., 1990; Rawlings and Hezareh, 1996). Accordingly, PACAP38 and PACAP27 both activate $PAC_1$ receptor with equivalent effectiveness, which is significantly higher than that of VIP, while PACAP and VIP both stimulate the VPAC receptors to the same degree (Rawlings and Hezareh, 1996; Vaudry et al., 2000).

*1.2.5 Post-receptor signal transduction*

In mammals, PACAP receptors operate through two signalling pathways: PLC/inositol-1,4,5-phosphate/PKC (PLC/$IP_3$/PKC) and AC/cAMP/PKA. $PAC_1$ efficiently uses both the AC and PLC pathways to elicit cellular effects; in contrast, $VPAC_1$ and $VPAC_2$ have been shown to operate through AC but the evidence is inconclusive as to whether they also use the PLC mechanism (Journot et al., 1994; Spengler et al., 1993). Because the $PAC_1$ receptor also has such a high affinity for the PACAP molecule and can be more effectively activated by PACAP38 and PACAP27 than by VIP to induce cAMP and $IP_3$ generation (Rawlings and Hezarah, 1996), this receptor is thought to be the most important in mediating PACAP action. Subsequent studies have confirmed the involvement of $PAC_1$-transduced PLC and/or AC activation in mediating PACAP

intracellular signalling (Basille et al., 1995; Coleman et al., 1996; Rene et al., 1996; Isobe et al., 1996; Zhou et al., 2001).

Further investigations into the signal transduction cascade indicate that PACAP also induces fluctuations in $[Ca^{2+}]_c$ levels. PACAP has been shown to facilitate extracellular $Ca^{2+}$ entry via VSCC, either through cAMP production and PKA activation (Isobe et al., 1993; Hezareh et al., 1997; Martinez-Fuentes et al., 1998a,b) or by diacylglycerol (DAG)/PKC via mechanisms independent of $IP_3$ (Chatterjee et al., 1996; Tanaka et al., 1998). Intracellular $Ca^{2+}$ mobilization following PACAP treatment involves the AC/PKA pathway (Canny et al., 1992; Payet et al., 2003) or $IP_3$ elevation following PLC activation (Rawlings et al., 1994; Schomerus et al., 1994; Barnhart et al., 1997; Martinez-Fuentes et al., 1998a,b; Pardi and Margiotta, 1999).

Specifically in somatotropes and gonadotropes, it has been reported that PACAP treatment induces a $[Ca^{2+}]_c$ rise but the mechanism by which this occurs may be different in each cell type. In somatotropes, $[Ca^{2+}]_c$ increases have been linked to the AC/cAMP pathway alone; whereas, in gonadotropes, elevations in $[Ca^{2+}]_c$ seems to be stimulated through $IP_3$/PLC (Canny et al., 1992; Rawlings et al., 1993, 1994, 1995; Schomerus et al., 1994; Martinez-Fuentes et al., 1998a,b; Alarcon and Garcia-Sancho, 2000), as well as the AC/cAMP pathways (Hezereh et al., 1997). It is through increases of $[Ca^{2+}]_c$ by entry from extracellular sources and release from intracellular stores that cellular responses, such as hormone secretion, are mediated.

*1.3 PACAP in non-mammalian vertebrates, and especially in fish*

*1.3.1 Identification of PACAP in non-mammalian vertebrates*

Both PACAP isoforms have been found in non-mammalian vertebrates and they share >85% amino acid similarity with mammalian forms (Vaudry et al., 2000; Wong et al., 2000). The non-mammalian vertebrate species from which PACAP mRNA has been identified include, chicken (Yasuhara et al., 1992), duck (Mirabella et al., 2001, 2002), turkey (Yoo et al., 2000), turtle (Reglodi et al., 2001), frog (Chartrel et al., 1991; Yoo et al., 2000), newt (Gobbetti et al., 1997), and lizard (Pohl and Wank, 1998). PACAP has also been identified in fish. It has been found in salmon (Parker et al., 1993); the Atlantic cod and rainbow trout (Olsson and Holmgren, 1994); catfish (McRory et al., 1995); stargazer (Matsuda et al., 1997); stingray (Matsuda et al., 1998); eel (Montero et al., 1998); goldfish (Wong et al., 1998); zebrafish (Wei et al., 1998; Fradinger and Sherwood, 2000; Wang et al., 2003); sturgeon, whitefish, grayling, flounder, and halibut (Adams et al., 2002); and grouper (Jiang et al., 2003).

*1.3.2 Tissue distribution in fish*

In fish, the tissue distribution of PACAP is not as well characterized as that in mammals, but there is a comparable amount of diversity in distribution. PACAP has been localized to the gut of the Atlantic cod and rainbow trout (Olsson and Holmgren, 1994); the large veins of rainbow trout (Johnsson et al., 2001); the brain and gut of the stargazer and stingray (Matsuda et al., 1997, 2002); the brain, testis, ovary, stomach, pituitary, fat, gastrointestinal tract, and muscle of catfish (McRory et al., 1995; Small and Nonneman, 2001); the CNS of

eels (Montero et al., 1998); the brain, eye, GI tract, ovary and testis of zebrafish (Fradinger and Sherwood, 2000); and the CNS, PNS, testes, ovary, pituitary, gill, kidney, GI tract, heart, and liver in goldfish (Wong et al., 2000).

*1.3.3 Functions in fish*

Functionally, PACAP has a similar variety of actions in fish as seen in mammals. It has been postulated to stimulate the secretion of mucus in goldfish gill (de Girolamo et al., 1998), to inhibit intestinal contractions in the Atlantic cod (Olsson and Holmgren, 1998, 2000), to promote the relaxation of the rectum of the stargazer (Matsuda et al., 2000), to directly stimulate the secretion of adrenalin from chromaffin cells in trout (Montpetit and Perry, 2000; Montpetit et al., 2003), and to mediate ovarian function in zebrafish (Wang et al., 2003).

*1.3.3.1 Hypophysiotropic actions in fish*

Of particular interest, PACAP has also been shown to affect adenohypophyseal cell functions in fish. PACAP can elevate the GnRH-stimulated pituitary glycoprotein hormone alpha subunit transcription in tilapia (Gur et al., 2001). It also stimulates GH secretion in several fish species including, catfish (McRory et al., 1995), salmon (Parker et al., 1997), goldfish (Wong et al., 1998), eel (Montero et al., 1998; Rousseau et al., 1999), turbot (Rousseau et al. 2001), rainbow trout (Rousseau et al., 2001), and carp (Xiao et al., 2002). It has also been shown to stimulate GTH-II secretion in goldfish (Wong et al., 2000; Chang et al., 2001).

In particular, the neuroendocrine actions of PACAP on teleost pituitary hormone secretion have been relatively well studied in goldfish. PACAP-immunoreactive neurons directly innervate the pars distalis, terminating in areas adjacent to the gonadotropes and somatotropes. Results from *in vitro* and *in vivo* experiments using PACAP and the PACAP antagonist PACAP6-38 indicate that PACAP enhances GH and GTH-II secretion by directly acting on the somatotropes and gonadotropes through a PACAP receptor (Wong et al., 2000; Wirachowsky et al., 2000).

*1.3.4 PACAP receptors in fish*

PACAP receptors have been cloned in several fish species, including zebrafish, rainbow trout and goldfish (Wei, et al., 1998; Wong et al.,1998; Montpetit et al., 2000). As in mammals, three PACAP receptors, $PAC_1$, $VPAC_1$, and $VPAC_2$, exist. In goldfish, $PAC_1$ has the greatest homology in amino acid composition relative to the mammalian receptors and the $PAC_1$ receptor is limited to the tissues of the CNS and the heart; whereas $VPAC_1$ and $VPAC_2$ have a much broader distribution (Wong et al., 2000; Wang et al., 2002). The functionality of the $PAC_1$ and $VPAC_1$ receptors closely resembles that seen in mammals as they have been shown to increase cAMP levels upon binding with PACAP in COS-7 cells (Chow et al., 1997; Wong et al., 1998). $PAC_1$ is selective for PACAP, whereas $VPAC_1$ can bind to PACAP or VIP equally (Chow et al., 1997). Interestingly, although the $VPAC_2$ receptors are activated by Helodermin, a ligand selective for mammalian $VPAC_2$, they are not affected by PACAP38 or

VIP stimulation (Wong et al., 2000) so their role in goldfish is uncertain at the present time.

### *1.3.5. Post-receptor transduction mechanisms of PACAP in fish pituitary hormone release*

The mechanisms of PACAP action on fish pituitary cells have largely been studied in cyprinids, and in the goldfish in particular. Preliminary results from early studies suggest that PACAP elevates cAMP, $IP_3$, and $[Ca^{2+}]_c$ levels in mixed populations of goldfish pituitary cells (Leung et al., 1997; Leung et al., 1999; Wong et al., 1996; cited in Wong et al., 2000). These results indicate that the AC/cAMP/PKA and the PLC/$IP_3$/$Ca^{2+}$ are potential pathways in mediating PACAP action in these cells. In goldfish somatotropes, PACAP-induced GH secretion is attenuated by inhibition of AC and PKA, showing that PACAP action on GH release in goldfish is mediated by the AC/cAMP/PKA pathway (Wirachowsky et al. 2000; Wong et al., 2000). The ability of the PKA inhibitor H89 to abolish PACAP-induced stimulation of GH secretion in carp confirms that the AC/cAMP/PKA pathway is an important transduction element in PACAP stimulation of GH release in fish (Xiao et al., 2002). A preliminary report (Leung, 1999; cited in Wong et al., 2000) suggests that GH secretion by PACAP may also involve the PLC/PKC pathway since the PLC inhibitor U73122 and the PKC inhibitors calphostin C and chelerythrine chloride reduced GH secretion responses in goldfish (Leung, 1999; cited in Wong et al., 2000). However, the same two PKC inhibitors were without effect in PACAP-induced GH release in another study with goldfish pituitary cells (Wirachowsky et al., 2000). Thus, it is

very likely that PACAP stimulation of GH secretion in goldfish is through coupling of the PAC1 receptor to the AC-cAMP-PKA pathway; however, the involvement of the PLC/$IP_3$/PKC pathway remains controversial. Likewise, PACAP stimulation of GTH-II secretion appears to involve the AC-cAMP-PKA pathway and not that of PLC-$IP_3$-PKC. The PKA inhibitor H89 suppressed PACAP- and forskolin-induced GTH-II release while the PKC inhibitors, calphostin C and chelerythrine chloride, had no effect (Chang et al., 2001).

Recent results show that PACAP increases $[Ca^{2+}]_c$ in identified goldfish somatotropes and gonadotropes (Wirachowsky et al., 2000; Chang et al., 2001). These findings suggest that $[Ca^{2+}]_c$ increases are important in mediating exocytotic responses to PACAP in these cells. The importance of extracellular $Ca^{2+}$ availability in PACAP-dependent GH secretion has also been confirmed by the reduction of GH responses to PACAP following incubation of goldfish pituitary cells with $Ca^{2+}$-deficient medium or inhibitors of VSCC (Leung et al., 1997; Wirachowsky et al., 2000). Furthermore, tetrodotoxin (TTX) has been reported to attenuate GH responses to PACAP (unpublished results, cited in Wong et al., 2000). Thus, it has been postulated that PACAP activates PKA (and/or PKC), which then either directly or indirectly affects TTX-sensitive $Na^+$ channels, leading to depolarization and opening of VSCC and $Ca^{2+}$ influx (Wong et al., 2000). Interestingly, PACAP stimulation of GTH-II release was shown to be unaffected by the VSCC inhibitors nifedipine and verapamil suggesting that $Ca^{2+}$ entry through VSCC may not play a dominant role in PACAP-stimulated GTH-II secretion (Chang et al., 2001).

In addition to extracellular $Ca^{2+}$ entry, release of $Ca^{2+}$ from intracellular stores is also an important source of $Ca^{2+}$ that contributes to changes in $[Ca^{2+}]_c$ leading to alterations in hormone secretion. Data suggest that PACAP-evoked hormone secretion shows a dependence on intracellular $Ca^{2+}$ stores as well. A broad-spectrum inhibitor of intracellular $Ca^{2+}$ release, TMB-8, abolished the GH release response to PACAP, and the SERCA inhibitor BHQ attenuated PACAP-elicited GH response (Chang et al., 2003). Although these data indicate the involvement of intracellular $Ca^{2+}$stores and BHQ-sensitive SERCA, the exact pharmacological properties of store(s) involved in PACAP stimulation of GH release remains to be determined. Whether intracellular $Ca^{2+}$ stores are used by PACAP in stimulating GTH-II secretion is currently unknown.

Given the known complexity of pharmacologically distinct stores and SERCA systems involved in the neuroendocrine regulation of GH and GTH-II secretion by GnRH and dopamine in the goldfish pituitary cell system, one must conclude that the present knowledge of the involvement of $Ca^{2+}$ stores in PACAP action on GH and GTH-II release is presently largely incomplete (Tables 1.1 & 1.2).

The transduction mechanism downstream of changes in $[Ca^{2+}]_c$ modulation in PACAP action has also been investigated in goldfish GH cells. Results show that activation of CaM and CaM kinase II are important in eliciting PACAP-stimulated release of GH (Wong et al., 2000; Chang et al., 2003).

*1.4 Proposed research*

PACAP is an important neuroendocrine regulator of goldfish GH and GTH-II secretion (see Section 1.1). Elucidating how PACAP exerts its hormone-releasing effects is important for understanding the neuroendocrine control of these two hormones. Evidence described in Section 1.3.5 indicates that changes in $[Ca^{2+}]_c$ and the use of intracellular $Ca^{2+}$ stores are important for PACAP action. However, the pharmacological identity of the store(s) involved is not well understood. Furthermore, the possible participation of PLC in PACAP action remains controversial.

In this study, the participation of intracellular $Ca^{2+}$ stores and $Ca^{2+}$-signalling in PACAP action on GH and GTH-II release from primary cultures of dispersed goldfish pituitary cells will be investigated using a pharmacological approach. In particular, the role of SERCA, and the involvement of ryanodine-, caffeine- and $IP_3$-sensitive $Ca^{2+}$ stores will be examined. The possible participation of mitochondria, an important $Ca^{2+}$ buffering organelle in many cell types (Hajnoczky et al., 1995; Park, et al., 1996; Hehl et al., 1996; Rizzuto et al., 1998, 2000; Montero et al., 2000) will also be evaluated. In addition, the participation of PLC and adenosine 5'-cyclic diphosphate-ribose (cADPR; endogenous ligand for ryanodine-sensitive stores in many systems; reviewed in Galione, 1993, 1998) will be tested. PACAP-induced GH and GTH-II release responses from primary cultures of dispersed goldfish pituitary cells will be studied using a perifusion system. Cell column perifusion is preferred over the static culture incubation as it measures acute responses, can more easily

account for the effects of drugs on basal release, and minimizes longer-term autocrine and/or paracrine effects (Chang et al., 1990b).

**Table 1.1.** Knowledge of the intracellular $Ca^{2+}$ store usage by sGnRH, cGnRH-II, DA, and PACAP to evoke GH secretion in goldfish somatotropes at the start of thesis work.

| Intracellular $Ca^{2+}$ stores & $Ca^{2+}$ modulating mechanisms | Stimulatory Regulator of GH secretion | | | |
|---|---|---|---|---|
| | sGnRH | cGnRH-II | dopamine | PACAP |
| | | | | |
| General (TMB-8-sensitive stores) | Yes | Yes | Yes | Yes |
| | | | | |
| $IP_3$ receptor | | | | |
| Xestospongin C-sensitive stores | Yes | No | ND | ND |
| | | | | |
| SERCA | | | | |
| Tg-sensitive | No | No | Mod | ND |
| BHQ-sensitive | No | No | Yes | Yes |
| CPA-sensitive | No | No | ND | ND |
| | | | | |
| Ryanodine Receptor | | | | |
| Ryanodine-sensitive stores | No | Yes | No | No |
| Dantrolene-sensitive stores | No | No | ND | ND |
| | | | | |
| Caffeine-sensitive stores | Yes | Yes | No | ND |
| | | | | |
| Mitochondria | | | | |
| CCCP-sensitive stores | ND | No | ND | ND |
| Ruthenium red-sensitive stores | Yes | No | ND | ND |

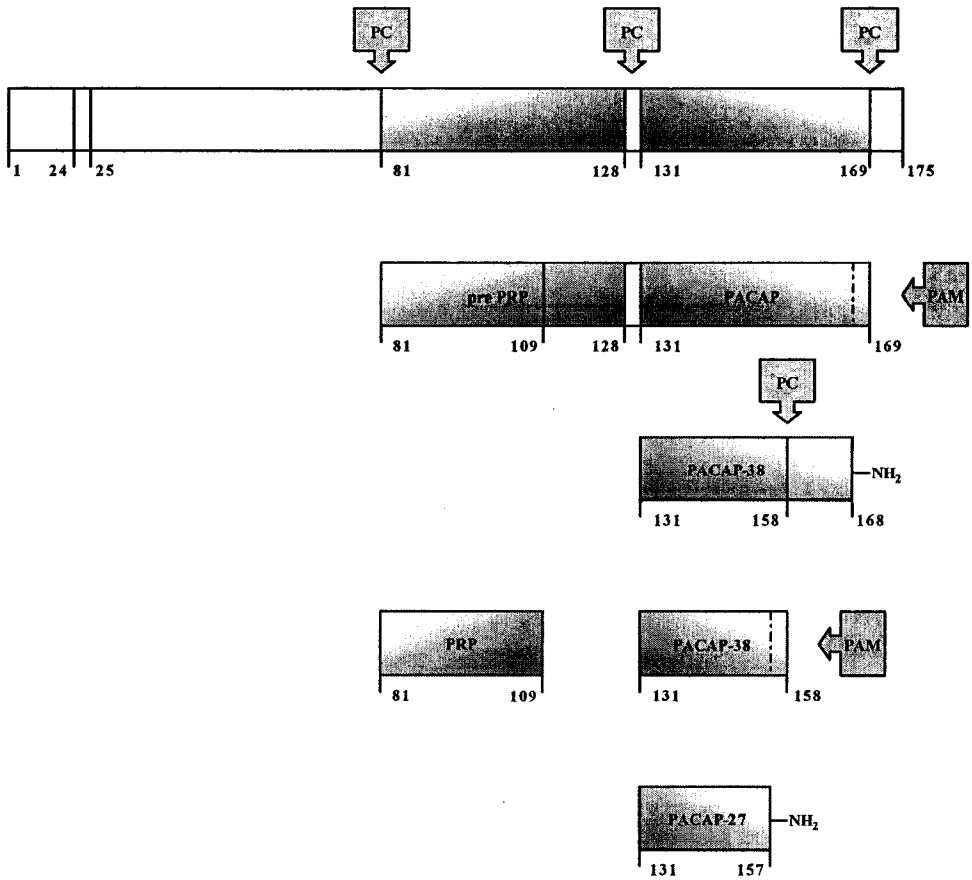
Yes = involved in mediating action
No = not involved in mediating action
ND = not determined
Mod = not directly involved but modulates

**Table 1.2.** Knowledge of the intracellular $Ca^{2+}$ store usage by sGnRH, cGnRH-II, DA, and PACAP to evoke GTH-II secretion in goldfish gonadotropes at the start of thesis work.

| Intracellular $Ca^{2+}$ stores & $Ca^{2+}$ modulating mechanisms | Stimulatory Regulator of GTH-II secretion | | |
|---|---|---|---|
| | sGnRH | cGnRH-II | PACAP |
| | | | |
| General (TMB-8-sensitive stores) | Yes | Yes | Yes |
| | | | |
| $IP_3$ receptor | | | |
| Xestospongin C-sensitive stores | Yes | No | ND |
| | | | |
| SERCA | | | |
| Tg-sensitive | No | No | ND |
| BHQ-sensitive | Yes | Yes | ND |
| CPA-sensitive | Yes | Yes | ND |
| | | | |
| Ryanodine Receptor | | | |
| Ryanodine-sensitive stores | Yes | Yes | No |
| Dantrolene-sensitive stores | No | No | ND |
| | | | |
| Caffeine-sensitive stores | Yes | No | ND |
| | | | |
| Mitochondria | | | |
| CCCP-sensitive stores | ND | ND | ND |
| Ruthenium red-sensitive stores | ND | ND | ND |

Yes = involved in mediating action
No = not involved in mediating action
ND = not determined

Figure 1.1. Schematic diagram of the posttranslational proteolysis of the PACAP precursor. PAM, peptidyl glycine alpha-amidating monooxygenase; PC, prohormone convertase; PRP, PACAP related peptide; SP, signal peptide. Adapted from Vaudry et al., (2000).



# 2 Materials and Methods

*2.1 Animals and cell preparation*

All animal maintenance and experimental protocols used have been approved by the animal care committee of the University of Alberta in accordance with national guidelines. Common goldfish (*Carassius auratus*; 8 – 13 cm in length) were purchased from Aquatic Imports (Calgary, AB) and maintained in flow-through aquaria (1800 L). Fish were maintained at 16 – 20°C on a simulated photoperiod (adjusted weekly to the times of sunrise and sunset in Edmonton AB, Canada) and fed to satiation with commercial fish food daily. Prior to use, fish were acclimated to the above conditions for at least 7 days. For all experiments, post-pubertal male and female fish from all stages of gonadal recrudescence/maturation were used. GH and GTH-II responses to neuroendocrine regulators are known to vary according to the seasonal reproductive conditions of the goldfish (Chang and Habibi, 2002; Lo and Chang, 1998). In order to minimize the variations caused by seasonal reproductive effects, replicate experiments in an experimental series were generally performed within a short time period where possible. Most of the experiments done in this study were performed in the spring and summer months (May through August). In some cases, additional replicates of experiments were performed between September and April if needed. Nonetheless, to facilitate future interpretation of possible seasonal effects, the times of year when the different sets of experiments were performed were indicated in the appropriate figure legends

*2.2 Drugs and reagents*

Mammalian PACAP38 was chosen to be used over that of goldfish PACAP38 in this study for several reasons. Mammalian PACAP has a high degree of primary amino acid sequence homology, similar binding properties for goldfish $PAC_1$ receptor, and similar effectiveness at stimulating pituitary hormone release in goldfish as goldfish PACAP (Wong et al., 1988). It is also readily available commercially. Mammalian PACAP38 was purchased from Peninsula Laboratories, Belmont, CA while most other chemicals and pharmacological reagents were purchased from either Calbiochem (San Diego, CA) or Sigma (St. Louis, MO). Stock solutions of PACAP38 (PACAP 1-38; Peninsula Laboratories, Belmont, CA), CPA, and 8-Br-cADPR (8-bromo-adenosine 5'-cyclic diphosphate-ribose) were made in distilled deionized water. Stock solutions of high purity ryanodine (99.5%), ionomycin, CCCP, dantrolene, Tg, xestospongin D, and tricyclodecan-9-yl-xanthogenate (D609) were all made in dimethyl sulphoxide (DMSO). Ethanol was used to prepare Edelfosine (1-O-Octadecyl-2-O-methyl-*rac*-glycero-3-phosphorylcholine; ET-18-$OCH_3$) and 1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione (U73122). The concentrated stock solutions were stored at –20°C with the exception of ryanodine and ionomycin which were stored at 4°C and U73122 which was kept at room temperature. Final concentrations were achieved by dilution in testing medium. Final concentrations of DMSO (0.1%) and ethanol (0.1% for all except for U73122, which was at 1% ethanol) had no effect on basal hormone release (Van Goor et al., 1997; unpublished data; this thesis). To minimize oxidation,

(u)[$(HCO_2)(NH_3)_4Ru]_2Cl_3$ (Ru360) was dissolved in boiled and degassed distilled deionized water and diluted in testing media immediately prior to use. Solutions of caffeine (1,3,7-trimethylxanthine) was prepared directly with testing medium just prior to use.

*2.3 Pituitary cell dispersion*

Fish were anaesthetized in 0.05% tricaine methane sulphonate (Syndel, Vancouver, Canada) prior to decapitation. Pituitaries from both male and female goldfish were removed and pituitary cells dispersed using a trypsin/DNase treatment procedure (Chang et al., 1990a). Following dispersion, cells were re-suspended in plating medium (Medium 199 with Earle's salts (Gibco, Grand Island, NY), 1% horse serum, 25 mM HEPES, 26.2 mM $NaHCO_3$, 100,000 U penicillin/l and 100 mg streptomycin/l, pH adjusted to 7.2 with 1N NaOH).

*2.4 Column perifusion experiments*

Cell column perifusion studies allow the determination of the kinetics of the hormone response. These were performed as previously described (Chang, et al., 1990b; Wong et al., 1992). Briefly, dispersed goldfish pituitary cells were cultured in plating media overnight on pre-swollen Cytodex-I beads at 28°C under saturated humidity and 5% $CO_2$, then loaded onto temperature-controlled (18°C) perifusion columns ($1.5 \times 10^6$ cells/column), and perifused with testing medium (Medium 199 with Hanks salts, 0.1% bovine serum albumin, 25 mM HEPES, 26.2 mM $NaHCO_3$, 100,000 U penicillin/l and 100 mg streptomycin/l, pH 7.2) at a rate of 15 ml/h. Cells were washed for 4 h to allow for stabilization of basal hormone secretion before the experiment began. Perifusates were

collected as 1- or 5-min fractions and stored at –20°C until being assayed for GH or GTH-II content by specific radioimmunoassay (Marchant, et al., 1987; Peter, et al., 1984).

Generally, PACAP was applied as a 5-min pulse at 45 min. The dose of PACAP used was 1 nM, which has previously been shown to be maximally effective in stimulating GH and GTH-II release in this system (Wirachowsky et al., 2000; Chang et al., 2001). Inhibitors or other pharmacological agents were applied at 25 min to 85 min. The flow rate and dead volume of the system are such that the hormone release response to a treatment will commence in the fraction collected roughly 5-6 min following drug application (Wong et al., 1992). A typical experimental perifusion treatment protocol and response is shown in Figures 2.1 & 2.2A.

Hormone secretion values from individual columns were expressed as a percentage of pretreatment, which was the average of the first 5 fractions collected at the beginning of an experiment. This conversion allowed for pooling of hormone-response data from different columns without distorting the shape of the response. Net hormone response to PACAP treatment (base-line subtracted; baseline for the reponse defined as the average hormone value in the 4 fractions collected prior to PACAP administration) was quantified as area under the curve (Wong et al., 1992; Chang et al., 2003). To facilitate determination of the temporal sensitivity of the PACAP-induced hormone release responses to manipulations, the net hormone responses were separated into two time-dependent phases. Under most experimental conditions, hormone responses to

a 5-min pulse of PACAP began in the 5-min fraction immediately after the commencement of PACAP treatment and maximal hormone response was observed in the following fraction (Chang et al., 2003). Accordingly, peak responses to PACAP were quantified as the net response observed within the first 10 min of the expected response. Plateau responses to PACAP were quantified as the net response observed for the 35 min following the peak response (Chang et al., 2003). Total response was defined as the sum of the peak and plateau responses (Figure 2.2A & B). Responses to drug alone treatments in the corresponding time frames were similarly quantified.

In some experiments, a modified fast fraction perifusion protocol was used (i.e., 1-min fraction collections). In this case, PACAP was applied as a 5-min pulse at 30 min into an experiment. Inhibitors or other pharmacological agents were applied at 20 min to 40 min. A fast fraction experimental perifusion treatment protocol and response is shown in Figures 2.3 & 2.4A. Hormone responses from individual columns were expressed as a percentage of basal release, which was the average of the first 5 fractions collected at the beginning of an experiment. This conversion allowed for pooling of hormone-response data from different columns without distorting the shape of the response. Net hormone response to PACAP treatment (base-line corrected) was quantified as area under the curve (Wong et al., 1992; Chang et al., 2003). In these experiments, only the total hormone response was quantified, which was the sum of the secretion response from 35 min to 45 min (Figure 2.4 A & B). Fast fraction perifusions were performed when long-term drug treatments were not economical.

All experiments were usually replicated a minimum of three times using different cell preparations. Results were then pooled for statistical analysis. Statistical analysis were performed by analysis of variance (ANOVA). When ANOVA showed that significant differences existed among the experimental groups, a Fisher's PLSD test was then used to identify differences between groups. Differences were considered significant when $P < 0.05$. All results are expressed as means ± SEM (standard error of mean).

Figure 2.1. An example of a typical perifusion protocol. Perifusate fractions were collected every 5-min over a 120-min experiment for a total of 24 fractions per column. Treatments were assigned as follows: columns A,B were treated with PACAP only, the drug control columns C,D received drug X alone, and columns E,F were treated with the combination of PACAP and drug X. M199, testing medium.

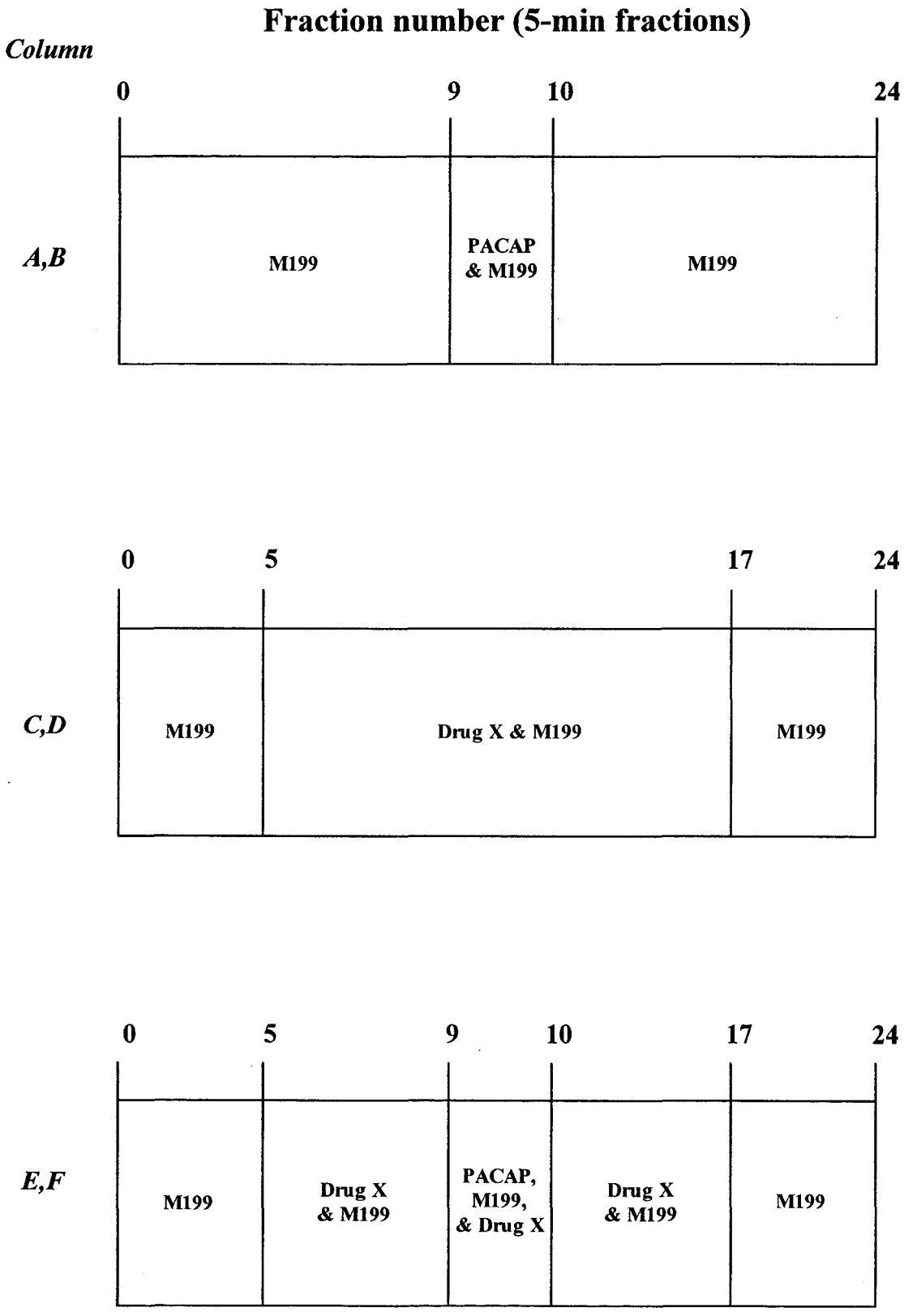
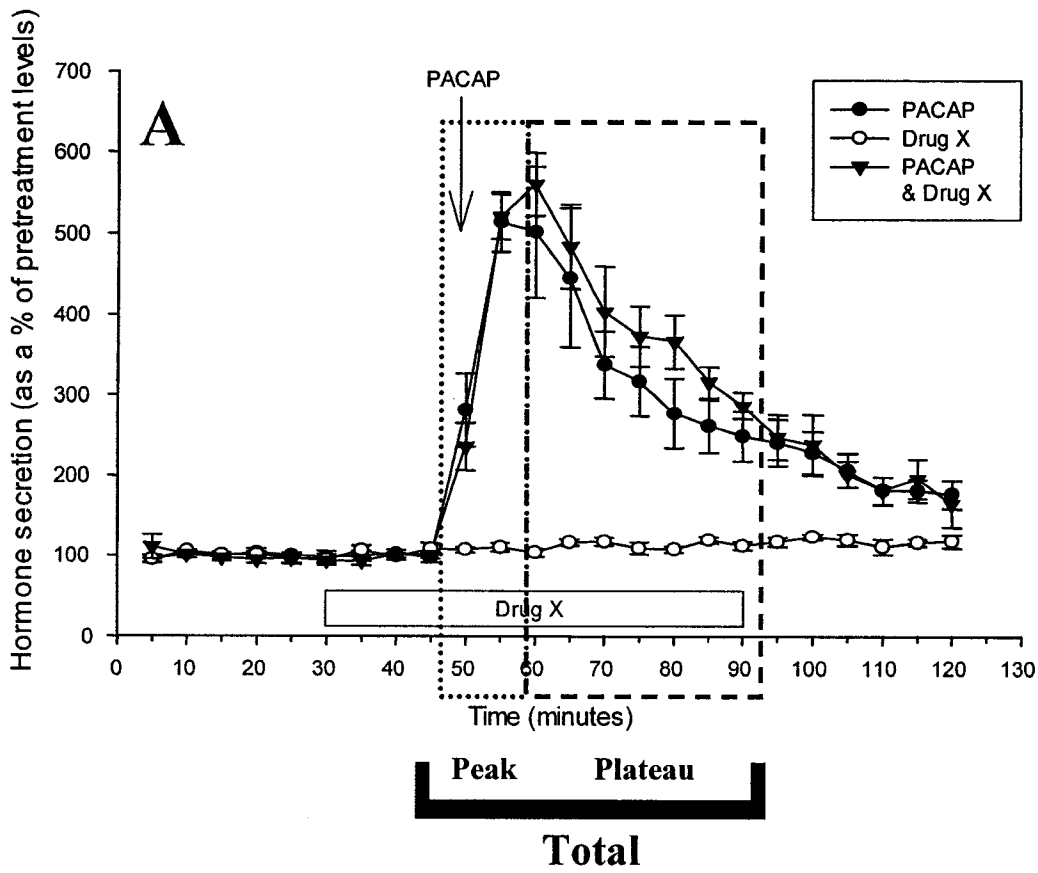


Figure 2.2. An example of the quantification of a typical 120 min perifusion experiment. **A**. Hormone release profiles are presented. Arrow indicates the estimated commencement of hormone response to the 5-min PACAP pulse. The open horizontal bar indicates duration of application of Drug X. **B**. Net hormone responses are presented.



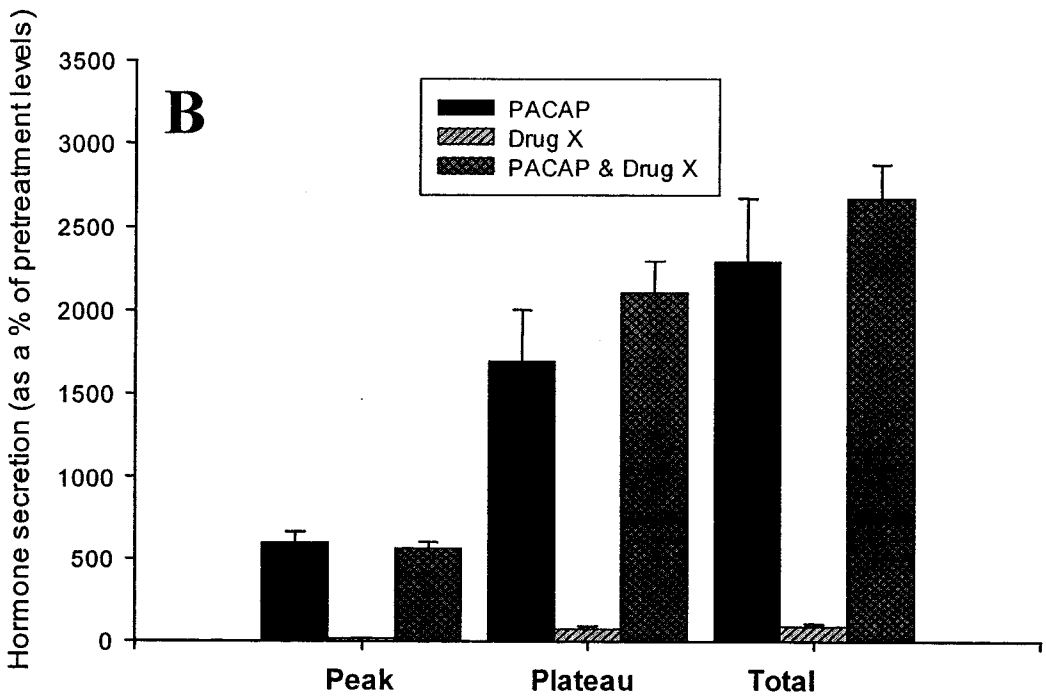



Figure 2.3. An example of a typical fast fraction perifusion protocol. Perifusate fractions were collected every 1-min over a 48-min experiment for a total of 48 fractions per column. Treatments were assigned as follows: columns A,B were treated with PACAP only, the drug control columns C,D received drug X alone, and columns E,F were treated with the combination of PACAP and drug X. M199, testing medium.

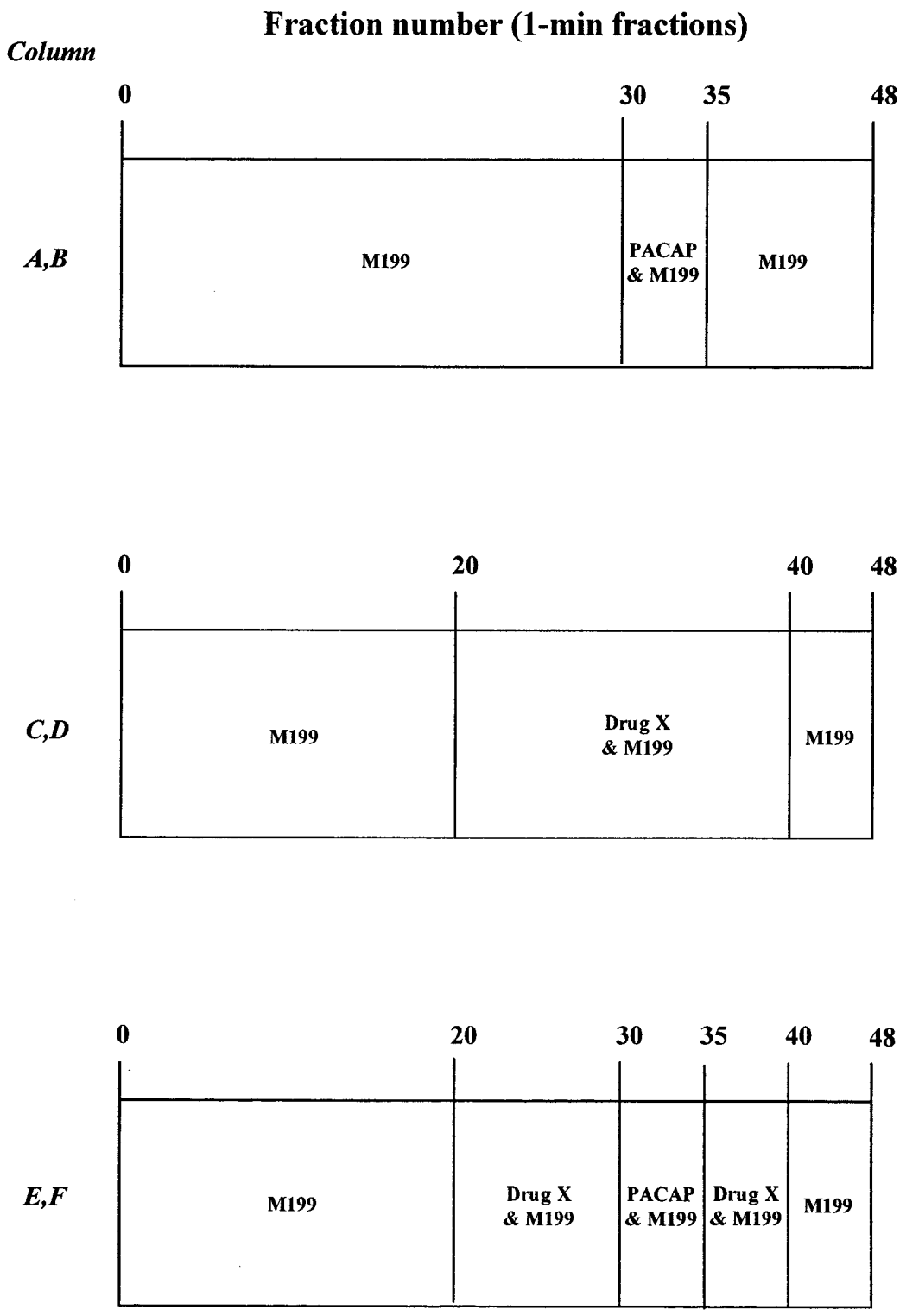
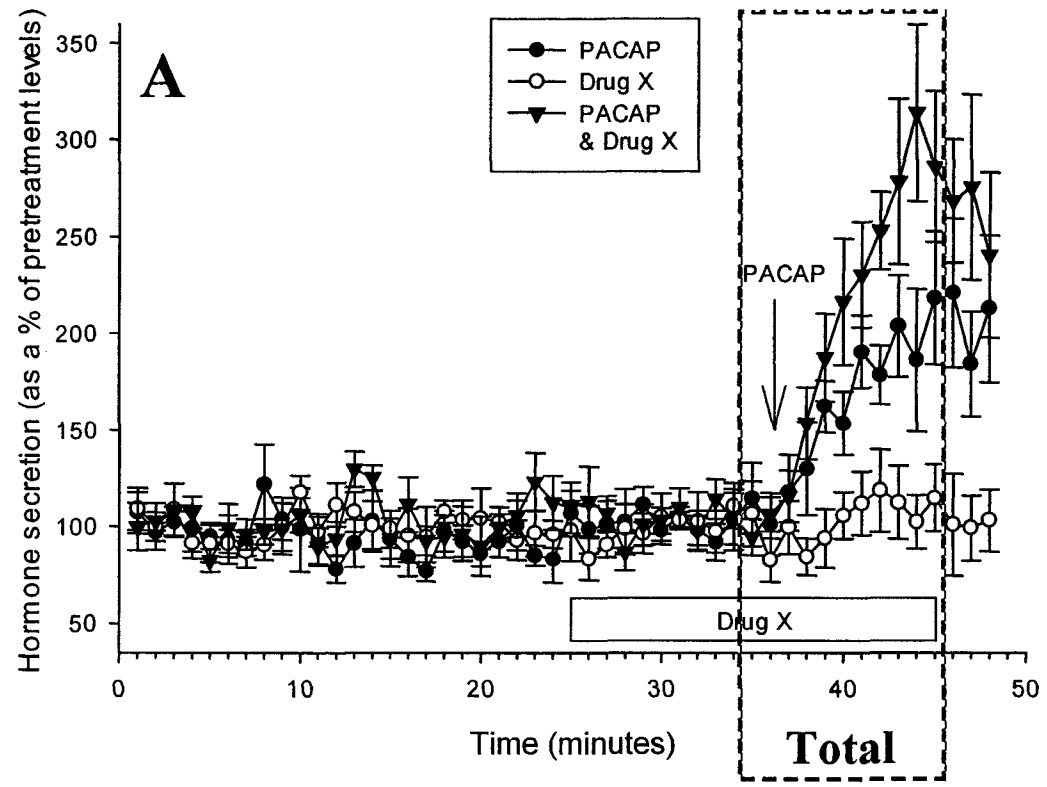
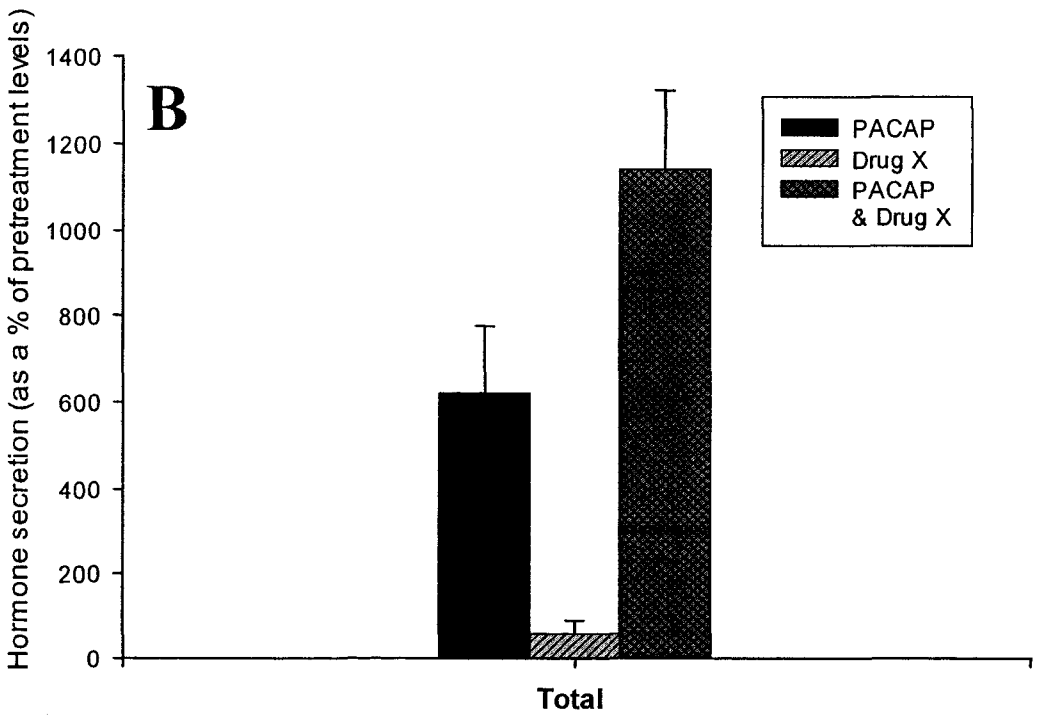


Figure 2.4. An example of the quantification of a typical 48 min fast fraction perifusion experiment. **A**. Hormone release profiles are presented. Arrow indicates the estimated commencement of hormone response to the 5-min PACAP pulse. The open horizontal bar indicates duration of application of Drug X. **B**. The total net hormone response is presented.



# 3 Results

## *3.1 PACAP-induced GH and GTH-II secretion*

Mammalian PACAP38 (PACAP) at 10 nM concentration has previously been shown to be maximally effective in stimulating GH and GTH-II release in static incubation experiments with goldfish pituitary cells (Wirachowsky et al., 2000; Chang et al., 2001). Similarly, 10 nM PACAP significantly increased GH and GTH-II secretion from perifused goldfish pituitary cells in all experiments performed in the present study.

## *3.2 The role of caffeine-sensitive $Ca^{2+}$ stores in PACAP-stimulated GH and GTH-II secretion*

Previous studies have shown that caffeine-sensitive (but relatively ryanodine-insensitive) intracellular $Ca^{2+}$ stores mediate GnRH-induced GH and GTH-II secretion (Johnson and Chang 2002; Johnson et al., 2002). To investigate the possible involvement of caffeine-sensitive $Ca^{2+}$ stores in mediating PACAP-stimulated GH and GTH-II release, we have chosen to examine the effects of exposure to a maximal stimulatory dose of caffeine (10 mM; Johnson and Chang 2000; Johnson et al., 2000) on the hormone responses to PACAP. We hypothesized that if caffeine-sensitive $Ca^{2+}$ stores are involved, depletion (or maximal mobilization) of this $Ca^{2+}$ source by continual exposure to caffeine should attenuate the PACAP-induced hormone responses. Conversely, if this intracellular $Ca^{2+}$ source is not involved, PACAP-induced hormone responses should be additive to those of caffeine.

Application of 10 mM caffeine produced a massive GH response (Figure 3.1A). Accordingly, the time of PACAP application was delayed to 65 min to allow for better visualization of the PACAP-induced response. In the presence of caffeine, the GH response to 10 nM PACAP was abolished. The peak, plateau and total GH responses to PACAP were all significantly reduced in caffeine-treated columns as compared to those treated with PACAP alone and not different from values observed with caffeine exposure alone (Figure 3.1A & B).

Similarly with GTH-II secretion, 10 mM caffeine produced a large hormone response (Figure 3.2A). In the presence of caffeine, the GTH-II response to 10 nM PACAP was abolished. The peak, plateau and total GTH-II responses in the PACAP with caffeine treatment group were significantly depressed from those seen with PACAP treatment only and not different from corresponding values in columns treated with caffeine alone (Figure 3.2A & B).

To confirm that hormone secretion could take place in the presence of 10 mM caffeine, a maximally effective dose of ionomycin (10 μM; Chang et al., 1990b), a known robust stimulator of GH and GTH-II secretion, was used during caffeine treatment. The GH and GTH-II responses to 10 μM ionomycin were largely unaffected by caffeine (Figures 3.3 & 3.4). The peak and total GH and GTH-II responses to ionomycin in the presence of caffeine were not significantly different from the hormone secretion induced by ionomycin alone; however, there was a slight reduction in the level of plateau response (Figures 3.3B & 3.4B).

The findings of this set of experiments point to PACAP operating through caffeine-sensitive $Ca^{2+}$ store(s) to elicit GH and GTH-II secretion.

*3.3 Activity of ryanodine-sensitive $Ca^{2+}$ stores in PACAP-stimulated GH and GTH-II hormone secretion*

Another distinct $Ca^{2+}$ store that has been identified in GH and GTH-II cells is termed, the ryanodine-sensitive store, which is the major $IP_3$-insensitive store in the endoplasmic reticulum (ER; reviewed in Pozzan et al., 1994). In the cell, the release of $Ca^{2+}$ from these pools is thought to be triggered through the activation of ryanodine receptors (RyR's) by cADPR (Meszaros et al., 1993; Lee, 1998; Walseth and Lee, 1993). The ryanodine-sensitive $Ca^{2+}$ store(s) have been shown to be involved in mediating the GH and GTH-II responses to GnRH in goldfish pituitary cell cultures (Johnson et al. 2002; Johnson and Chang, 2002). To investigate the possible involvement of ryanodine-sensitive stores in PACAP-induced GH and GTH-II secretion, we tested the effects of three inhibitors of ryanodine receptor $Ca^{2+}$ channels, ryanodine, dantrolene and 8-Br-cADPR.

Application of a dose of ryanodine that has previously been shown to inhibit ryanodine receptors (10 μM, Johnson and Chang, 2002) caused a small but steady reduction in basal GH and GTH-II release (Figures 3.5A & 3.6A). 10 nM PACAP stimulated GH and GTH-II release with similar effectiveness, both alone and in the presence of ryanodine (Figures 3.5B & 3.6B). This inability of 10 μM ryanodine to affect PACAP-induced GH release was similar to previously published results (Chang et al., 2003).

The drug, dantrolene, is reported to block the release of $Ca^{2+}$ through RyR channels by binding to the receptor with maximal effectiveness at concentrations in the range of $10^{-5}$ M (Parness and Palnitkar, 1995). Dantrolene (50 μM) slightly

elevated basal GH secretion but had no effects on the peak, plateau and total GH response to PACAP (Figure 3.7). On the other hand, dantrolene induced a rapid large rise in GTH-II secretion; this was followed by an extended decay lasting until the end of the experiment (Figure 3.8 A) PACAP-induced GTH-II secretion was adversely affected by dantrolene treatment; the plateau phase, as well as the total GTH-II response to PACAP in the presence of dantrolene were significantly less than those seen with PACAP alone (Figure 3.8).

Next, the membrane permeant analog of cADPR was used to block the release of $Ca^{2+}$ from the RyR channel (Walseth and Lee, 1993; Iino et al., 1997). Based on effective doses reported for use in several animal models (guinea pig, Iino et al., 1997; sea urchin, Chini and Dousa, 1999; rat, Zhang et al., 1999; bovine, Geiger et al., 2000; rat, Yusufi et al., 2002; bovine, Ge et al., 2003) a concentration of 30 μM was chosen as the dose of 8-Br-cADPR for the present experiments. Application of 8-Br-cADPR alone did not alter basal GH and GTH-II secretion (Figures 3.9A & 3.10A). Co-treatment with 8-Br-cADPR did not effect the GH and GTH-II responses to PACAP (Figures 3.9 & 3.10).

Taken together, these results indicate that there may not be a role for ryanodine-sensitive $Ca^{2+}$ stores in PACAP-induced GH secretion but it may be important for GTH-II release induced by PACAP, especially in the prolonged release ("plateau") phase.

*3.4 SERCA-refilled $Ca^{2+}$ stores and PACAP-induced GH and GTH-II release*

It is known that $Ca^{2+}$ in the ER store(s) is refilled by $Ca^{2+}$ ATPases, the so-called SERCA enzyme pumps (reviewed in Carafoli, 2003). Hormone release

studies using SERCA inhibitors generally operates on the rationale that blocking the refilling of a specific store will eventually empty it due to a slow leakage of $Ca^{2+}$ across the ER membrane and thus rendering it nonfunctional in a signalling cascade. Studies have demonstrated that SERCA-refilled stores, especially those sensitive to inhibition by BHQ and CPA, mediate sGnRH- and cGnRH-II-stimulated GH and GTH-II secretion in goldfish (Johnson et al., 2000; 2003). Similarly, the participation of SERCA in PACAP action on GH release in goldfish pituitary cells is suggested by the ability of BHQ to attenuate the GH secretion elicited by this peptide (Chang et al., 2003). To further examine the involvement of SERCA-refilled $Ca^{2+}$ stores in PACAP-induced GH and GTH-II secretion, the effects of two SERCA inhibitors, Tg (2 μM; Chang et al., 2003; Johnson et al., 2003) and CPA (10 μM; Johnson, 2000) were examined.

Tg treatment by itself did not alter basal GH and GTH-II release until the cessation of treatment with this drug after which hormone levels rose sharply (Figures 3.11A & 3.12A). In the presence of Tg, the GH and GTH-II responses to PACAP were unexpectedly elevated above levels seen with PACAP alone. The peak, plateau and total GH and GTH-II responses to PACAP in the presence of Tg were all significantly increased above those to PACAP alone (Figures 3.11 & 3.12).

Similarly, basal GH and GTH-II release were not drastically altered during CPA treatment (Figures 3.13A & 3.14A). CPA slightly increased the plateau and total GH response to PACAP but the differences were not significant (Figure

3.13B). On the other hand, the peak, plateau and total GTH-II responses were significantly enhanced above their counterparts to PACAP alone (Figure 3.14B).

The above results suggest that Tg- and CPA-sensitive SERCA-refilled $Ca^{2+}$ stores do not directly mediate PACAP-induced GH and GTH-II secretion but they, and especially Tg-sensitive SERCA, may modulate the hormone release responses to PACAP.

*3.5 Mitochondrial $Ca^{2+}$ buffering in PACAP-stimulated GH and GTH-II secretion*

Since the discovery that the mitochondria could sequester $Ca^{2+}$ by Vasington and Murphy (1962), research has shown that this pool of $Ca^{2+}$ plays a role in the control of many cellular functions, including exocytosis (reviewed in Duchen, 1999; Rizutto et al., 2000; Gunter and Gunter, 2001; Brini, 2003; Carafoli, 2003). The mitochondrial $Ca^{2+}$ pool is established by $Ca^{2+}$-specific uniporters, which are coupled to the mitochondrial $H^+$ gradient on the surface of this organelle (Gunter and Pfeiffer, 1990). In goldfish, mitochondrial $Ca^{2+}$ transport has been shown to be an important component in pituitary hormone secretion. Two inhibitors of mitochondrial $Ca^{2+}$ transport, ruthenium red and CCCP (Moore, 1971; Duchen, 1999; Hehl et al., 1996; Peng, 1998; Tang and Zucker, 1997), were shown to potentiate sGnRH-induced GH release (Johnson, 2000). To examine the importance of mitochondrial $Ca^{2+}$ buffering in PACAP-induced GH and GTH-II secretion, the effects of two inhibitors of mitochondrial $Ca^{2+}$ transport, CCCP (Hehl et al., 1996; Peng, 1998; Tang and Zucker, 1997) and Ru360 (Matlib et al., 1998; Zhou et al., 1998; Sanchez, et al. 2001) were examined. CCCP uncouples the $Ca^{2+}$ uniporter from the mitochondrial $H^+$

gradient driving it preventing its function, whereas Ru360 has been reported to antagonize the action of the uniporter without uncoupling the proton gradient.

Application of 10 μM CCCP, which has previously been shown to be effective in goldfish pituitary cells (Johnson, 2000), produced an elevation in GH secretion which reversed to normal after termination of drug administration (Figure 3.15A). A GH response to PACAP was observed in the presence of CCCP treatment but the peak and total responses were significantly reduced from that observed with PACAP alone (Figure 3.15). CCCP also produced a reversible increase in basal GTH-II hormone secretion (Figure 3.16A). PACAP effectively stimulated GTH-II release in the presence of CCCP. The GTH-II response to PACAP treatment in the presence of CCCP was quantitatively similar to that seen with PACAP alone (Figure 3.16).

Next, we looked at the effects of the more specific mitochondrial $Ca^{2+}$ uniport inhibitor Ru360 on PACAP-stimulated GH and GTH-II release. Based on the common concentration used in other systems (Smaili and Russel, 1999; Matlib et al., 1998; Sanchez et al., 2001; Frantseva et al., 2001; Zhou and Bers, 2002; Isaeva and Shirokova, 2003), 10 μM was chosen to be the concentration of Ru360 used in the present study. Ru360 treatment had no effect on basal GH levels and did not significantly alter the GH response to PACAP (Figure 3.17). On the other hand, Ru360 treatment alone produced an initial momentary rise in basal GTH-II secretion (Figure 3.18A). GTH-II response in the PACAP with Ru360 treatment group mimicked that seen with PACAP alone; no significant differences were seen in the quantified peak, plateau and total GTH-II values

between the PACAP plus Ru360 and PACAP alone treatment groups (Figure 3.18).

Taken together, it would seem that there may be some mitochondrial contribution to PACAP-induced GH, but not GTH-II, secretion in goldfish.

*3.6 PLC and $IP_3$ signalling in PACAP-stimulated GH and GTH-II secretion*

Another intracellular $Ca^{2+}$ pool important in mediating hormone secretion is the $IP_3$-sensitive $Ca^{2+}$ stores. They are discharged when $IP_3$ receptors are activated by $IP_3$ formed by the hydrolysis of phosphatidylinositol 4,5-bisphosphate by PLC (reviewed in Michikawa et al., 1996; Marks, 1997; Taylor and Thorn, 2001). $IP_3$-sensitive $Ca^{2+}$ stores have been implicated in mediating GnRH-induced hormone release in goldfish (see section 1.1.1.1), but the role of PLC/$IP_3$ pathway in mediating PACAP-induced GH secretion in goldfish is unclear (see section 1.3.5), To examine the possible participation of PLC and $IP_3$-sensitive $Ca^{2+}$ stores in PACAP action on GH and GTH-II release, the effects of PLC inhibitors were first examined.

The first PLC inhibitor tested was U73122, which has been shown to block $IP_3$ formation (Bleasdale et al, 1990). Although there was no known effective dose for goldfish, we chose to use a drug concentration of 10 μM which has been shown to be effective in studies involving a related cyprinid, the grass carp (Choi et al., 2000). Treatment with U73122 alone did not immediately affect basal GH levels although a rapid increase in hormone secretion was seen when the drug treatment was stopped (Figure 3.19A). In the presence of U73122, PACAP was still able to elicit a GH response; interestingly, the plateau and total GH response

to PACAP were actually greater in the presence than in the absence of U73122 although the differences were not significant (Figure 3.19). In contrast to GH secretion, basal GTH-II was greatly elevated by U73122 and with the increase starting immediately upon treatment (Figure 3.20A). The quantified plateau and total GTH-II response to PACAP were significantly greater in the presence than in the absence of U73122; however, plateau and total values observed in the PACAP plus U73122 treatment group were not different from those observed for U73122 alone in the corresponding time frames (Figure 3.20).

Due to the solubility characteristics of U73122, the final concentration of the solvent, ethanol, needed to be at 1%, which was 10 times the normal acceptable value of 0.1% in goldfish hormone release studies. We wondered if this higher concentration of ethanol, rather than the actions of U73122, had affected basal and PACAP-stimulated hormone secretion. When the effect of 1% ethanol was directly examined, neither basal hormone release nor PACAP-induced GH and GTH-II secretion were significantly affected by this concentration of ethanol (Figures 3.21 & 3.22). Therefore, the observed changes in basal and stimulated hormone secretion with U73122 application cannot be attributed to the effects of a high concentration the carrier solvent alone.

To further examine the role of phosphatidylinositol-specific PLC, the effects of another phosphatidylinositol-specific PLC inhibitor, ET-18-$OCH_3$ (Powis et al., 1992), were tested. Based on information available regarding the effective concentration of this inhibitor in a variety of animal systems (human, Oishi et al., 1988; rat, Llansola et al., 2000; cockroach, Steele et al., 2001; bovine; Donald et

al., 2002), a dose of 30 μM was chosen. Application of 30 μM ET-18-$OCH_3$ resulted in a large initial increase in basal GH and GTH-II release, which were followed by a slow reduction in hormone release and another rapid increase in secretion upon termination of drug application (Figure 3.23A & 3.24A). In the presence of ET-18-$OCH_3$, PACAP was unable to elicit GH and GTH-II responses (Figures 3.23 & 3.24).

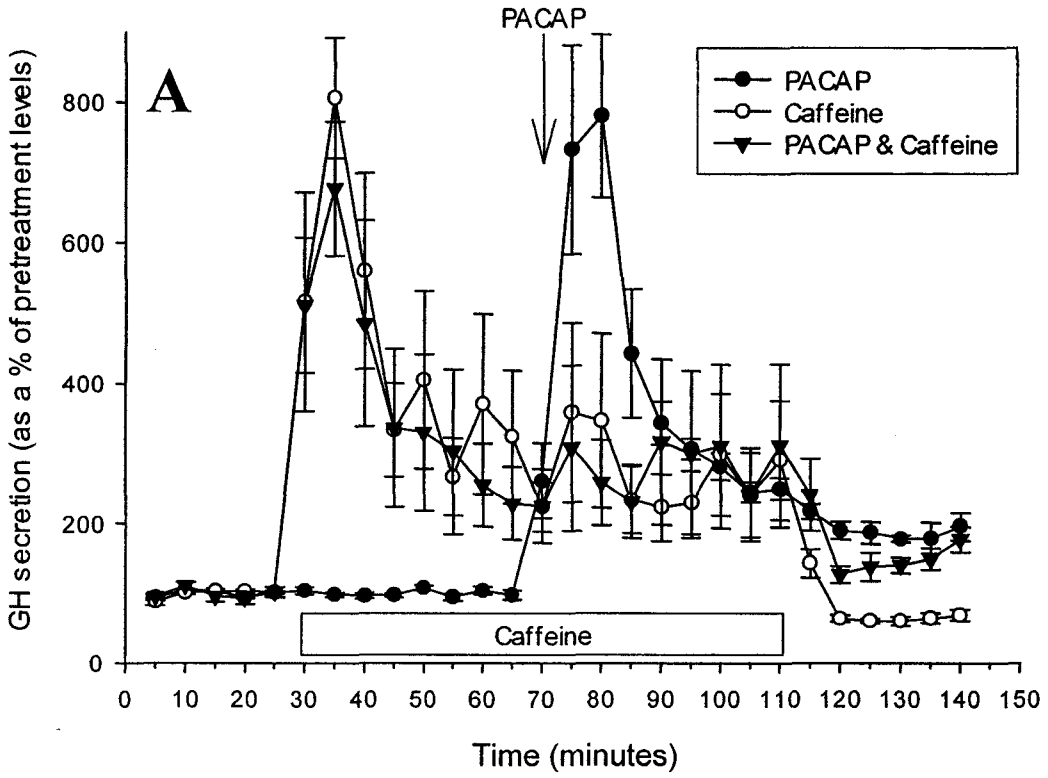
The final PLC inhibitor used was D609, which has been reported to specifically inhibit phosphatidylcholine-specific PLC (Muller-Decker, 1989). As with ET-18-$OCH_3$, the effective dose for use in goldfish was unknown. After examining previous studies (frog, Wilson et al., 1998; human, Antony et al., 2001; rat, Ito et al., 2002 and Shum et al., 2002), a concentration of 200 μM was chosen. On its own, D609 treatment did not affect basal GH secretion (Figure 3.25A). When it was combined with PACAP, the GH response was significantly increased when compared to that to PACAP alone (Figure 3.25). Unlike GH secretion, basal GTH-II secretion was greatly enhanced by D609 treatment (Figure 3.26A). Nevertheless, PACAP was able to elicit a GTH-II response even in the presence of the D609-induced elevation in basal GTH-II secretion. The GTH-II secretion response to the combined treatment of PACAP and D609 was greater than the corresponding quantified values observed with PACAP alone and D609 alone (Figure 3.26).

From the results described above, it appears that phosphatidylcholine-specific PLC does not directly mediate PACAP-stimulated hormone secretion, whereas results with phosphatidylinositol-specific PLC inhibitors are inconsistent.

While results with ET-18-$OCH_3$ suggest that phosphatidylinositol-specific PLC (and presumably an $IP_3$-sensitive $Ca^{2+}$ store) mediates PACAP-induced GH and GTH-II responses, results with U73122 are either contradicting (in the case for GH) or inconclusive (in the case of GTH-II). To more directly evaluate the possible participation of $IP_3$-sensitive $Ca^{2+}$ stores in mediating the ability of PACAP to stimulate GH and GTH-II secretion, the effects of $IP_3$ receptor antagonists were examined.

Xestospongin C and D are two commonly used antagonists of the $IP_3$ receptor $Ca^{2+}$ channel with effective concentrations around $10^{-6}$ M (Johnson, 2000). Application of 1 μM Xestospongin D did not alter basal GH and GTH-II secretion (Figures 3.27A & 3.28A). In the presence of Xestospongin D, PACAP was able to elicit GH and GTH-II responses (Figures 3.27 & 3.28). The magnitude of the quantified GH response to PACAP was actually significantly greater in the presence than in the absence of Xestospongin D while the GTH-II response was not different regardless of the presence or absence of the $IP_3$ receptor antagonist (Figures 3.27B & 3.28B). Similarly, application of 1 μM Xestospongin C, which has previously been shown to be effective in reducing sGnRH-stimulated hormone secretion in goldfish (Johnson, 2000), did not affect basal and PACAP-elicited GH and GTH-II secretion in a preliminary experiment (data not shown). These results with $IP_3$ receptor antagonists suggest that $IP_3$-sensitive intracellular $Ca^{2+}$ stores are unlikely to be involved in mediating the stimulated GH and GTH-II release in response to PACAP.

Figure 3.1. Effects of caffeine on PACAP-stimulated GH secretion. **A**. Temporal characteristics of the GH response to PACAP (10 nM), either alone or in the presence of caffeine (10 mM). Arrow indicates the estimated commencement of hormone response to the 5-min PACAP pulse and the open bar indicates the duration of exposure to caffeine. Pooled results (mean ± SEM; n=6 columns each) from 3 replicate experiments (performed once in May and twice in June) are presented. Average pre-treatment GH values for this set of experiments are 28.4 ± 1.4 ng/ml. **B**. Quantified GH responses are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only group.



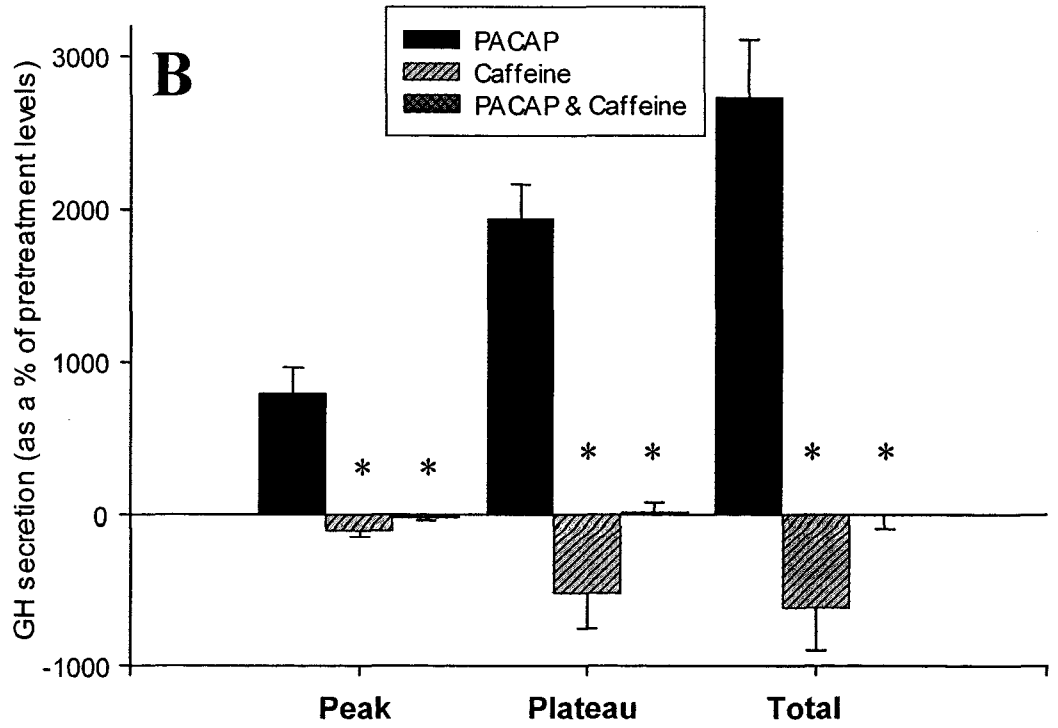
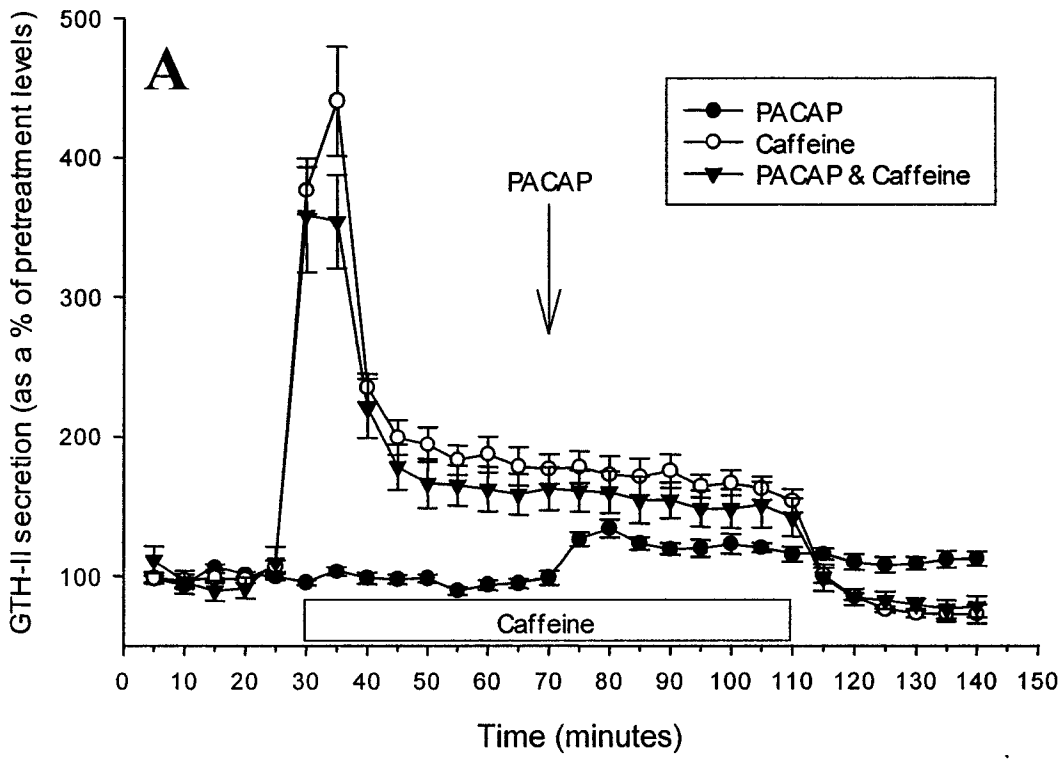



Figure 3.2. Effects of caffeine on PACAP-stimulated GTH-II secretion. **A.** Temporal characteristics of the GTH-II response to PACAP (10 nM), either alone or in the presence of caffeine (10 mM). Arrow indicates the estimated commencement of hormone response to the 5-min PACAP pulse and the open bar indicates the duration of exposure to caffeine. Pooled results (mean ± SEM; n=6 columns each) from 3 replicate experiments (performed once in May and twice in June) are presented. Average pre-treatment GTH-II values for this set of experiments are 2.8 ± 0.1 ng/ml. **B.** Quantified GTH-II responses are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only group.



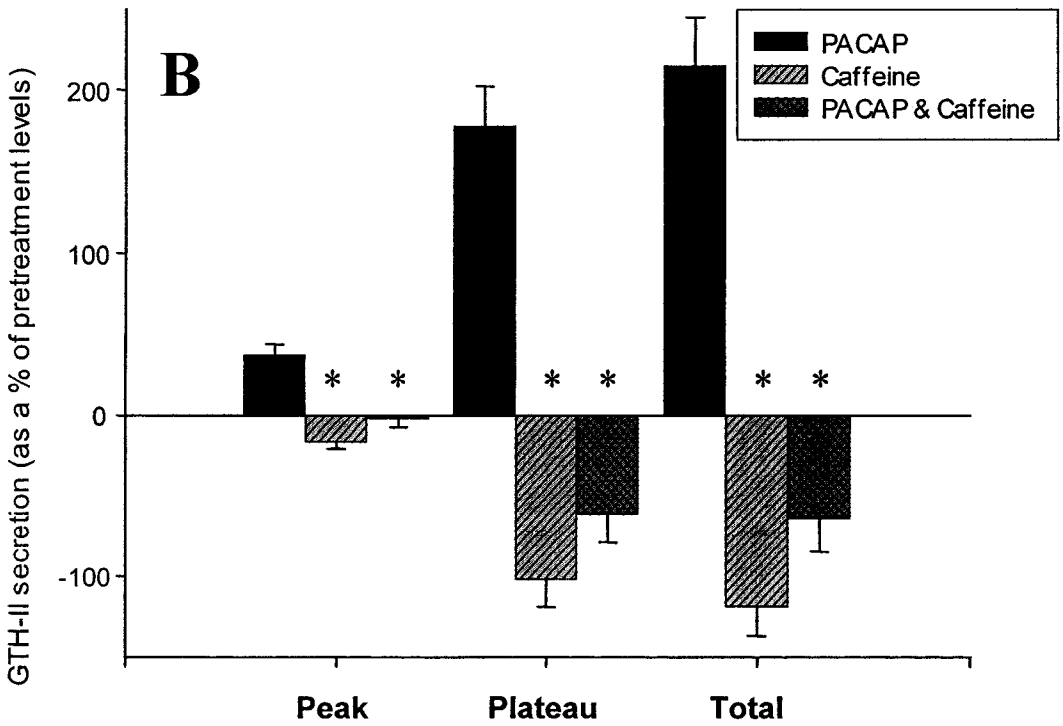
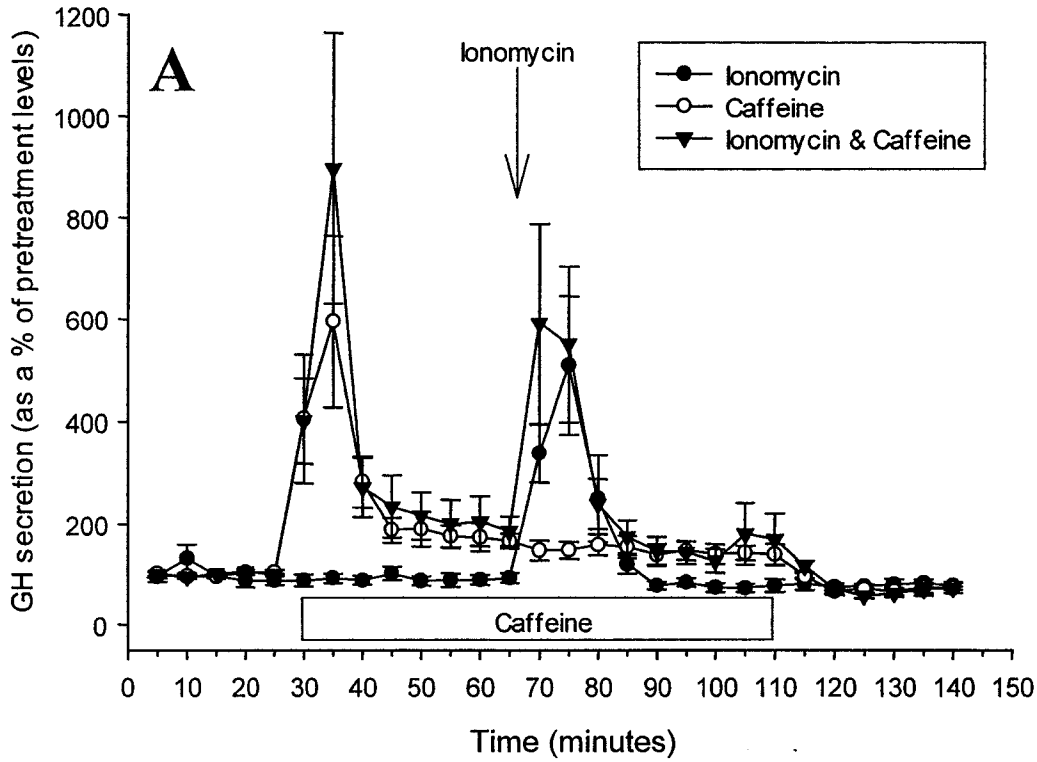



Figure 3.3. Effects of caffeine on ionomycin-induced GH secretion. **A**. Temporal characteristics of the GH response to ionomycin (10 μM), either alone or in the presence of caffeine (10 mM). Arrow indicates the estimated commencement of hormone response to the 5-min ionomycin pulse and the open bar indicates the duration of exposure to caffeine. Pooled results (mean ± SEM; n=6 columns each) from 3 replicate experiments (performed in June) are presented. Average pre-treatment GH values for this set of experiments are 30.3 ± 2.1 ng/ml. **B**. Quantified GH responses are presented. A star indicates a significant difference ($p < 0.05$) from the ionomycin only group. A number sign denotes a significant difference ($p < 0.05$) from the caffeine only treatment group.



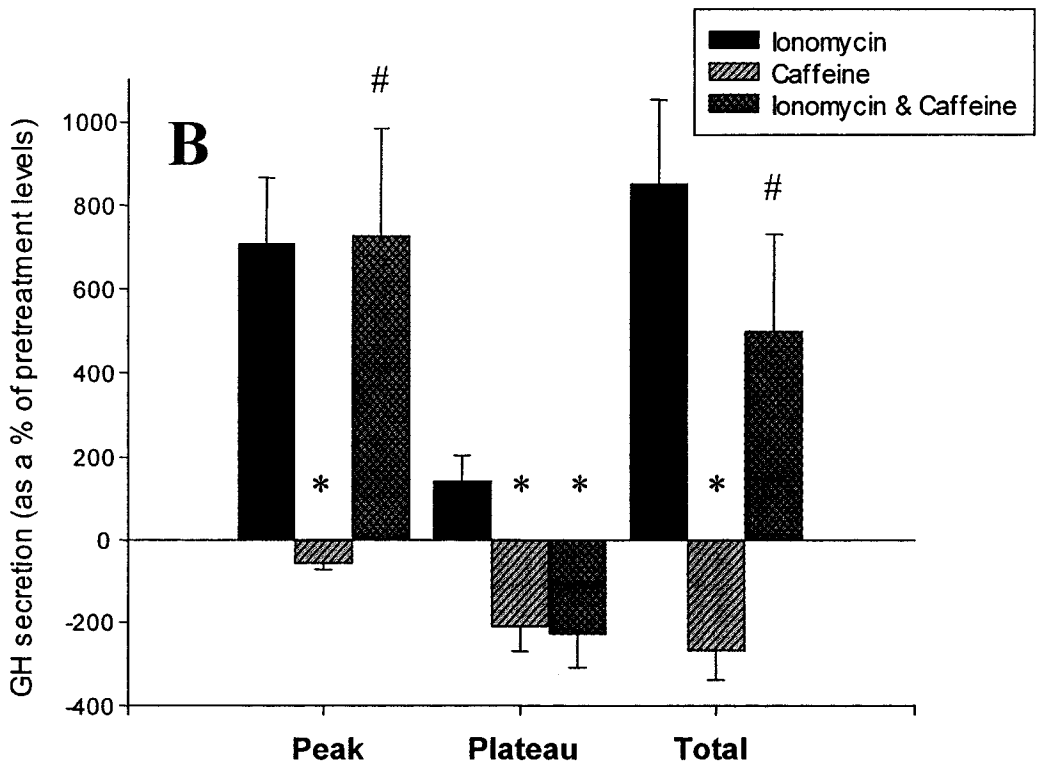
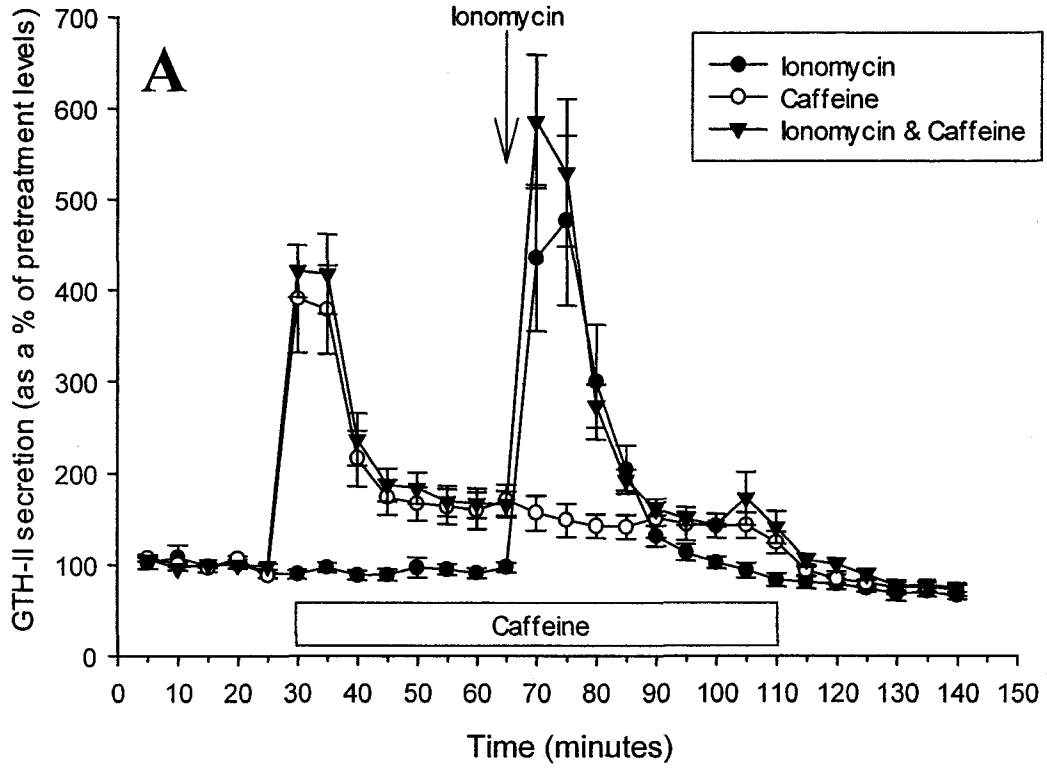



Figure 3.4. Effects of caffeine on ionomycin-induced GTH-II secretion. **A.** Temporal characteristics of the GTH-II response to ionomycin (10 μM), either alone or during treatment with caffeine (10 mM). Arrow indicates the estimated commencement of hormone response to the 5-min ionomycin and the open bar indicates the duration of caffeine exposure. Pooled results (mean ± SEM; n=6 columns each) from 3 replicate experiments (performed in June) are presented. Average pre-treatment GTH-II values for this set of experiments are 2.6 ± 0.9 ng/ml. **B.** Quantified GH responses are presented. A star indicates a significant difference ($p < 0.05$) from the ionomycin only group. A number sign denotes a significant difference ($p < 0.05$) from the caffeine only treatment group.



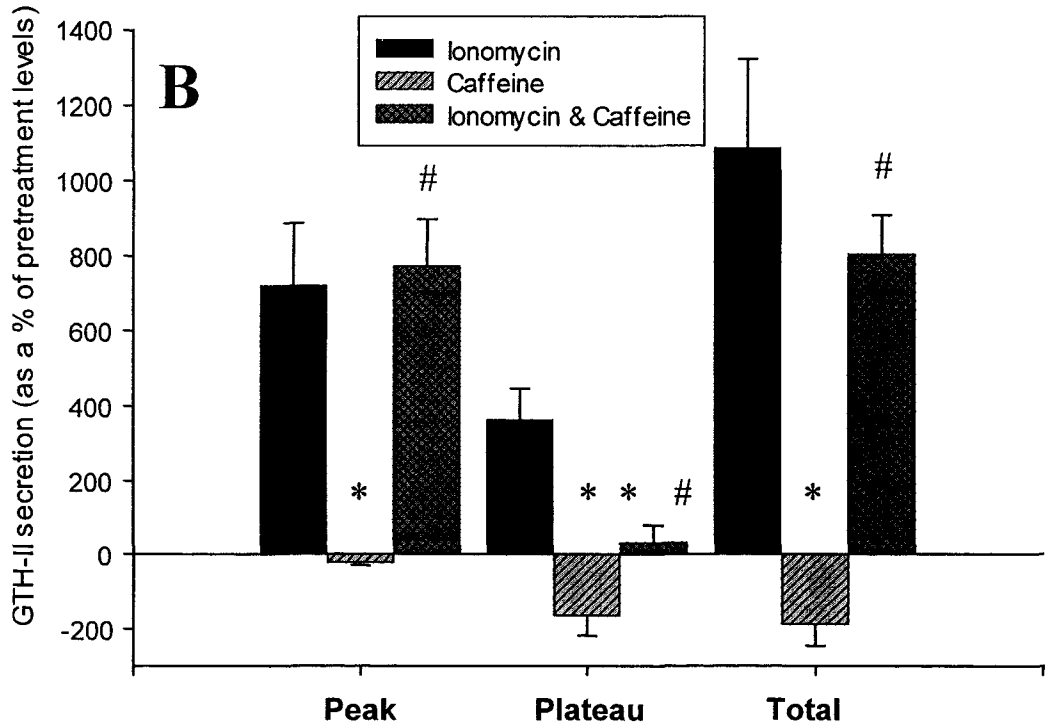
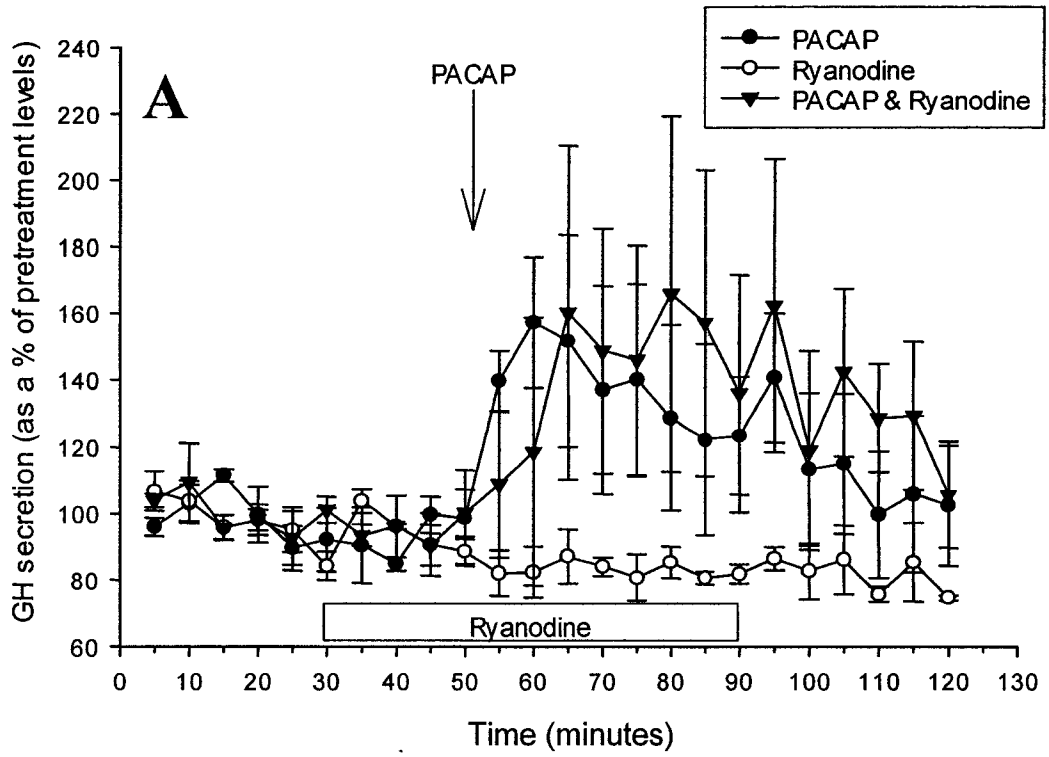



Figure 3.5. Effects of ryanodine on PACAP-induced GH secretion. **A**. Temporal characteristics of the GH response to PACAP (10 nM), either alone or in the presence of ryanodine (100 μM). Arrow indicates the estimated commencement of hormone response to the 5-min PACAP pulse and the open bar indicates the duration of exposure to ryanodine. Pooled results (mean ± SEM; n=4 columns each) from 2 replicate experiments (performed once in December and once in January) are presented. Average pre-treatment GH values for this set of experiments are 33.8 ± 1.8 ng/ml. **B**. Quantified GH responses are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only group.



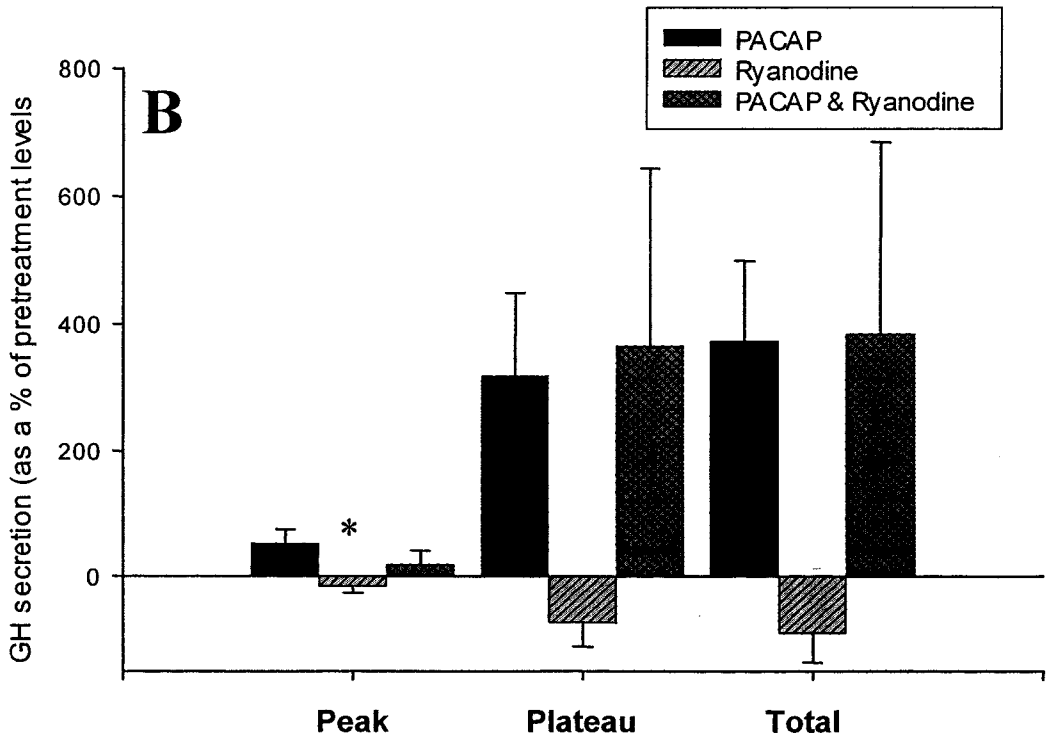
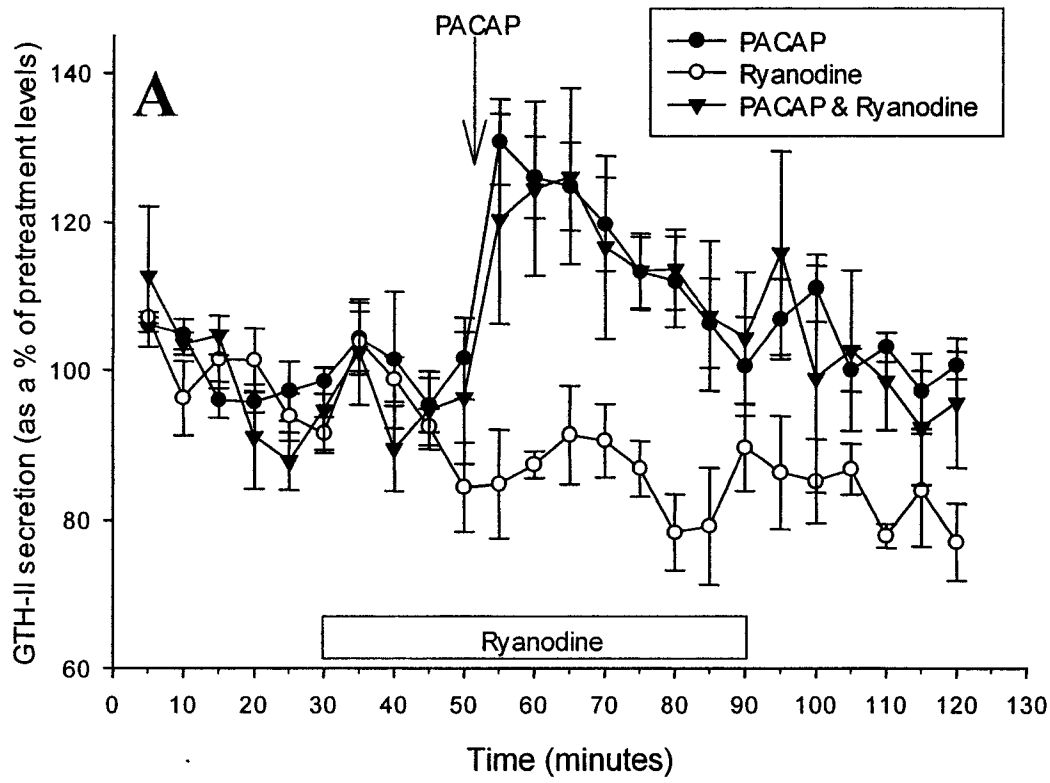



Figure 3.6. Effects of ryanodine on PACAP-induced GTH-II secretion. **A**. Temporal characteristics of the GTH-II response to PACAP (10 nM), either alone or in the presence of ryanodine (100 -μM). Arrow indicates the estimated commencement of hormone response to the 5-min PACAP pulse and the open bar indicates the duration of exposure to ryanodine. Pooled results (mean ± SEM; n=4 columns each) from 2 replicate experiments (performed once in December and once in January) are presented. Average pre-treatment GTH-II values for this set of experiments are 2.5 ± 0.2 ng/ml. **B**. Quantified GTH-II responses are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only group. A number sign indicates a significant difference ($p < 0.05$) from the ryanodine only treatment group.



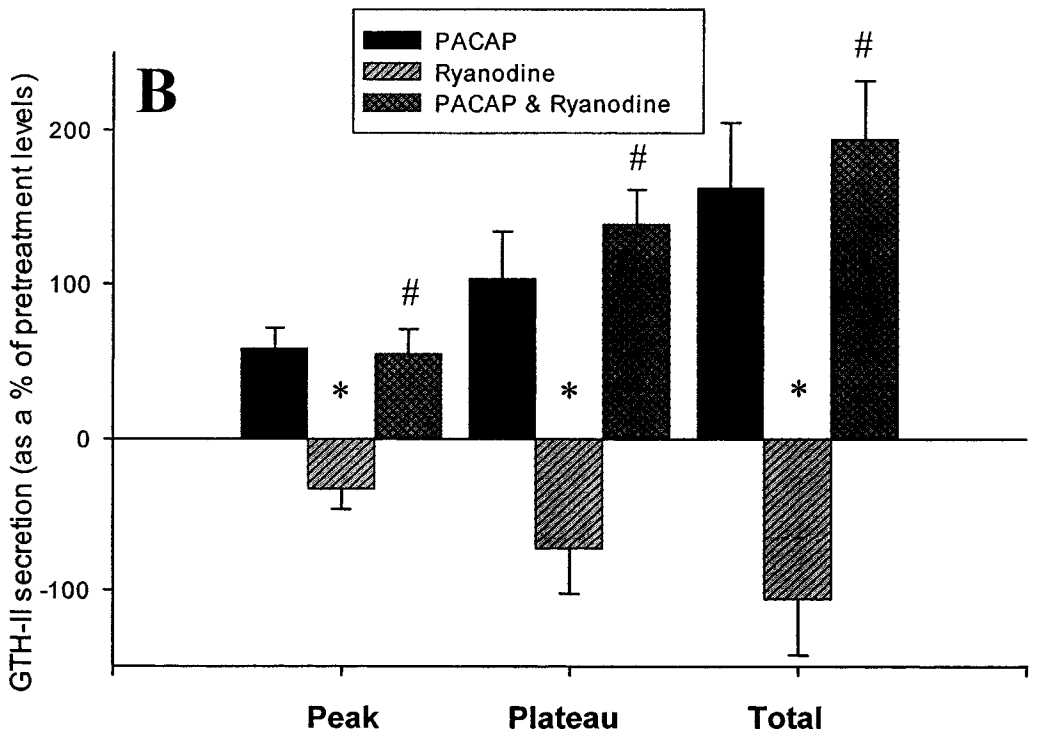
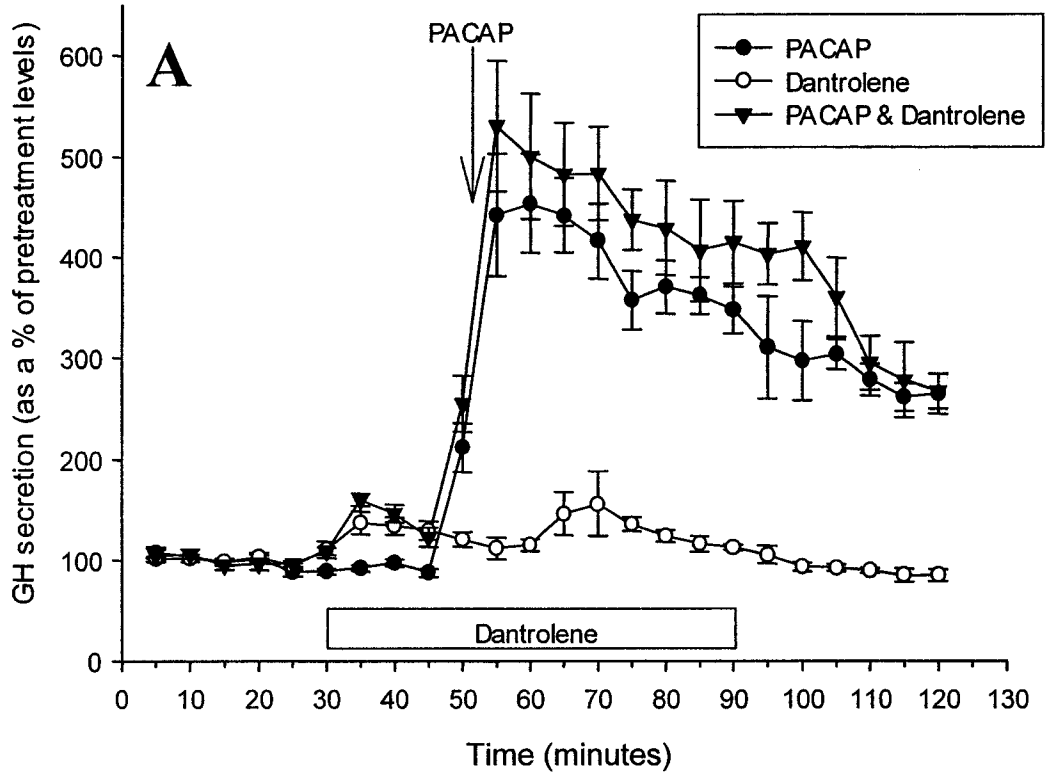



Figure 3.7. Effects of the RyR antagonist dantrolene on PACAP-stimulated GH secretion. **A**. Temporal characteristics of the GH response to PACAP (10 nM), either alone or in the presence of dantrolene (50 μM). Arrow indicates the estimated commencement of hormone response to the 5-min PACAP pulse and open bar indicates the duration of exposure to dantrolene. Pooled results (mean ± SEM; n=6 columns each) from 3 replicate experiments (performed twice in February and once in April) are presented. Average pre-treatment GH values for this set of experiments are 29.0 ± 1.2 ng/ml. **B**. Quantified GH responses are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only group. A number sign indicates a significant difference ($p < 0.05$) from the dantrolene only treatment group.



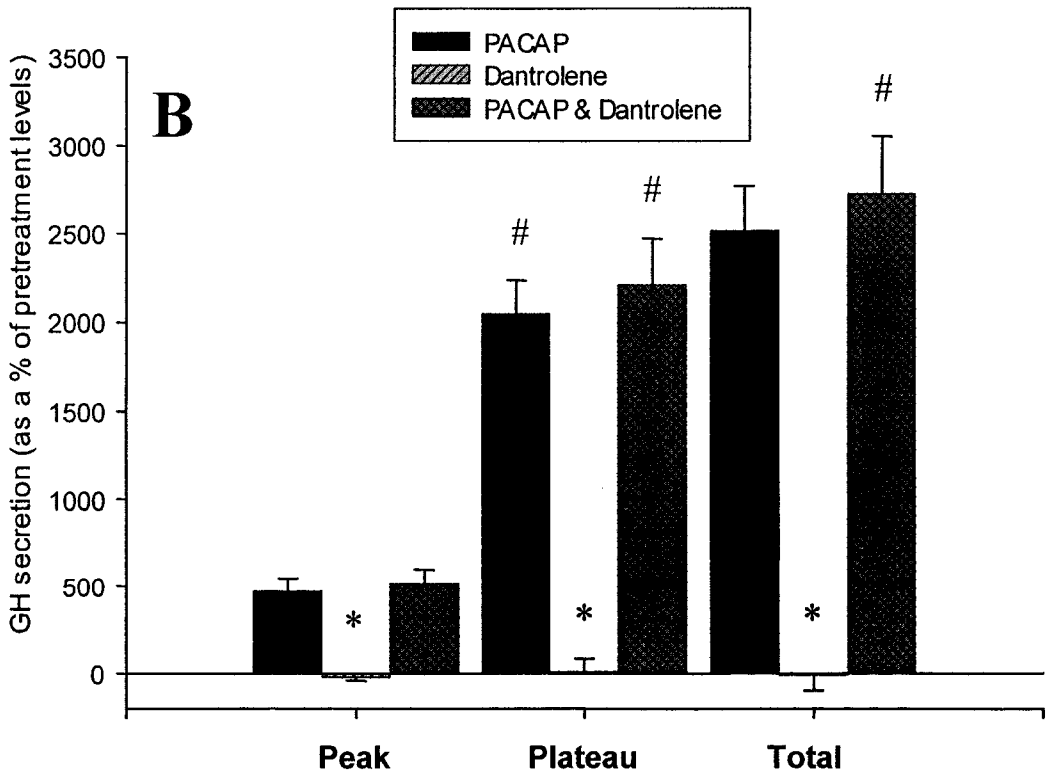
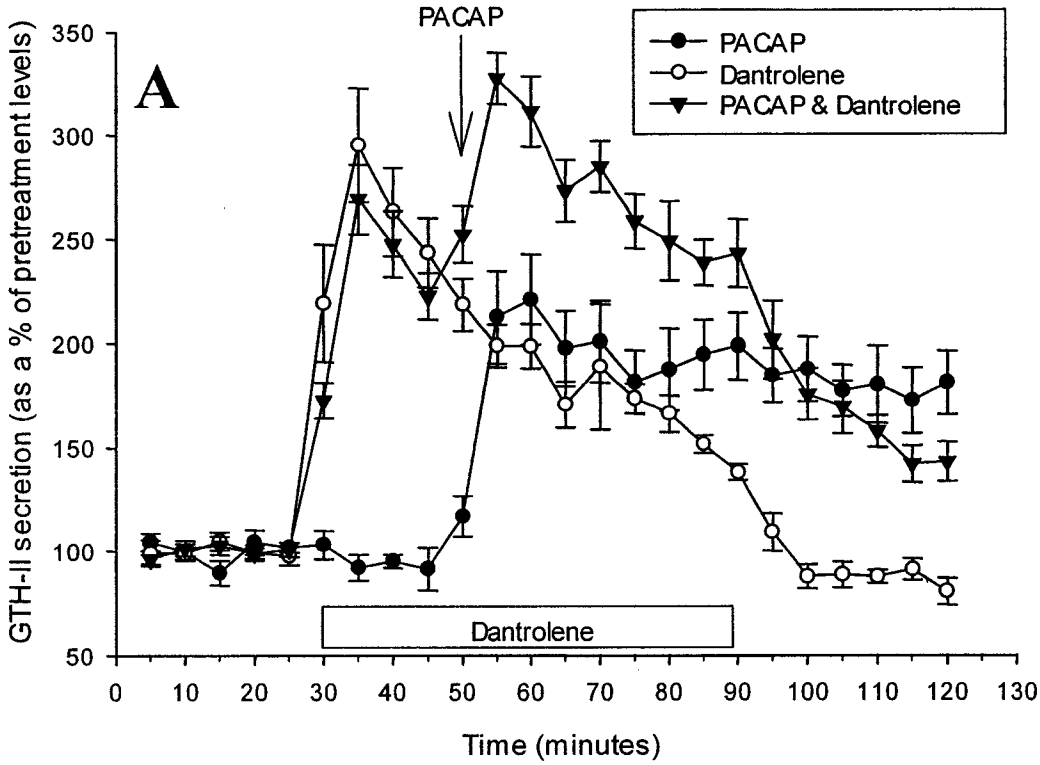



Figure 3.8. Effects of the RyR antagonist dantrolene on PACAP-stimulated GTH-II secretion. **A**. Temporal characteristics of the GTH-II response to PACAP (10 nM), either alone or in the presence of dantrolene (50 μM). Arrow indicates the estimated commencement of hormone response to the 5-min PACAP pulse and the open bar indicates the duration of exposure to dantrolene. Pooled results (mean ± SEM; n=6 columns each) from 3 replicate experiments (performed twice in February and once in April) are presented. Average pre-treatment GTH-II values for this set of experiments are 1.6 ± 0.4 ng/ml. **B**. Quantified GTH-II responses are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only group. A number sign indicates a significant difference ($p < 0.05$) from the dantrolene only treatment group.



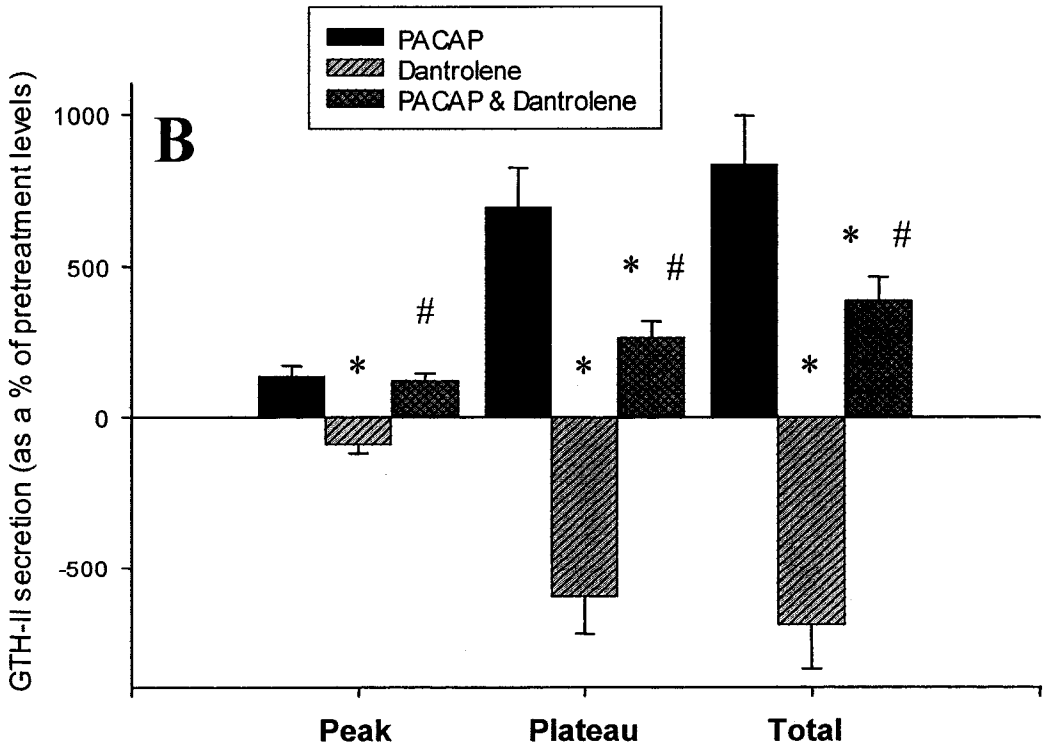
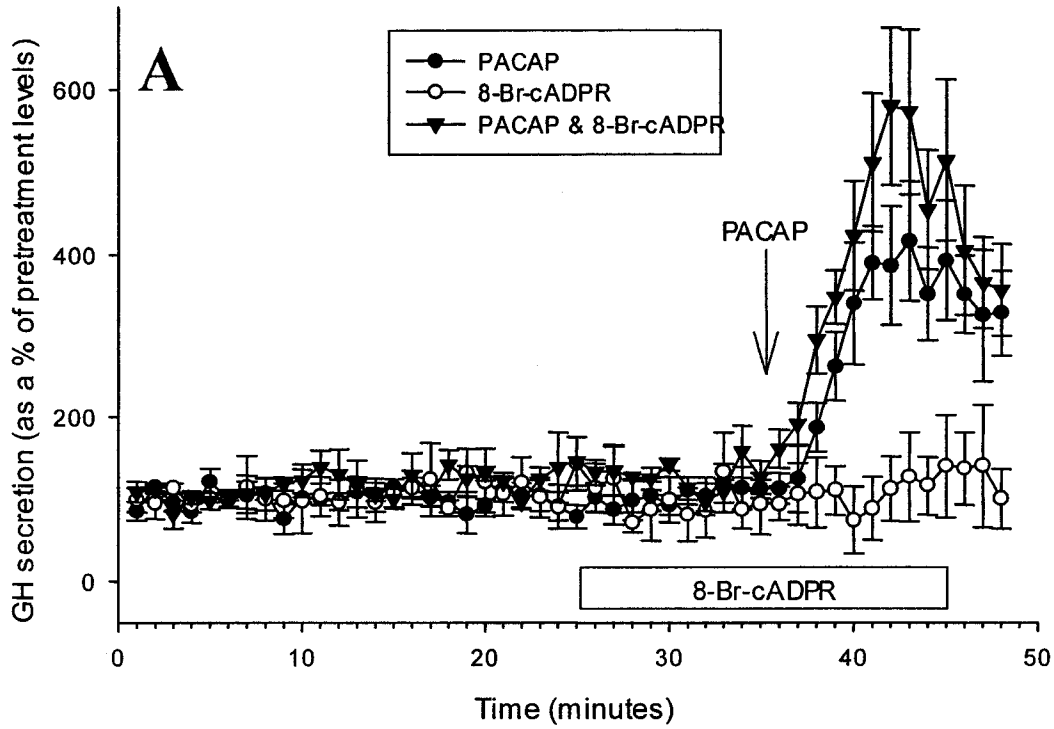



Figure 3.9. Effects of the RyR antagonist 8-Br-cADPR on PACAP-stimulated GH secretion. **A**. Temporal characteristics of the GH response to PACAP (10 nM), alone or in the presence of 8-Br-cADPR (100 μM). Arrow indicates the estimated commencement of hormone response to the 5-min PACAP pulse and open bar indicates the duration of exposure to 8-Br-cADPR. Pooled results (mean ± SEM; n=4 columns each) from 2 replicate experiments (performed once in July and once in August) are presented. Average pre-treatment GH values for this set of experiments are 14.4 ± 0.4 ng/ml. **B**. Quantified GH responses are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only group. A number sign indicates a significant difference ($p < 0.05$) from the 8-Br-cADPR only treatment group.



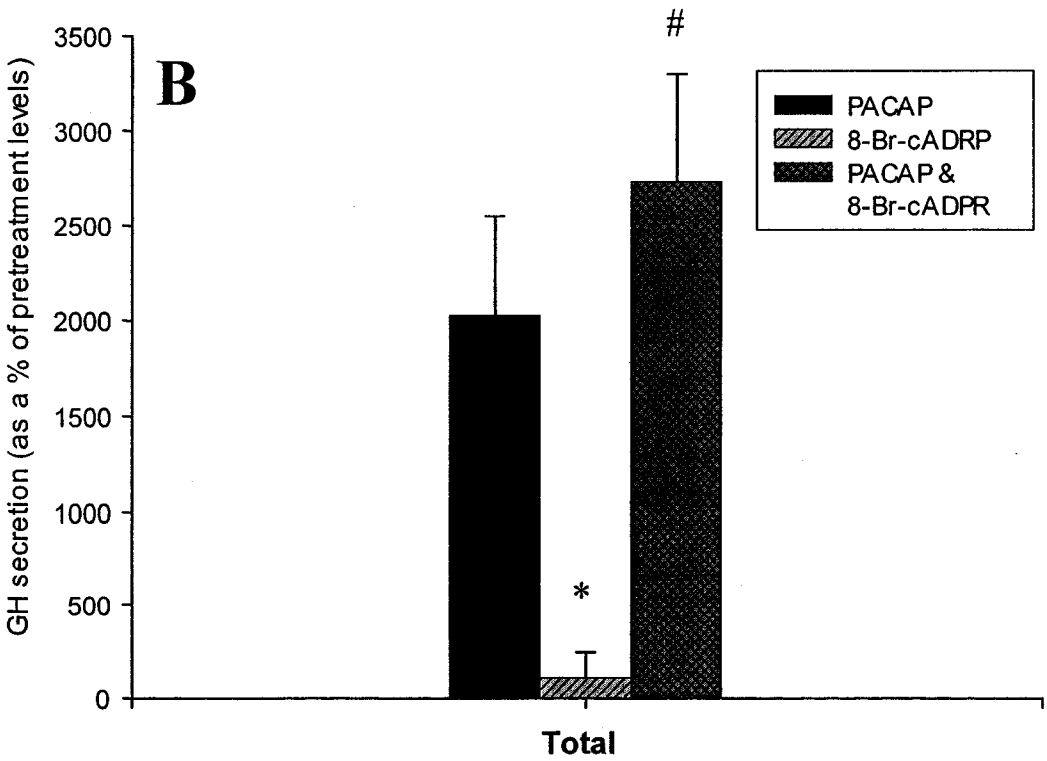
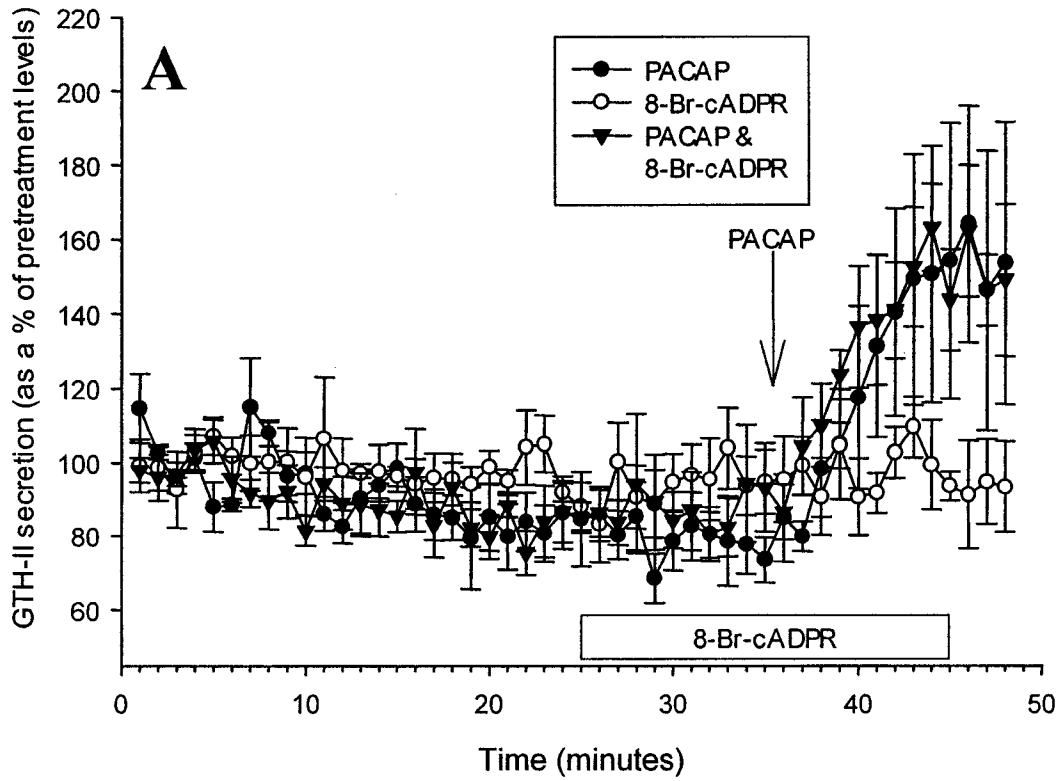



Figure 3.10. Effects of RyR antagonist 8-Br-cADPR on PACAP-stimulated GTH-II secretion. **A**. Temporal characteristics of the GTH-II response to PACAP (10 nM), either alone or in the presence of 8-Br-cADPR (100 μM). Arrow indicates the estimated commencement of hormone response to the 5-min PACAP pulse and the open bar indicates the duration exposure to 8-Br-cADPR. Pooled results (mean ± SEM; n=4 columns each) from 2 replicate experiments (performed once in July and once in August) are presented. Average pre-treatment GTH-II values for this set of experiments are 1.5 ± 0.1 ng/ml. **B**. Quantified GTH-II responses are presented. A star indicates a significant different ($p < 0.05$) from the PACAP alone group. A number sign indicates a significant difference ($p < 0.05$) from the 8-Br-cADPR only treatment group.



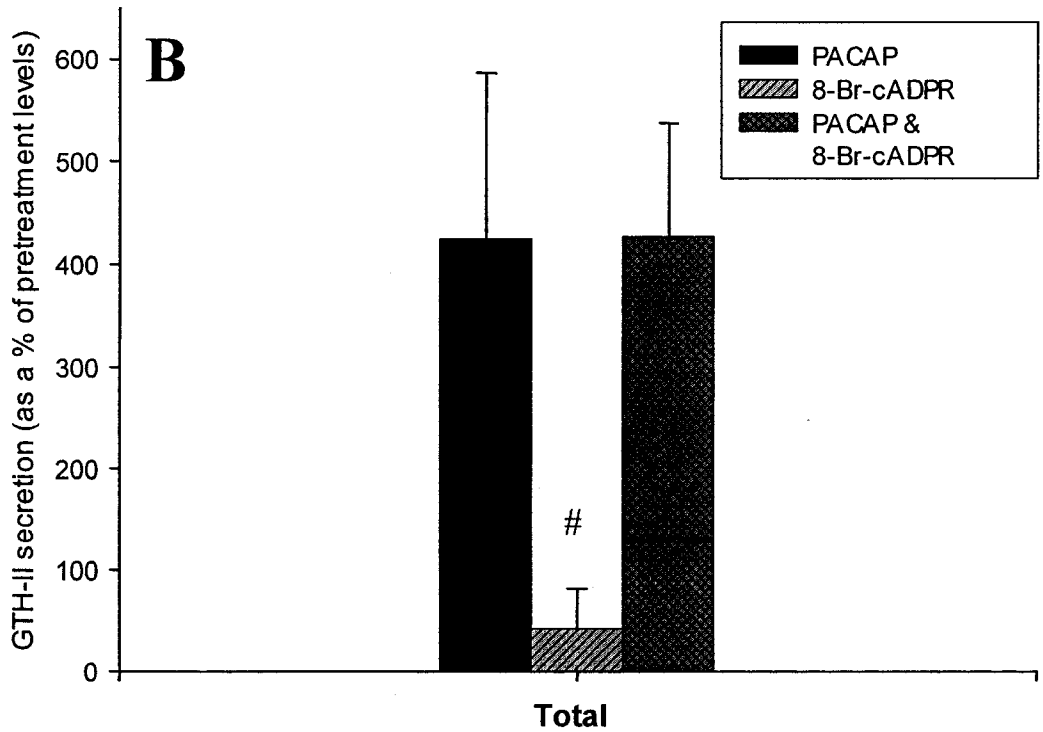
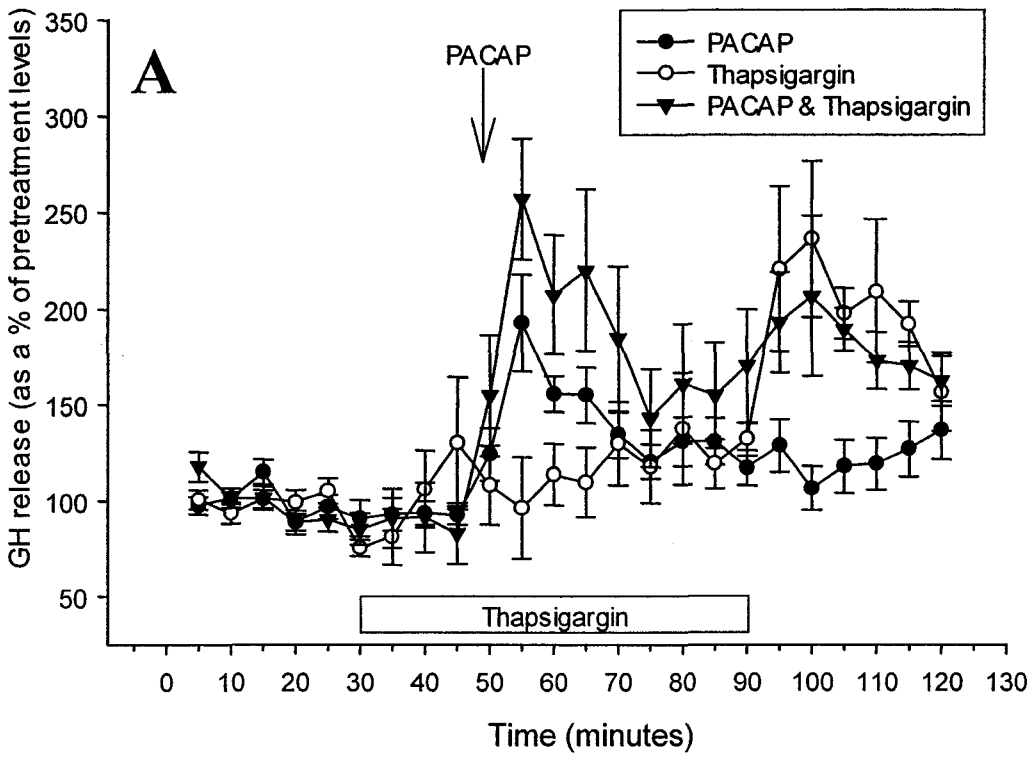



Figure 3.11. Effects of the SERCA inhibitor thapsigargin (Tg) on PACAP-stimulated GH secretion. **A**. Temporal characteristics of the GH response to PACAP (10 nM), either alone or in the presence of Tg (2 μM). Arrow indicates the estimated commencement of hormone response to the 5-min PACAP pulse and the open bar indicates the duration of exposure to Tg. Pooled results (mean ± SEM; n=8 columns each) from 4 replicate experiments (performed twice in January, once in February and once in May) are presented. Average pre-treatment GH values for this set of experiments are 39.7 ± 3.4 ng/ml. **B**. Quantified GH responses are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only group. A number sign indicates a significant difference ($p < 0.05$) from the Tg only treatment group.



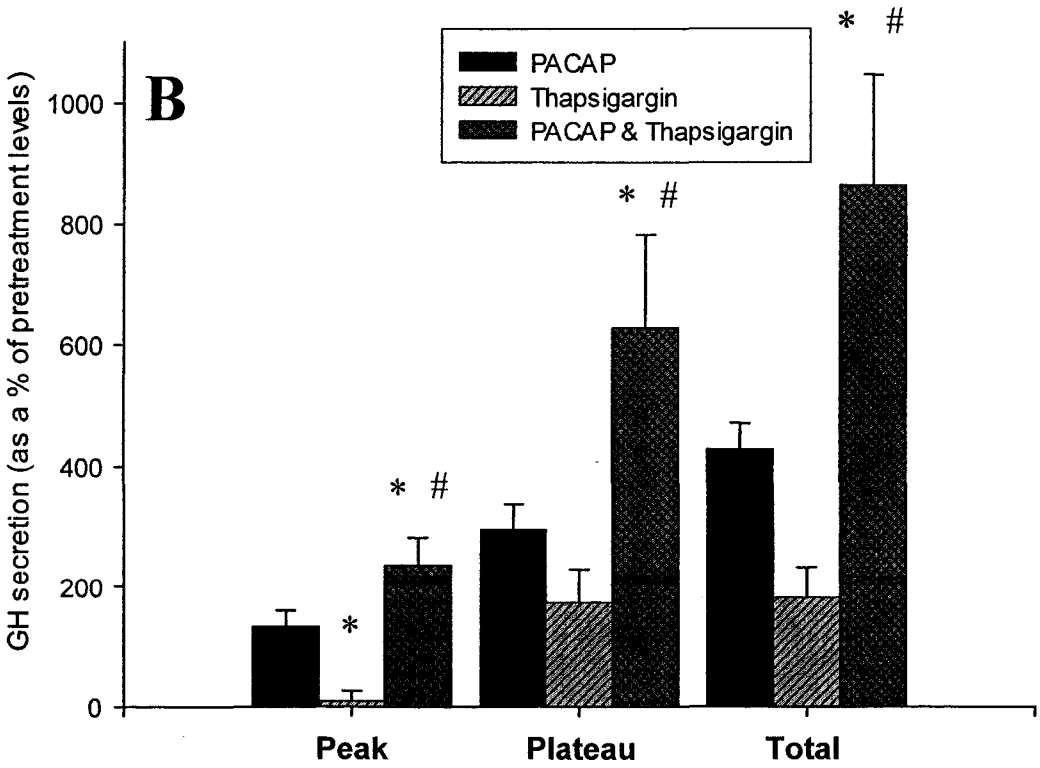
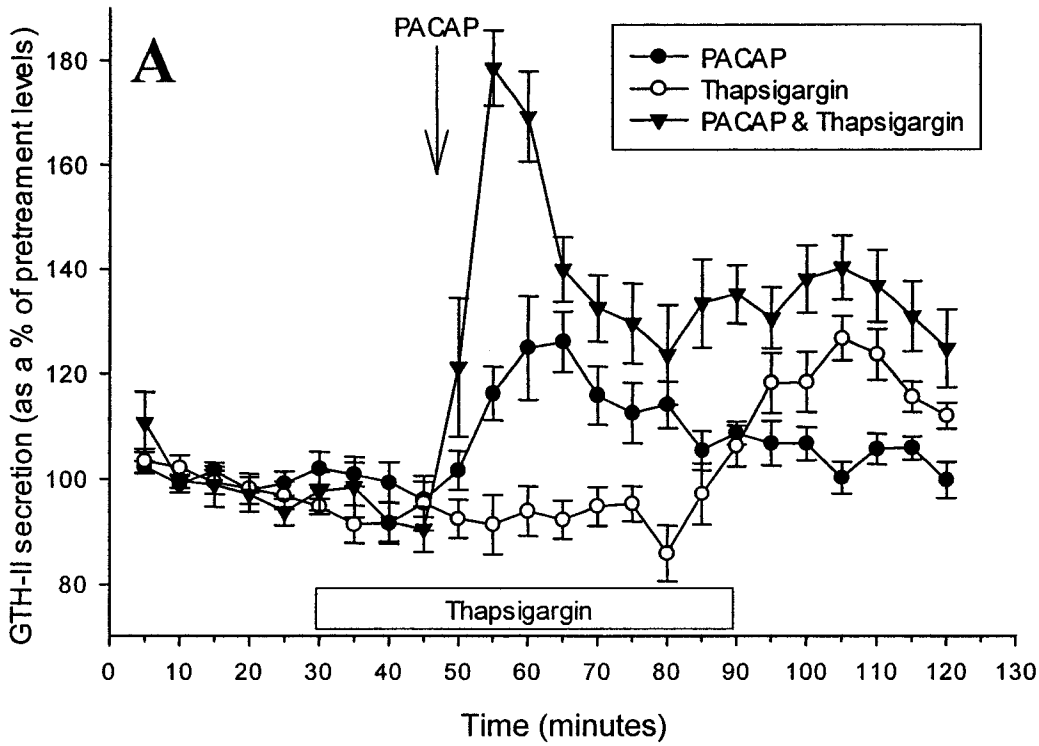



Figure 3.12. Effects of the SERCA inhibitor thapsigargin (Tg) on PACAP-stimulated GTH-II secretion. **A**. Temporal characteristics of the GTH-II response to PACAP (10 nM), either alone or in the presence of Tg (2 μM). Arrow indicates the estimated commencement of hormone response to the 5-min PACAP pulse and the open bar indicates the duration of exposure to Tg. Pooled results (mean ± SEM; n=8 columns each) from 4 replicate experiments (performed twice in January, once in February and once in May) are presented. Average pre-treatment GTH-II values for this set of experiments are 2.6 ± 0.1 ng/ml. **B**. Quantified GTH-II responses are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only group. A number sign indicates a significant difference ($p < 0.05$) from the Tg only treatment group.



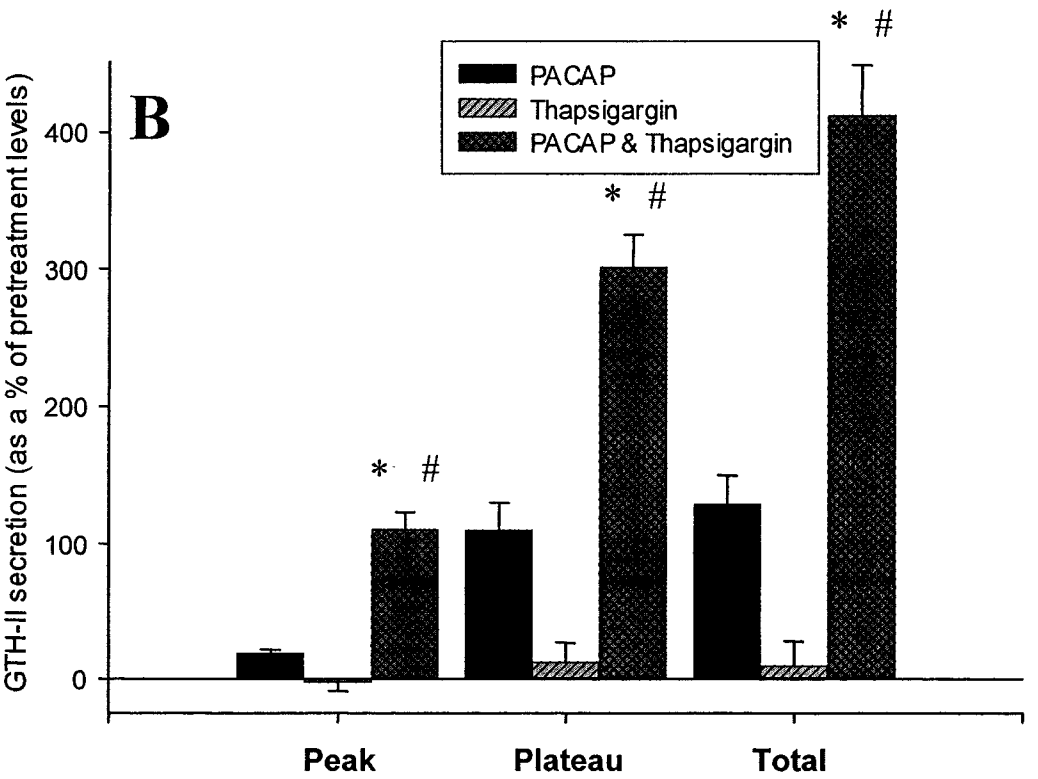
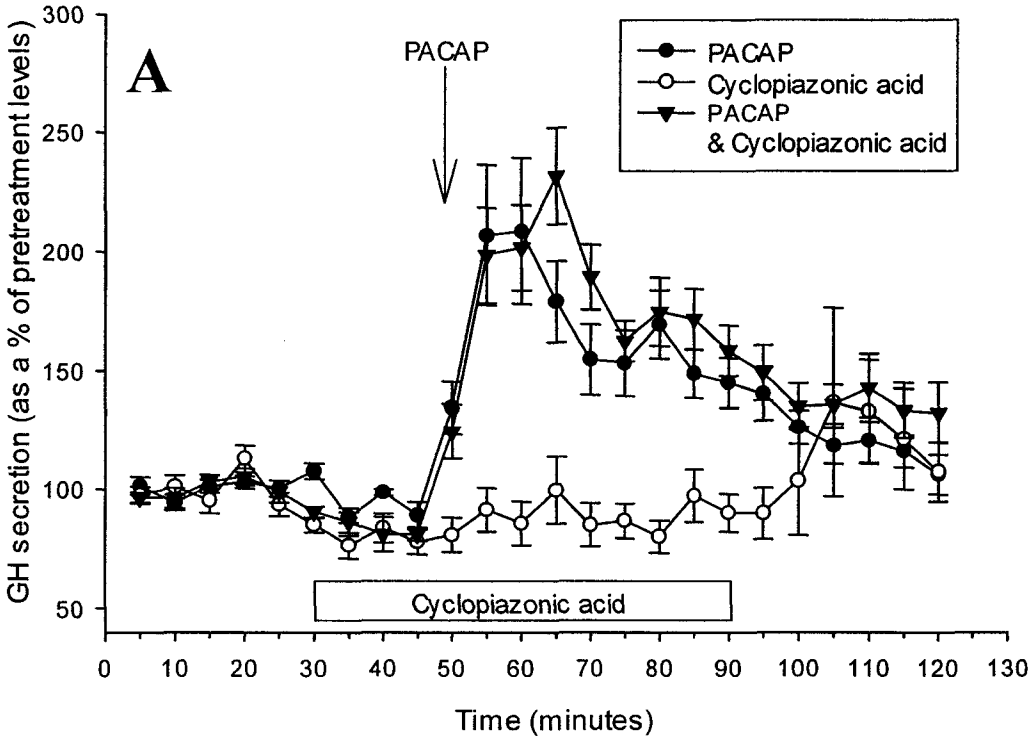



Figure 3.13. Effects of the SERCA inhibitor cyclopiazonic acid (CPA) on PACAP-stimulated GH secretion. **A**. Temporal characteristics of the GH response to PACAP (10 nM), either alone or in the presence of CPA (10 μM). Arrow indicates the estimated commencement of hormone response to the 5-min PACAP pulse and the open bar indicates the duration of exposure to CPA. Pooled results (mean ± SEM; n=6 columns each) from 3 replicate experiments performed in January are presented. Average pre-treatment GH values for this set of experiments are 39.0 ± 1.4 ng/ml. **B**. Quantified GH responses are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only group. A number sign indicates a significant difference ($p < 0.05$) from the CPA only treatment group.



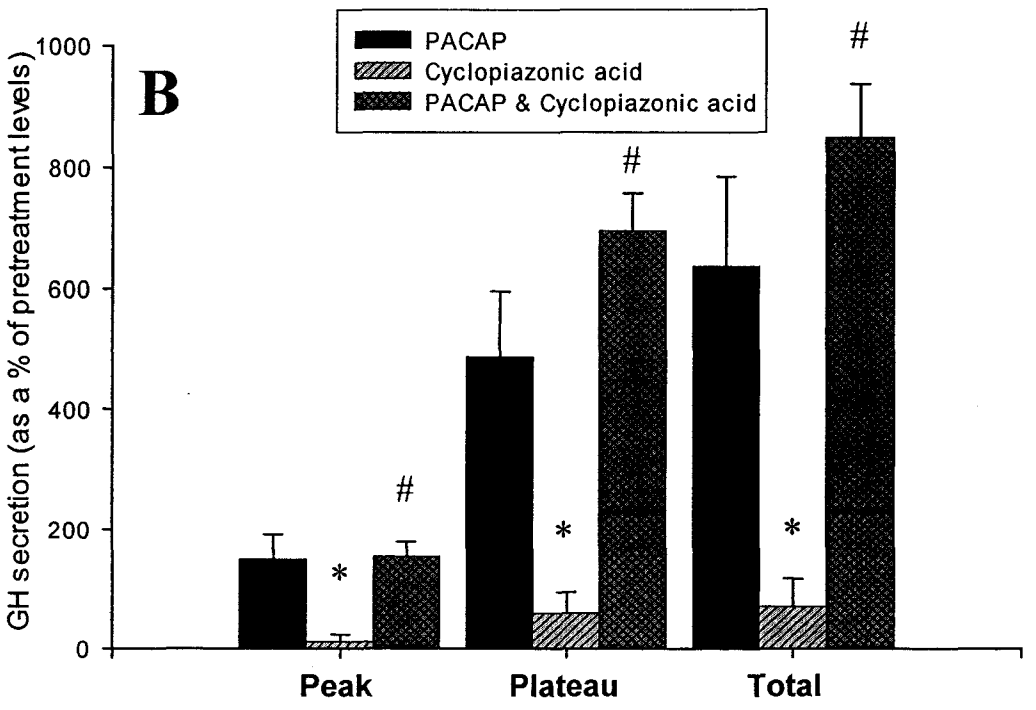
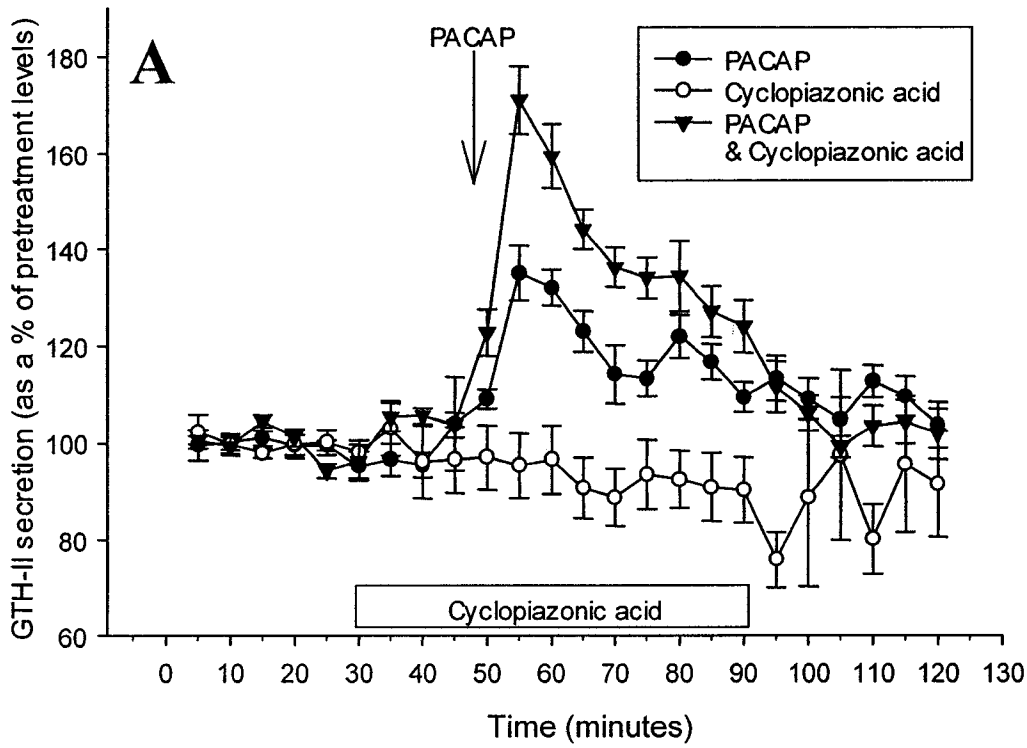



Figure 3.14. Effects of the SERCA inhibitor cyclopiazonic acid (CPA) on PACAP-stimulated GTH-II secretion. **A**. Temporal characteristics of the GTH-II response to PACAP (10 nM), alone or in the presence of CPA (10 μM). Arrow indicates the estimated commencement of hormone response to the 5-min PACAP pulse and the open bar indicates the duration of exposure to CPA. Pooled results (mean ± SEM; n=6 columns each) from 3 replicate experiments performed in January are presented. Average pre-treatment GTH-II values for this set of experiments are 2.8 ± 0.1 ng/ml. **B**. Quantified GTH-II response are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only group. A number sign indicates a significant difference ($p < 0.05$) from the CPA only treatment group.



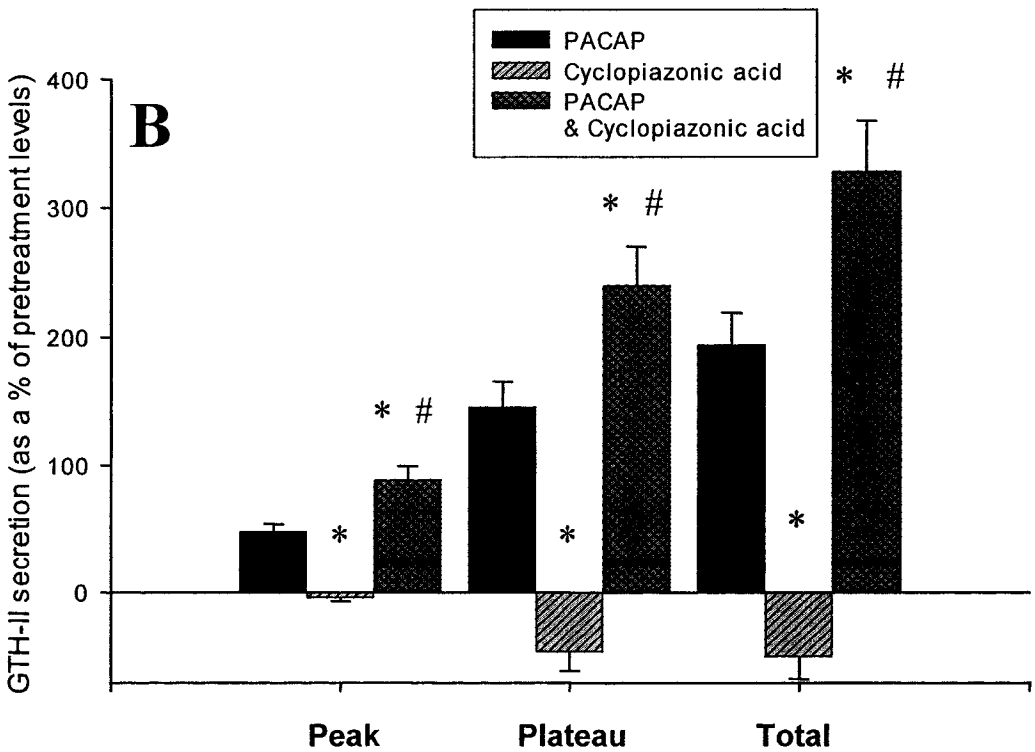
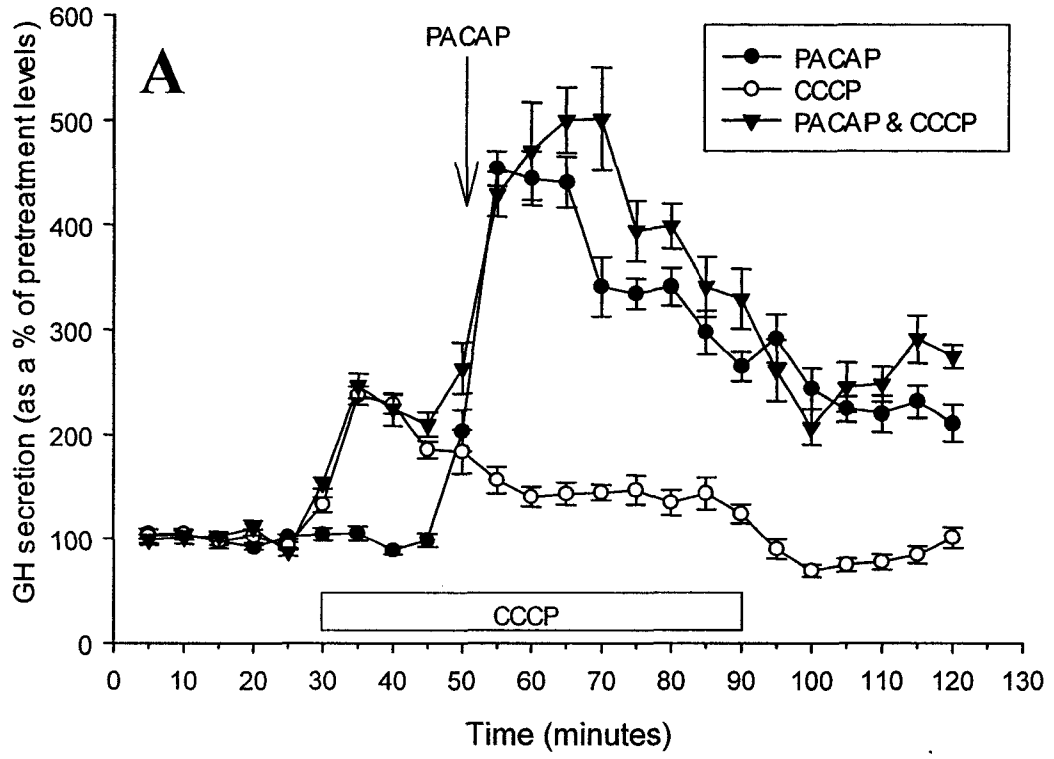



Figure 3.15. Effects of mitochondrial $Ca^{2+}$ uniport uncoupler CCCP on PACAP-stimulated GH secretion. **A**. Temporal characteristics of the GH response to PACAP (10 nM) alone or in the presence of CCCP (10 μM). Arrow indicates the estimated commencement of hormone response to the 5-min PACAP pulse and the bar indicates the duration of exposure to CCCP. Pooled results (mean ± SEM; n=6 columns each) from 3 replicate experiments performed in April are presented. Average pre-treatment GH values for this set of experiments are 29.2 ± 1.3 ng/ml. **B**. Quantified GH responses are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only group. A number sign indicates a significant difference ($p < 0.05$) from the CCCP only treatment group.



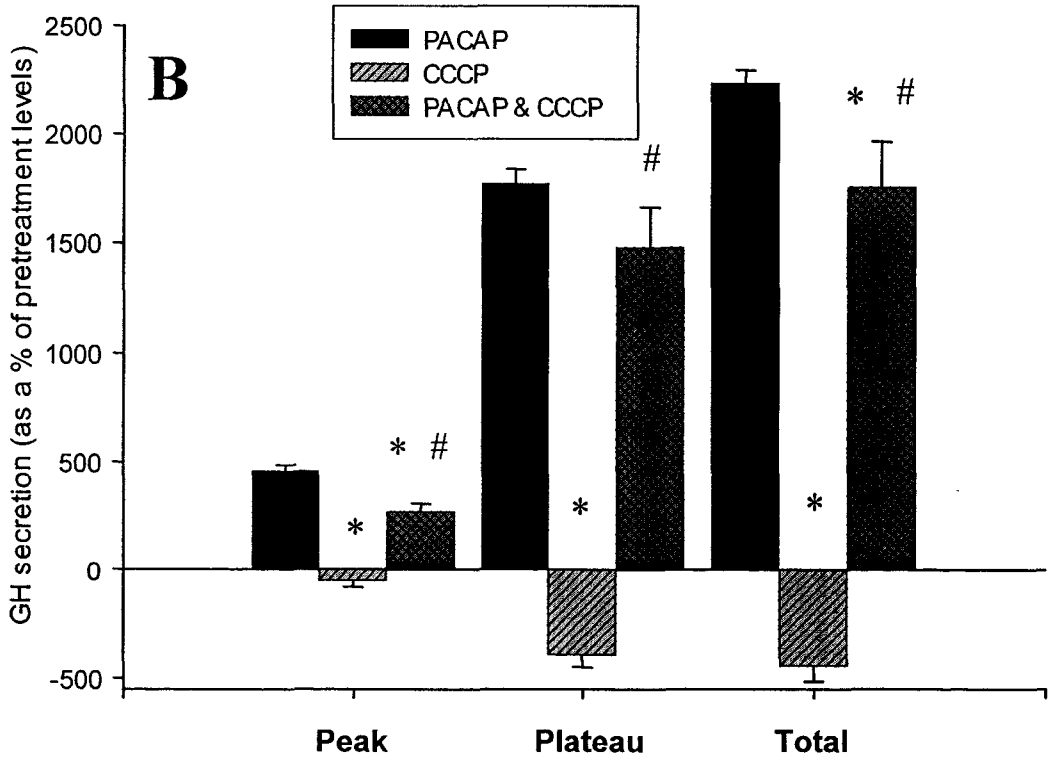
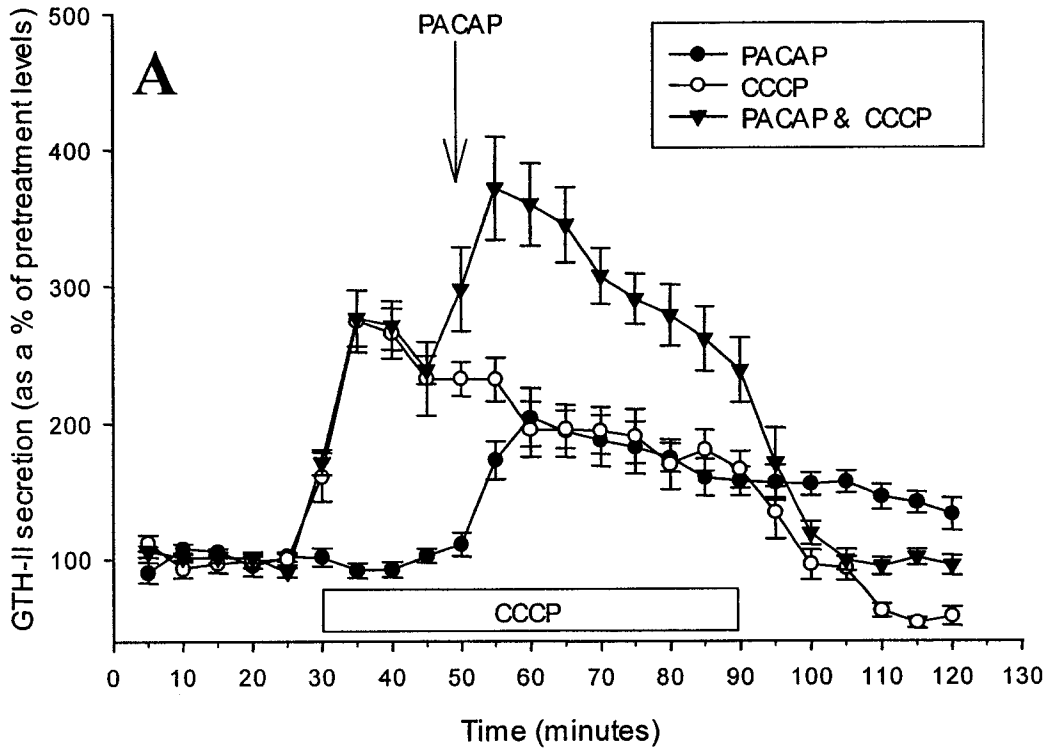



Figure 3.16. Effects of mitochondrial $Ca^{2+}$ uniport uncoupler CCCP on PACAP-stimulated GTH-II secretion. **A**. Temporal characteristics of the GTH-II response to PACAP (10 nM), either alone or in the presence of CCCP (10 μM). Arrow indicates the estimated commencement of hormone response to the 5-min PACAP pulse and the open bar indicates the duration of exposure to CCCP. Pooled results (mean ± SEM; n=6 columns each) from 3 replicate experiments performed in April are presented. Average pre-treatment GTH-II values for this set of experiments are 1.5 ± 0.1 ng/ml. **B**. Quantified GTH-II responses are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only group. A number sign indicates a significant difference ($p < 0.05$) from the CCCP only treatment group.



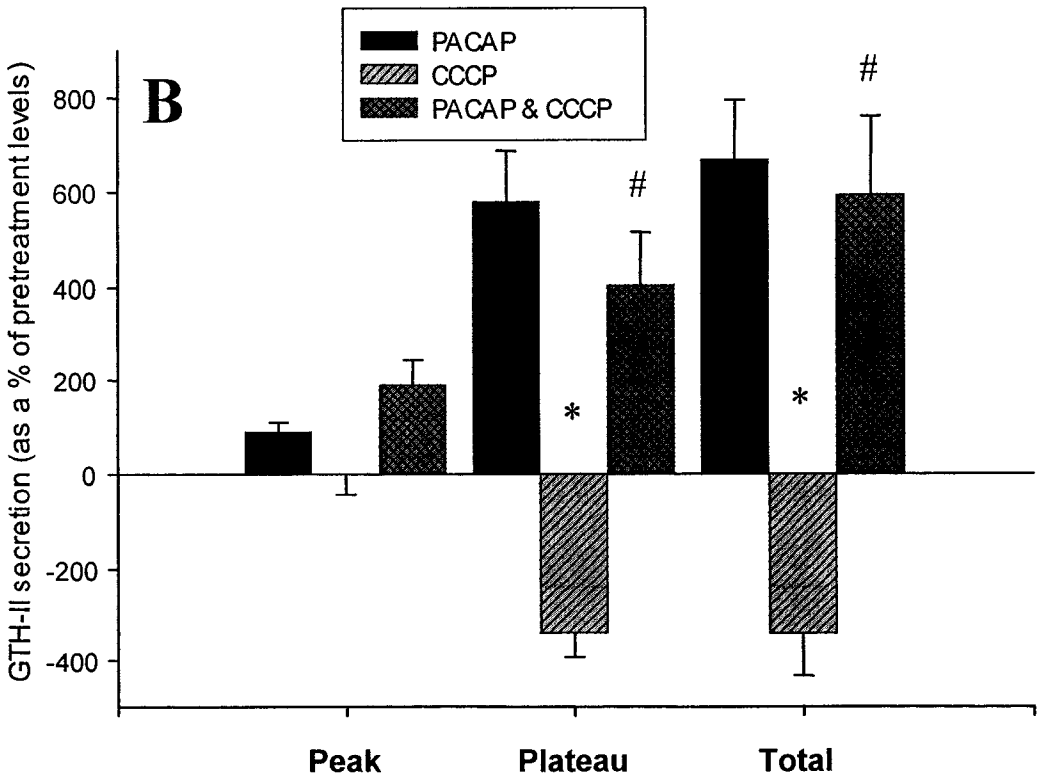
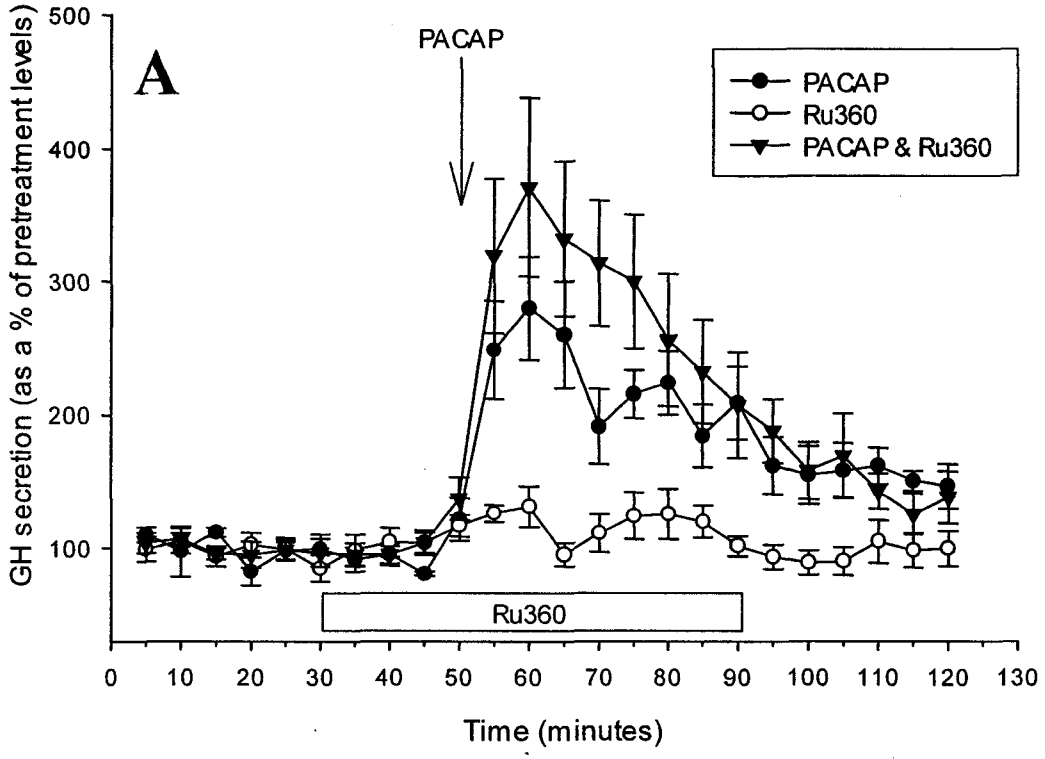



Figure 3.17. Effects of mitochondrial $Ca^{2+}$ uniport inhibitor Ru360 on PACAP-stimulated GH secretion. **A**. Temporal characteristics of GH response to PACAP (10 nM), either alone or in the presence of Ru360 (10 μM). Arrow indicates the estimated commencement of hormone response to the 5-min PACAP pulse and the open bar indicates the duration of exposure to Ru360. Pooled results (mean ± SEM; n=6 columns each) from 3 replicate experiments performed in July are presented. Average pre-treatment GH values for this set of experiments are 28.2 ± 0.4 ng/ml. **B**. Quantified GH responses are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only group. A number sign indicates a significant difference ($p < 0.05$) from the Ru360 only treatment group.



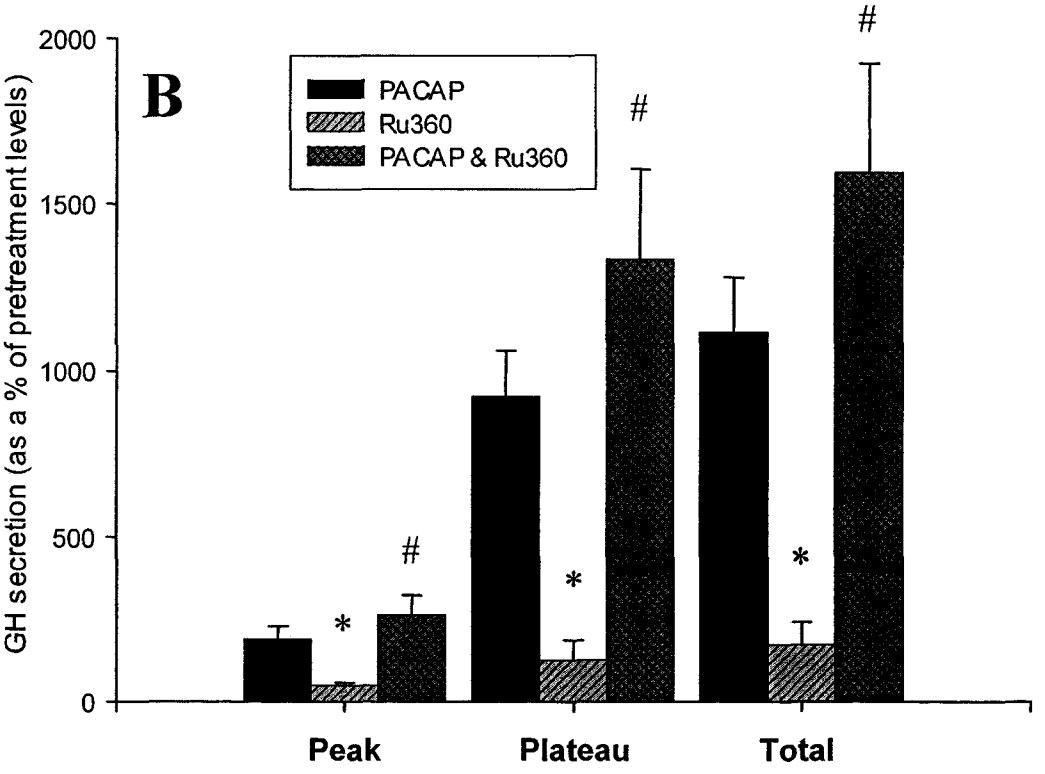
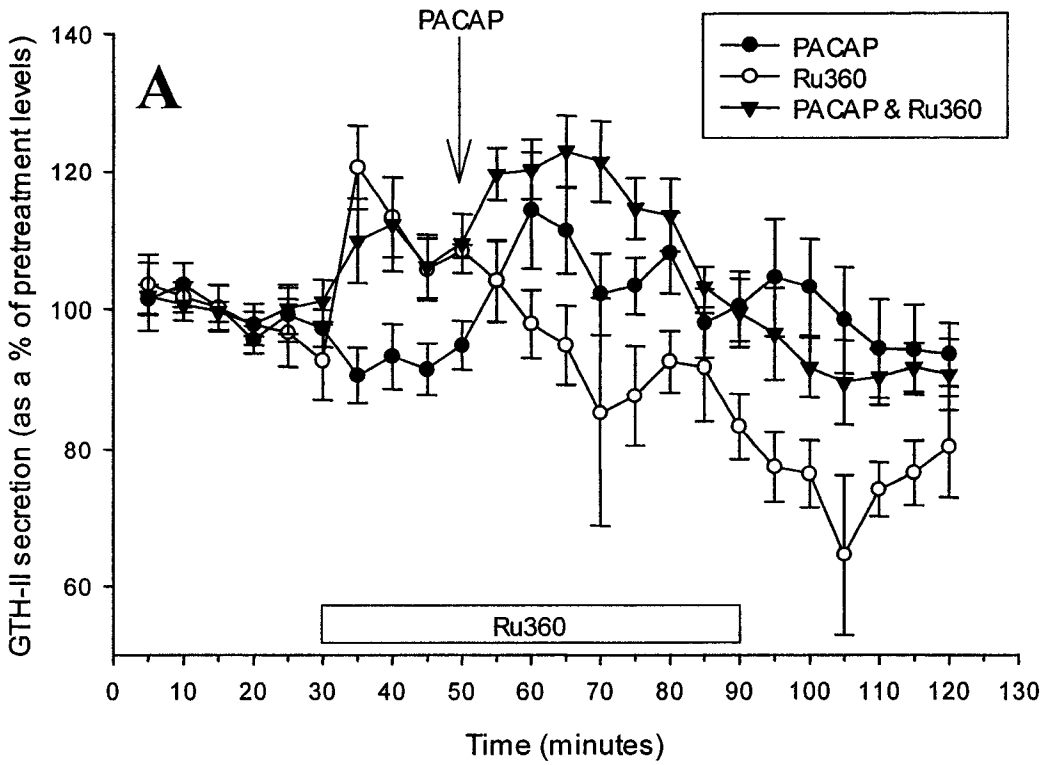



Figure 3.18. Effects of the mitochondrial $Ca^{2+}$ uniport inhibitor Ru360 on PACAP-stimulated GTH-II secretion. **A**. Temporal characteristics of GTH-II response to PACAP (10 nM), either alone or in the presence of Ru360 (10 μM). Arrow indicates the estimated commencement of hormone response to the 5-min PACAP pulse and the open bar indicates the duration exposure to Ru360. Pooled results (mean ± SEM; n=6 columns each) from 3 replicate experiments performed in July are presented. Average pre-treatment GTH-II values for this set of experiments are 3.0 ± 0.1 ng/ml. **B**. Quantified GTH-II responses are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only group. A number sign indicates a significant difference ($p < 0.05$) from the Ru360 only treatment group.



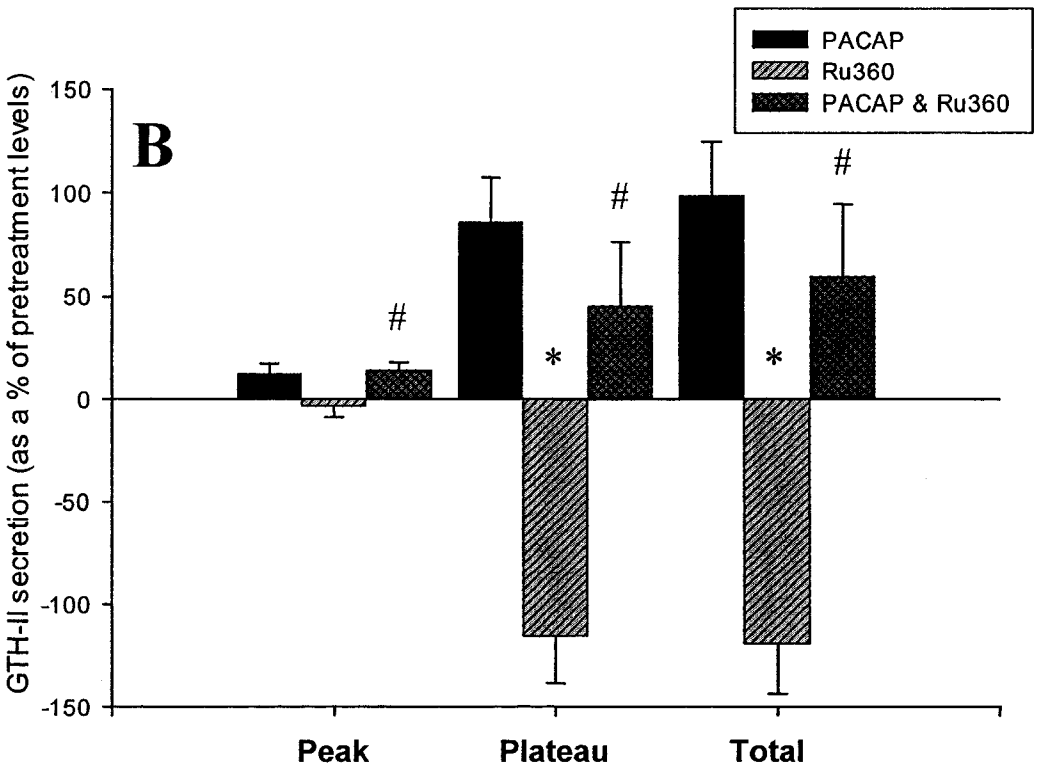
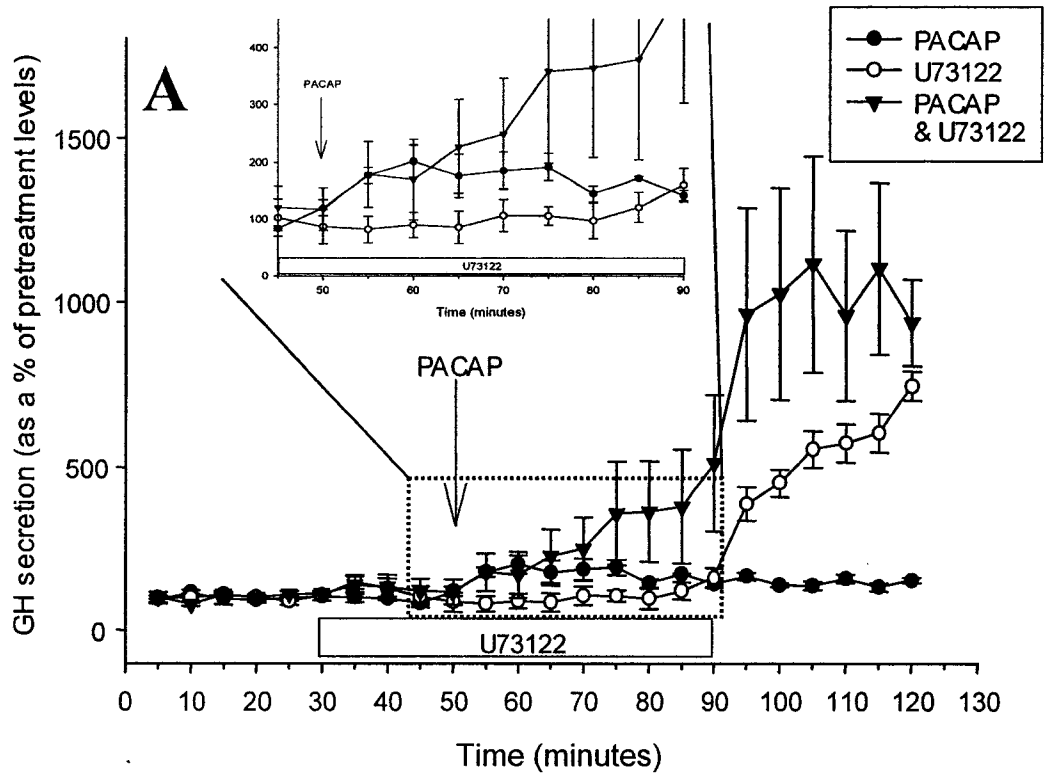



Figure 3.19. Effects of the phosphatidylinositol-specific PLC inhibitor, U73122, on PACAP-stimulated GH release. **A**. Temporal characteristics of the GH response to PACAP (10 nM), either alone or in the presence of U73122 (10 μM). Arrow indicates the estimated commencement of hormone response to the 5-min PACAP pulse and the open bar indicates the duration of exposure to U73122. Pooled results (mean ± SEM; n=6 columns each) from replicate 3 experiments performed in July are presented. Average pre-treatment GH values for this set of experiments are 27.4 ± 0.6 ng/ml. **B**. Quantified GH responses are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only group. A number sign indicates a significant difference ($p < 0.05$) from the U73122 only treatment group.



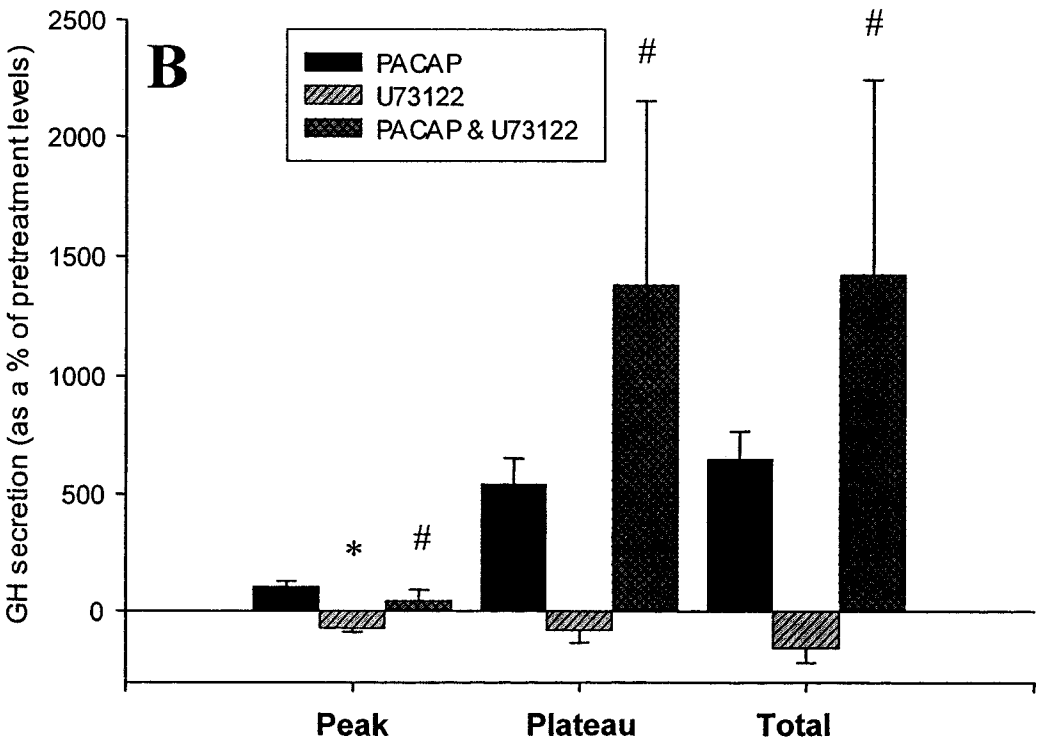
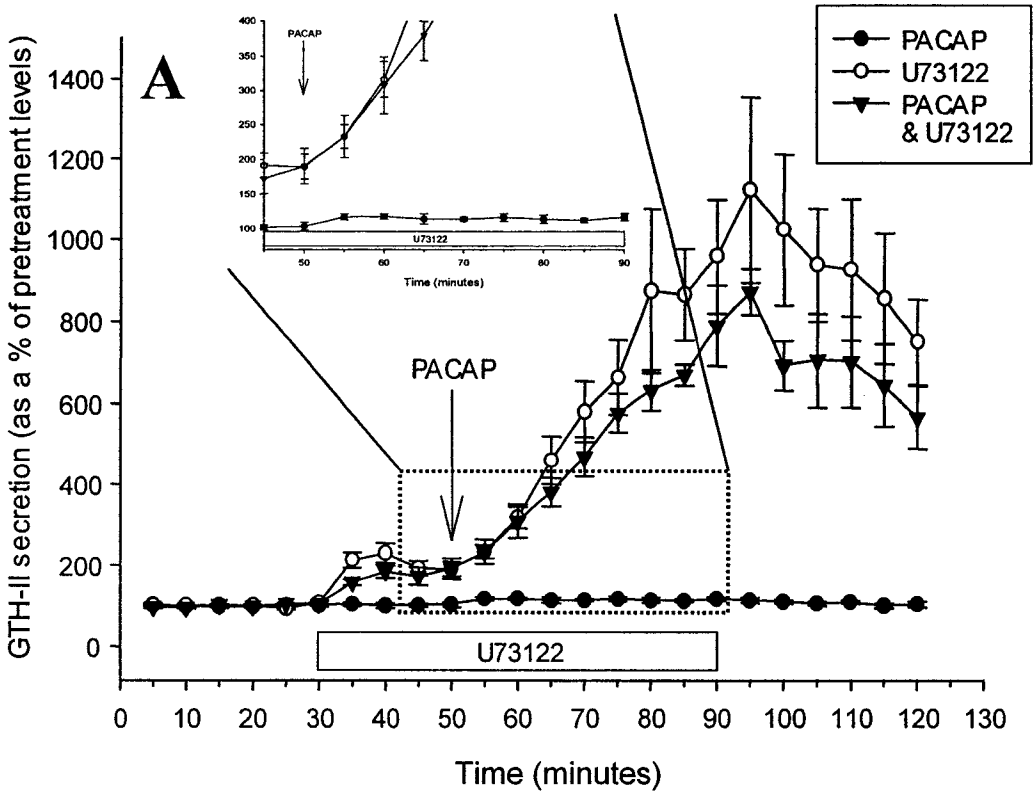



Figure 3.20. Effects of the phosphatidylinsositol-specific PLC inhibitor U73122 on PACAP-stimulated GTH-II secretion. **A**. Temporal characteristics of the GTH-II response to PACAP (10 nM), either alone or in the presence of U73122 (10 μM). Arrow indicates the estimated commencement of hormone response to the 5-min PACAP pulse and the open bar indicates the duration of exposure to U73122. Pooled results (mean ± SEM; n=6 columns each) from 3 replicate experiments performed in July are presented. Average pre-treatment GTH-II values for this set of experiments are 3.5 ± 0.6 ng/ml. **B**. Quantified GTH-II responses are presented as a mean ± SEM (% pre-treatment). A star indicates a significant difference from PACAP alone group.



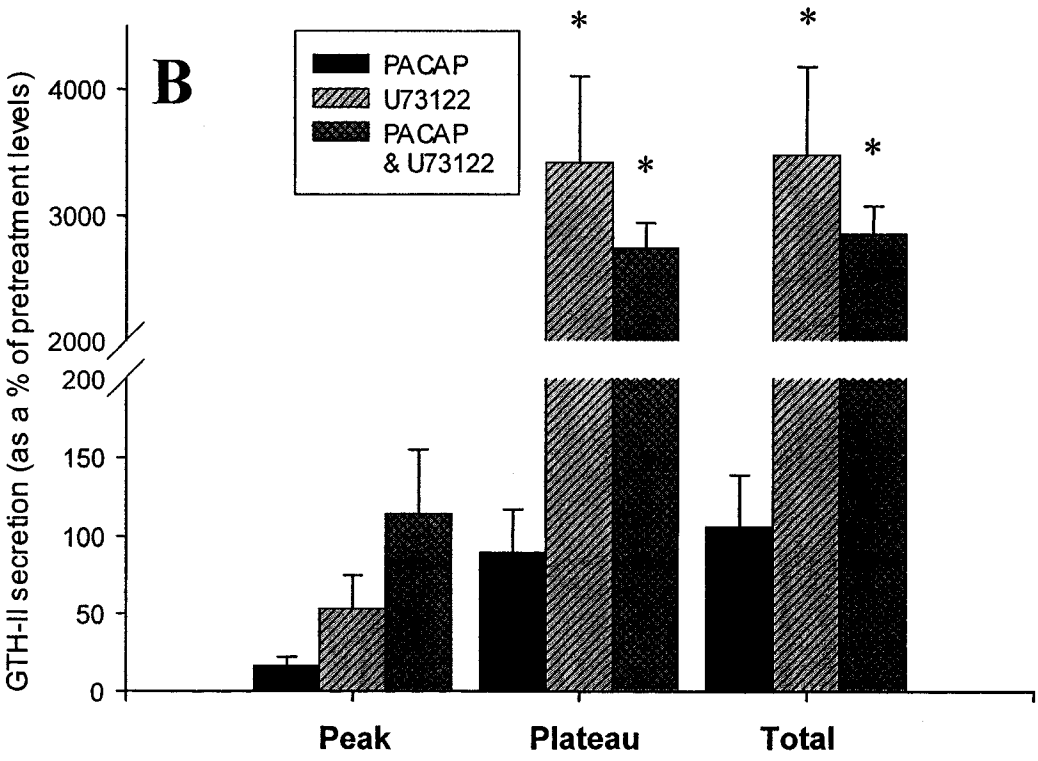
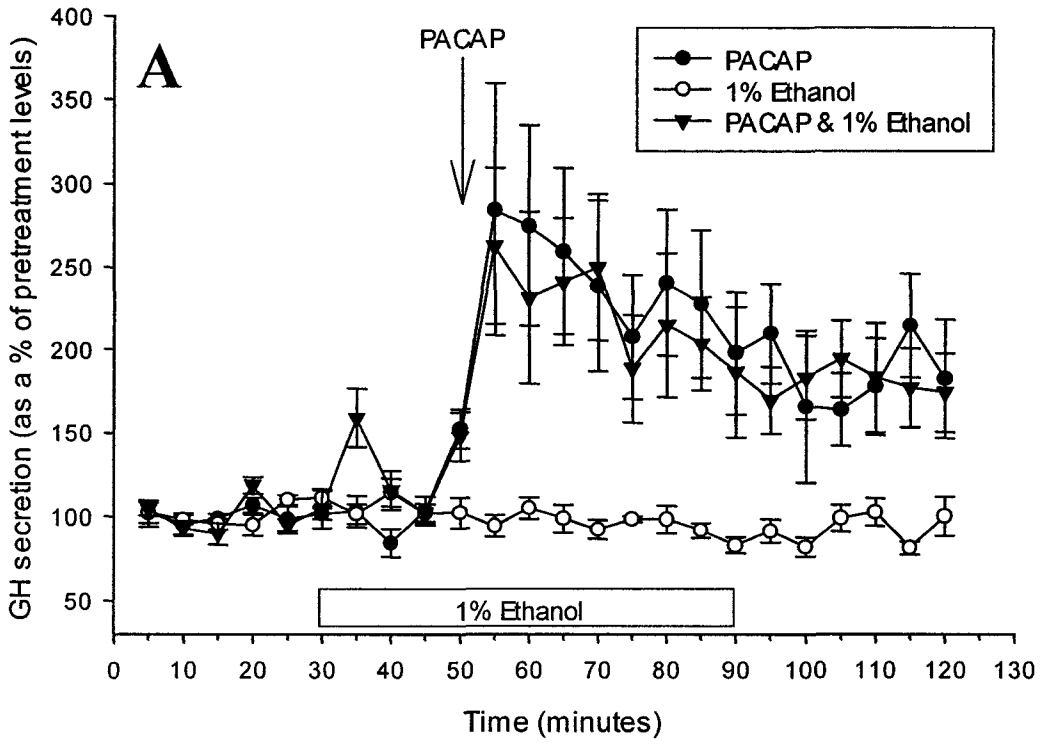



Figure 3.21. Effects of 1% ethanol on PACAP-stimulated GH secretion. **A.** Temporal characteristics of the GH response to PACAP (10 nM), either alone or in the presence of 1% ethanol. Arrow indicates the estimated commencement of hormone response to the 5-min PACAP pulse and the open bar indicates the duration exposure to 1% ethanol =. Pooled results (mean ± SEM; n=6 columns each) from 3 replicate experiments (performed once in September and in once in November) are presented. Average pre-treatment GH values for this set of experiments are 28.3 ± 1.0 ng/ml. **B**. Quantified GH responses are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only group. A number sign indicates a significant difference ($p < 0.05$) from the 1% ethanol only treatment group.



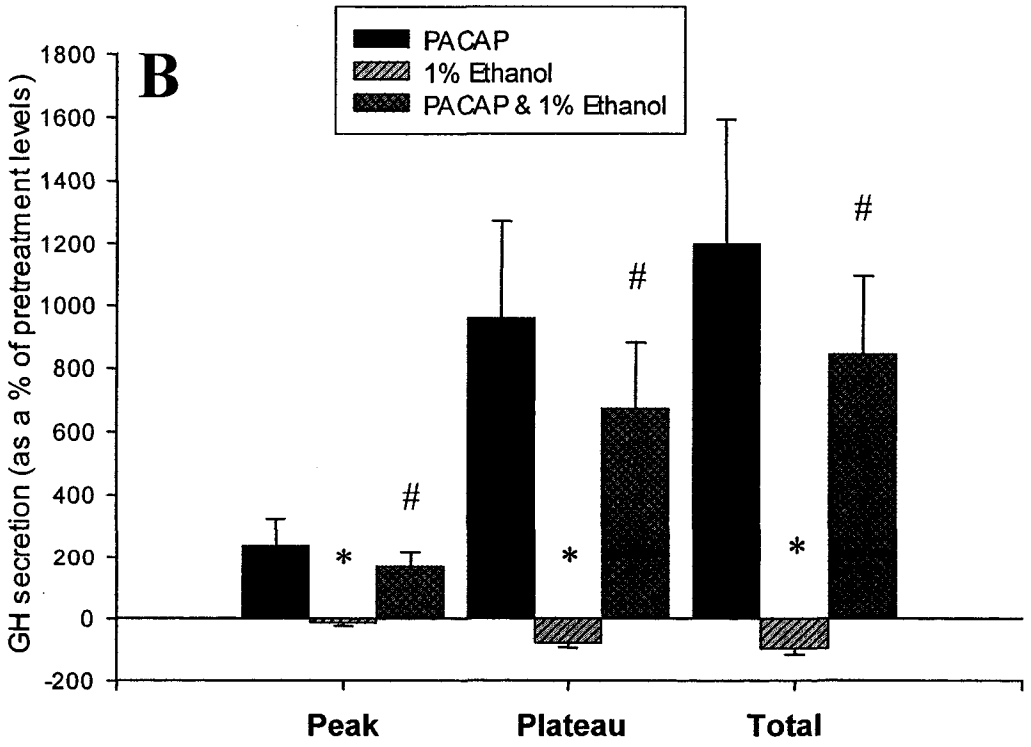
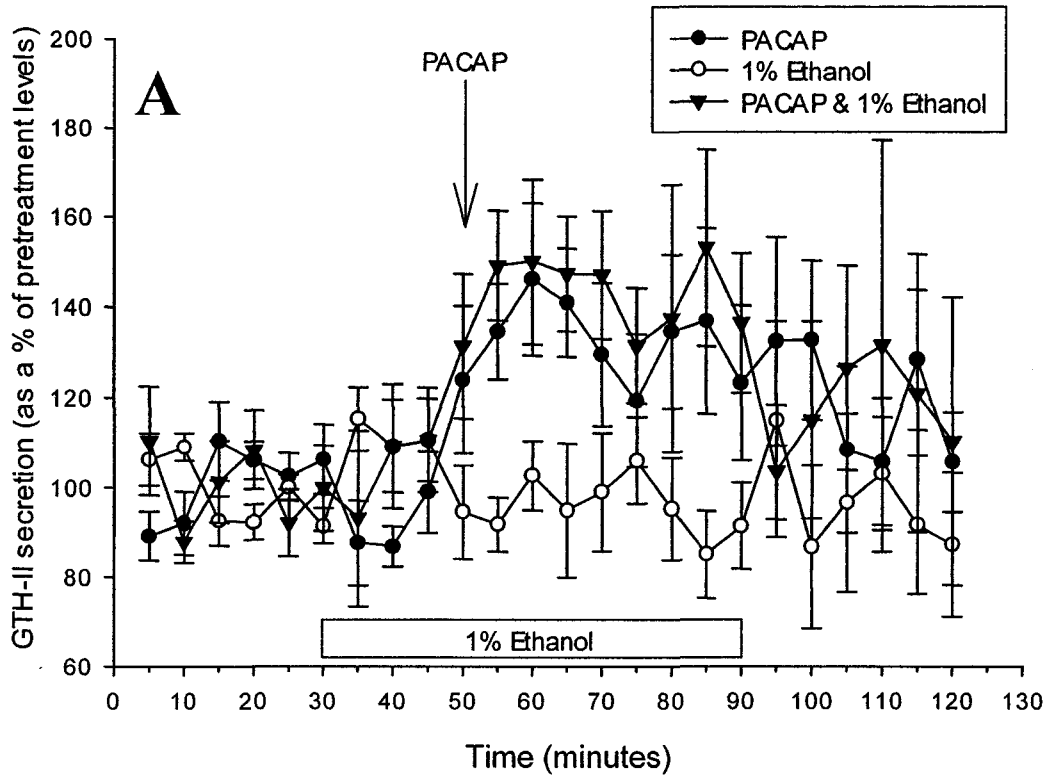



Figure 3.22. Effects of 1% ethanol on PACAP-stimulated GTH-II secretion. **A.** Temporal characteristics of the GTH-II response to PACAP (10 nM), either alone or in the presence of 1% ethanol. Arrow indicates the estimated commencement of hormone response to the 5-min PACAP pulse and the open bar indicates the duration of exposure to 1% ethanol. Pooled results (mean ± SEM; n=6 columns each) from 3 replicate experiments (performed once in September and twice in November) are presented. Average pre-treatment GTH-II values for this set of experiments are 1.4 ± 0.1 ng/ml. **B.** Quantified GTH-II responses are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only group. A number sign indicates a significant difference ($p < 0.05$) from the 1% ethanol only treatment group.



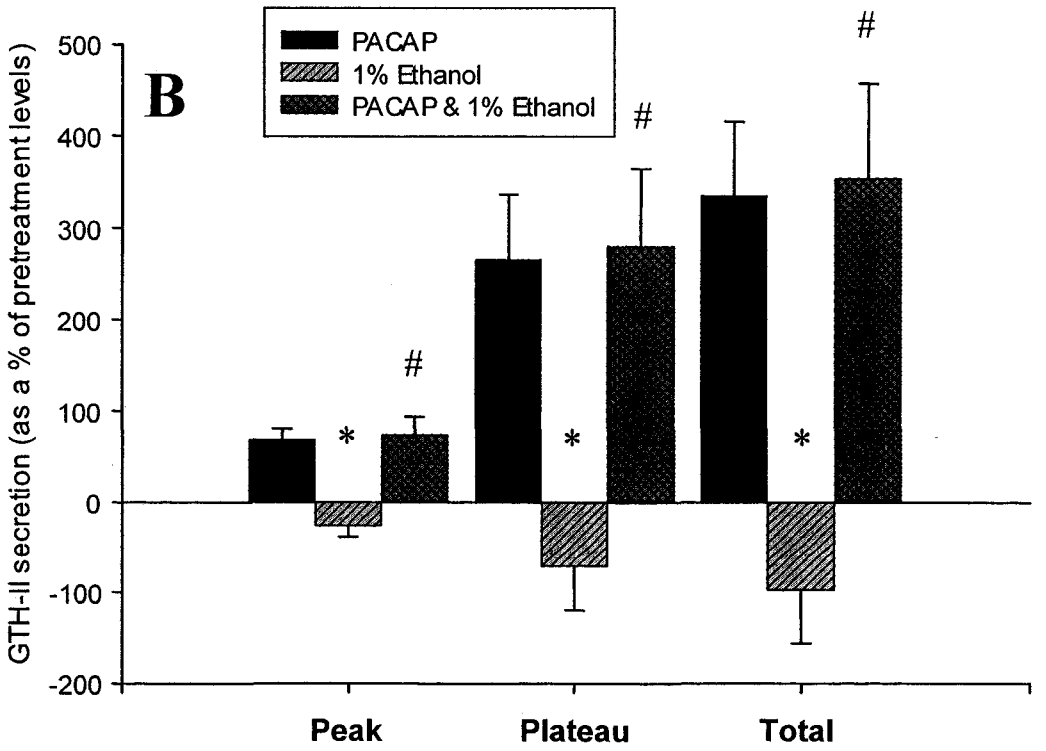
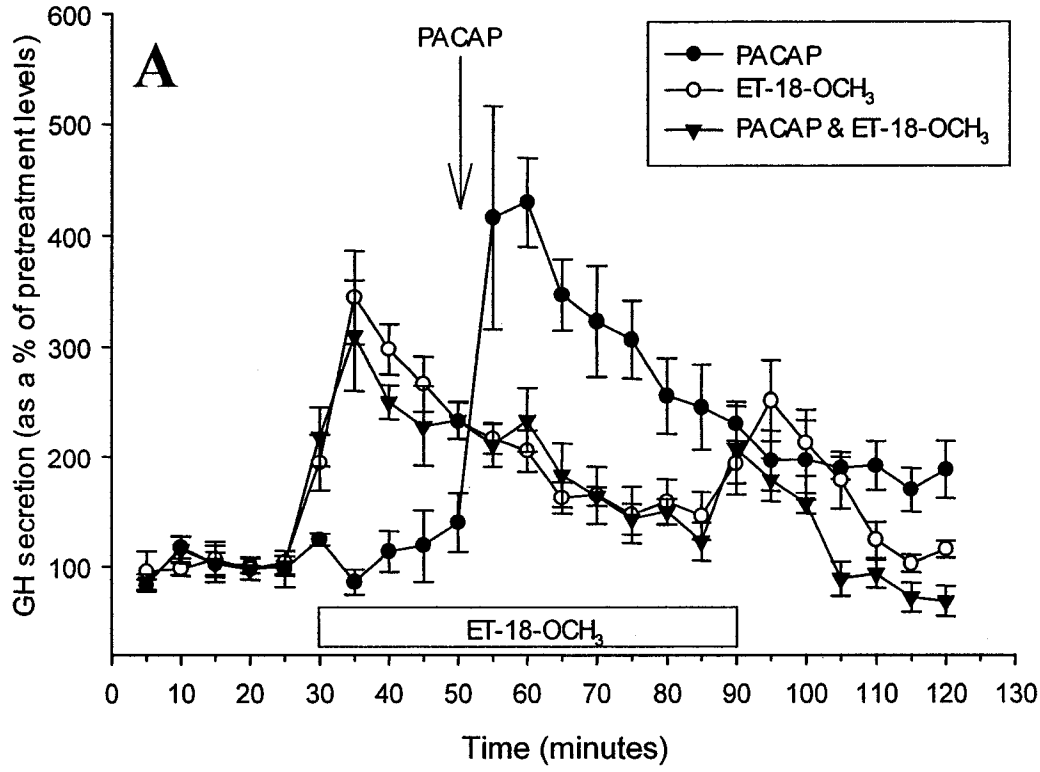



Figure 3.23. Effects of the phosphatidylinositol-specific PLC inhibitor ET-18-$OCH_3$ on PACAP-stimulated GH secretion. **A**. Temporal characteristics of the GH response to PACAP (10 nM), either alone or in the presence of ET-18-$OCH_3$ (30 μM). Arrow indicates the estimated commencement of hormone response to the 5-min PACAP pulse and the open bar indicates the duration of exposure to ET-18-$OCH_3$. Pooled results (mean ± SEM; n=4 columns each) from 2 replicate experiments performed in July are presented. Average pre-treatment GH values for this set of experiments are 32.2 ± 0.6 ng/ml. **B**. Quantified GH responses are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only group. A number sign indicates a significant difference ($p < 0.05$) from the ET-18-$OCH_3$ only treatment group.



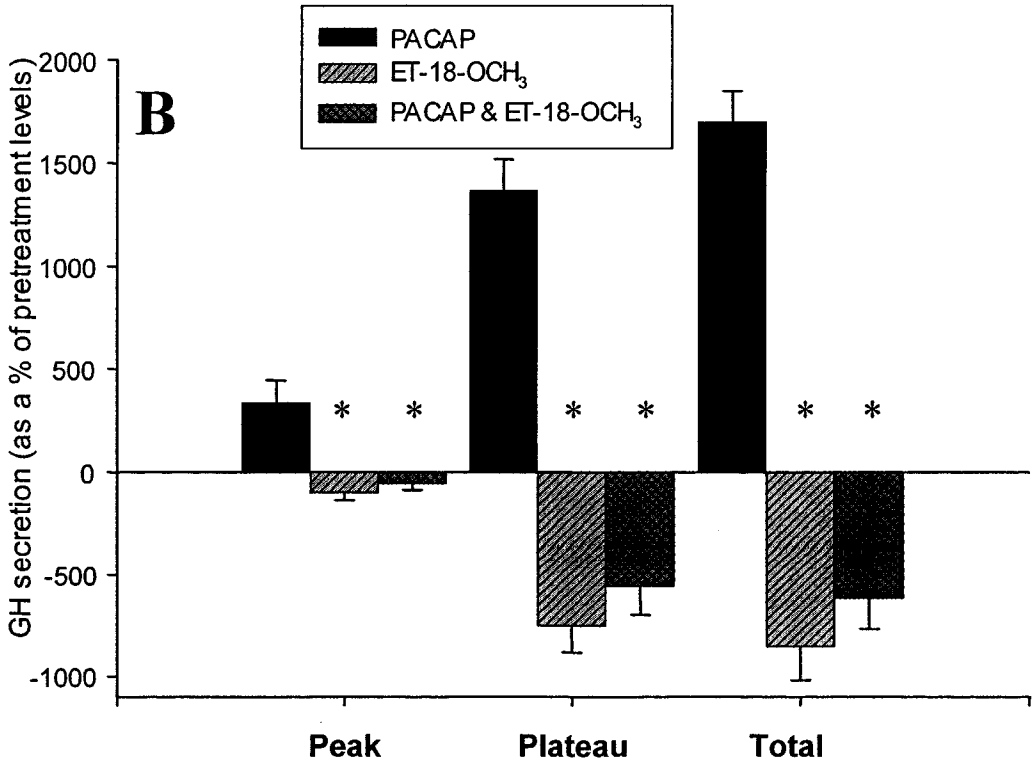
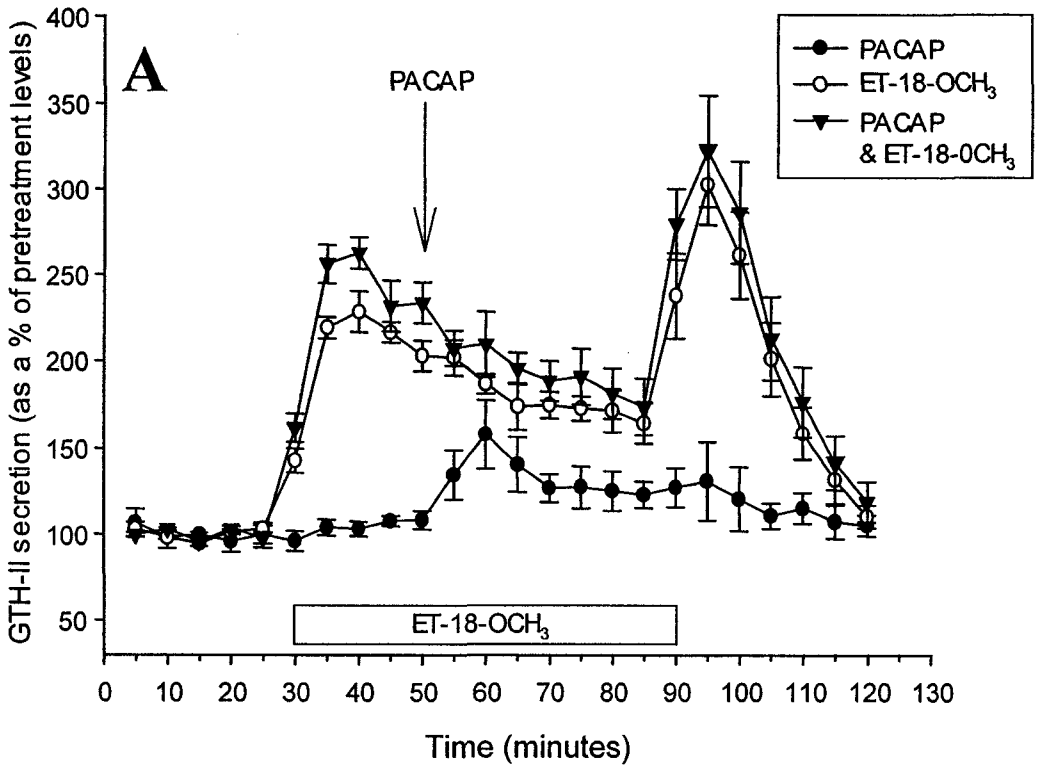



Figure 3.24. Effects of the phosphatidylinositol-specific PLC inhibitor ET-18-$OCH_3$ on PACAP-stimulated GTH-II secretion. **A**. Temporal characteristics of the GTH-II response to PACAP (10 nM), either alone or in the presence of ET-18-$OCH_3$ (30 μM). Arrow indicates the estimated commencement of hormone response to the 5-min PACAP pulse the open bar indicates the duration of exposure to ET-18-$OCH_3$. Pooled results (mean ± SEM; n=4 columns each) from 2 replicate experiments performed in July are presented. Average pre-treatment GTH-II values for this set of experiments are 3.4 ± 0.1 ng/ml. **B**. Quantified GTH-II response are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only control GTH-II levels.



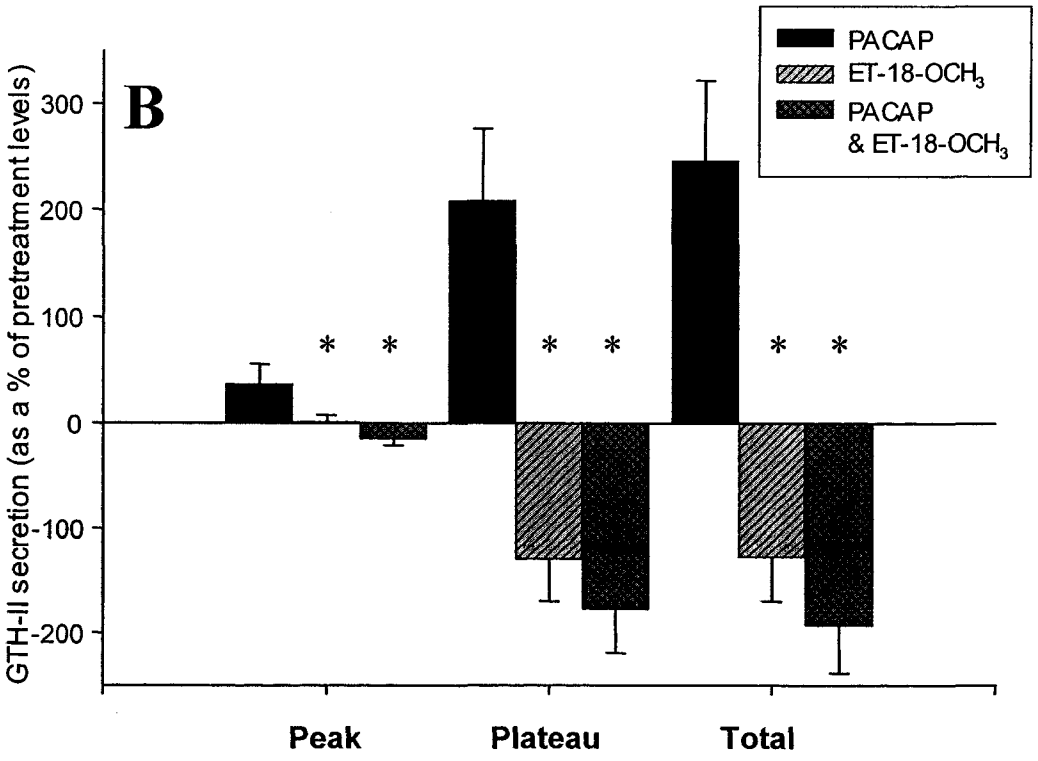
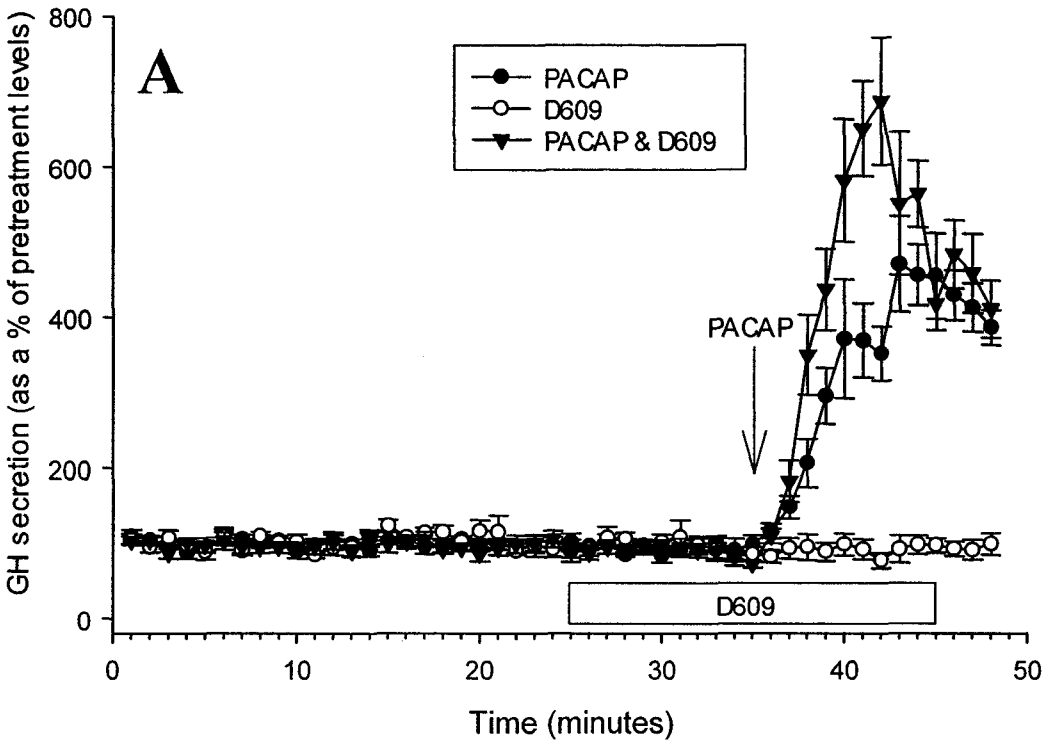



Figure 3.25. Effects of the phosphatidylcholine-specific PLC inhibitor D609 on PACAP-stimulated GH secretion. **A**. Temporal characteristics of the GH response to PACAP (10 nM), either alone or in the presence of D609 (200 μM). Arrow indicates the estimated commencement of hormone response to the 5-min PACAP pulse and the open bar indicates the duration of exposure to D609. Pooled results (mean ± SEM; n=6 columns each) from 3 replicate experiments performed in July are presented. Average pre-treatment GH values for this set of experiments are 31.0 ± 1.3 ng/ml. **B**. Quantified GH responses are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only group. A number sign indicates a significant difference ($p < 0.05$) from the D609 only treatment group.



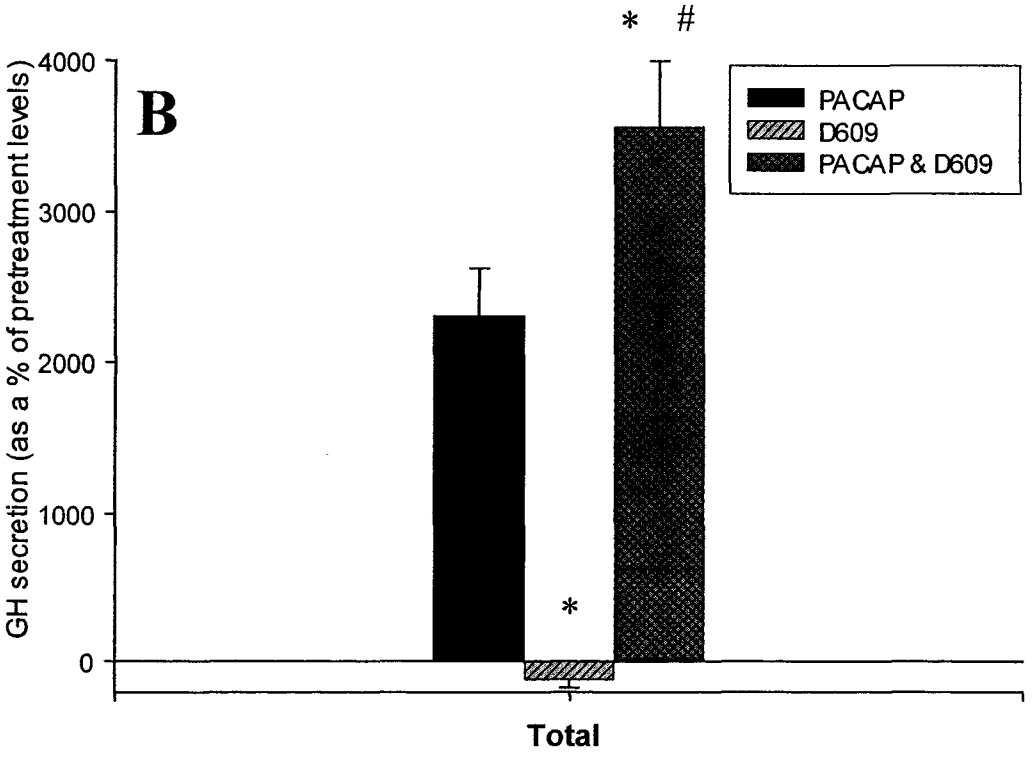
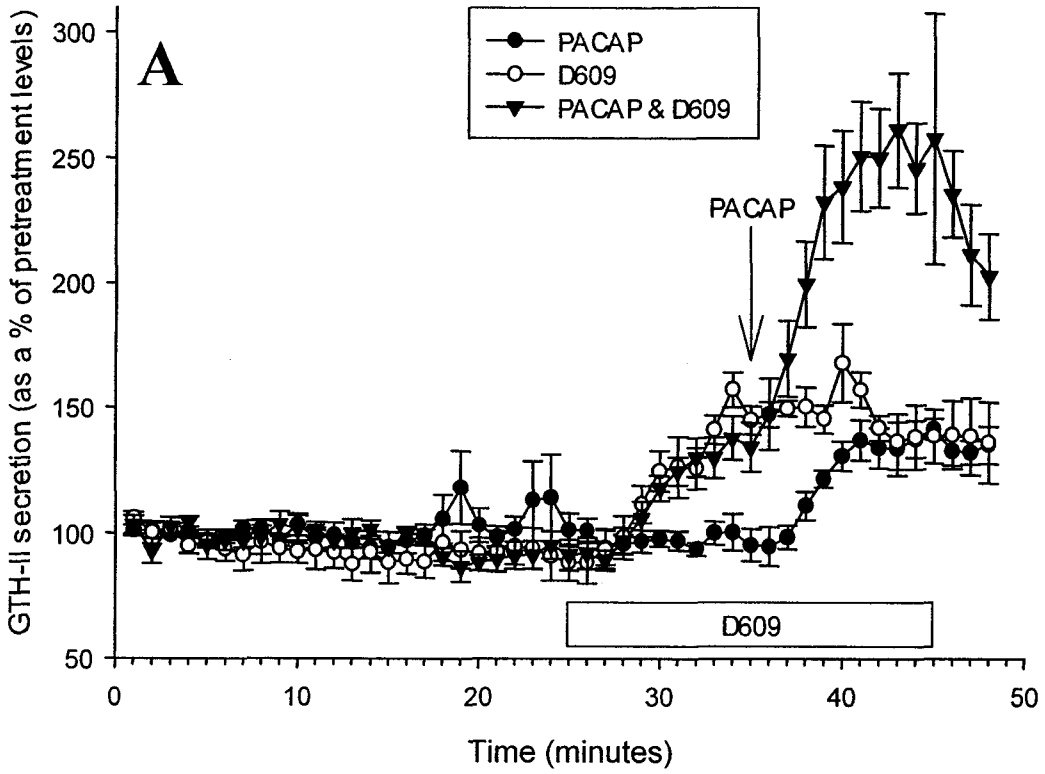



Figure 3.26. Effects of the phosphatidylcholine-specific PLC inhibitor D609 on PACAP-stimulated GTH-II secretion. **A**. Temporal characteristics of the GTH-II response to PACAP (10 nM), either alone or in the presence of D609 (200 μM). Arrow indicates the estimated commencement of hormone response to the 5-min PACAP pulse and the open bar indicates the duration of exposure to D609. Pooled results (mean ± SEM; n=6 columns each) from 3 replicate experiments performed in July are presented. Average pre-treatment GTH-II values for this set of experiments are 3.1 ± 0.1 ng/ml. **B**. Quantified GTH-II responses are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only group. A number sign indicates a significant difference ($p < 0.05$) from the D609 only treatment group.



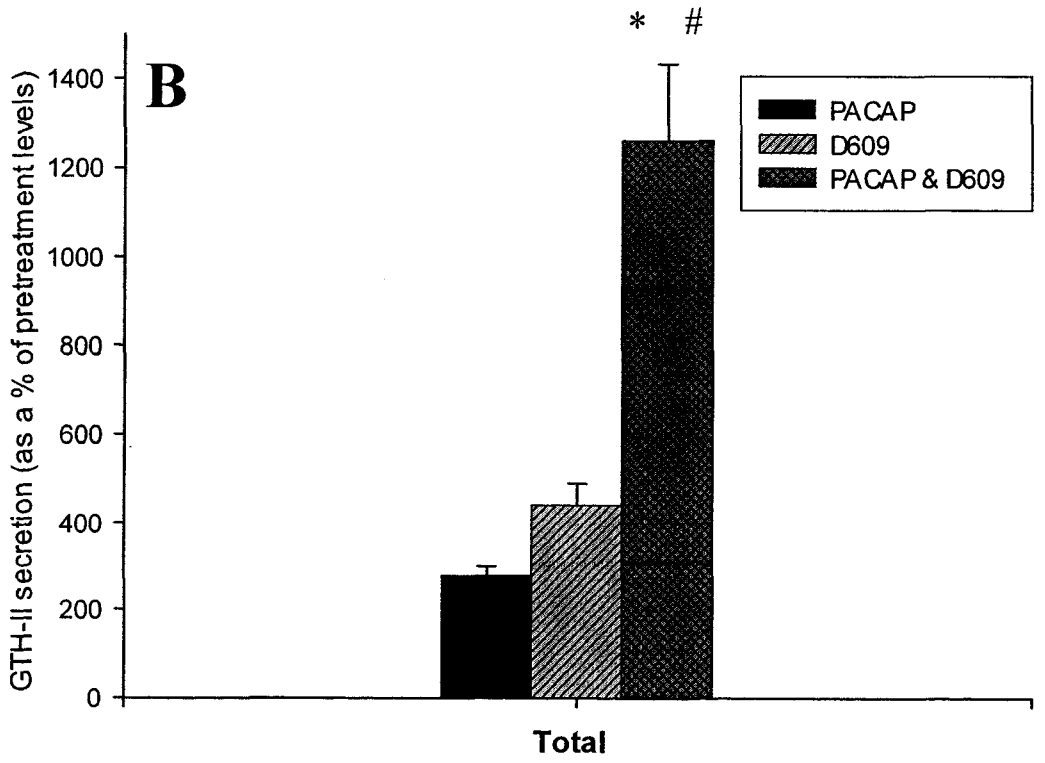
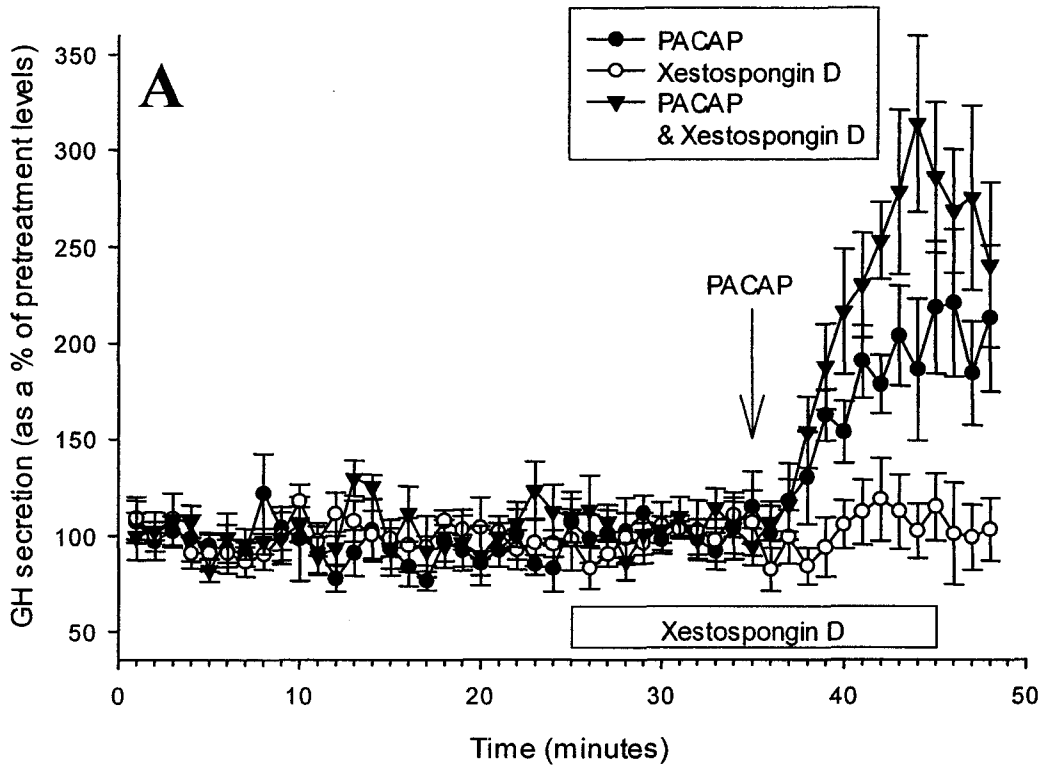



Figure 3.27. Effects of Xestospongin D on PACAP-induced GH release. **A.** Temporal characteristics of the GH response to PACAP (10 nM), either alone or in the presence of Xestospongin D (1 μM). Arrow indicates the estimated commencement of hormone response to the 5-min PACAP pulse and the open bar indicates the duration of exposure to Xestospongin D. Pooled results (mean ± SEM; n=6 columns each) from 3 replicate experiments performed in July are presented. Average pre-treatment GH values for this set of experiments are 21.0 ± 0.3 ng/ml. **B.** Quantified GH responses are presented. The star indicates a significant difference ($p < 0.05$) from the PACAP group. The number sign indicates a significant difference ($p < 0.05$) from the xestospongin D only treatment group.



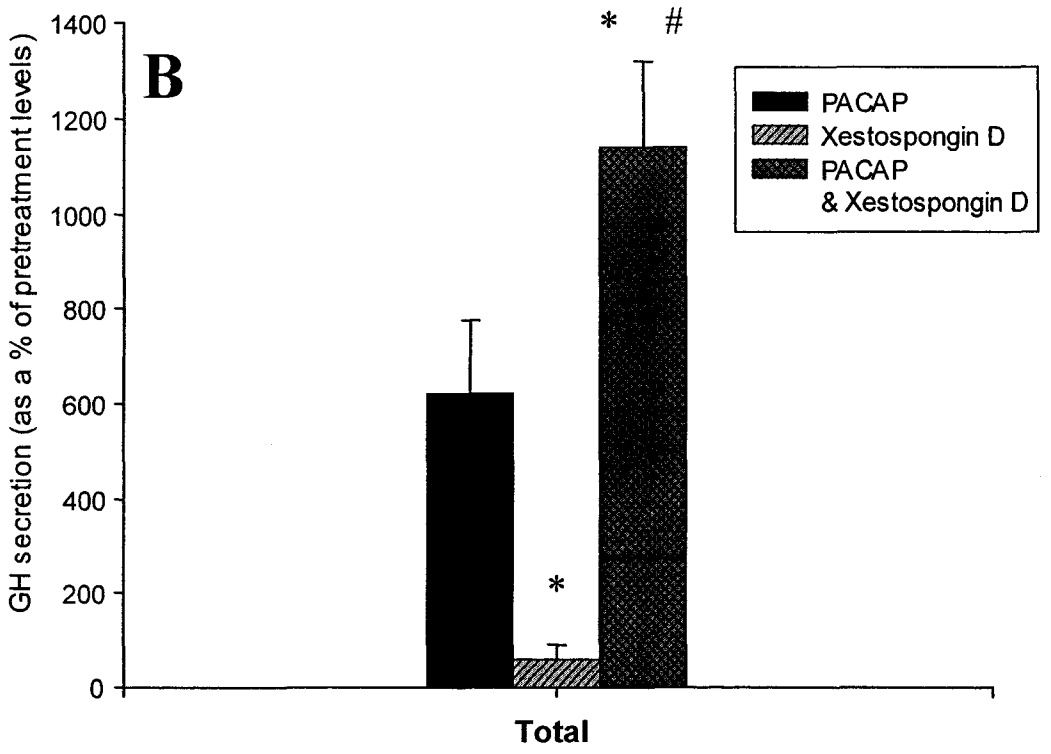
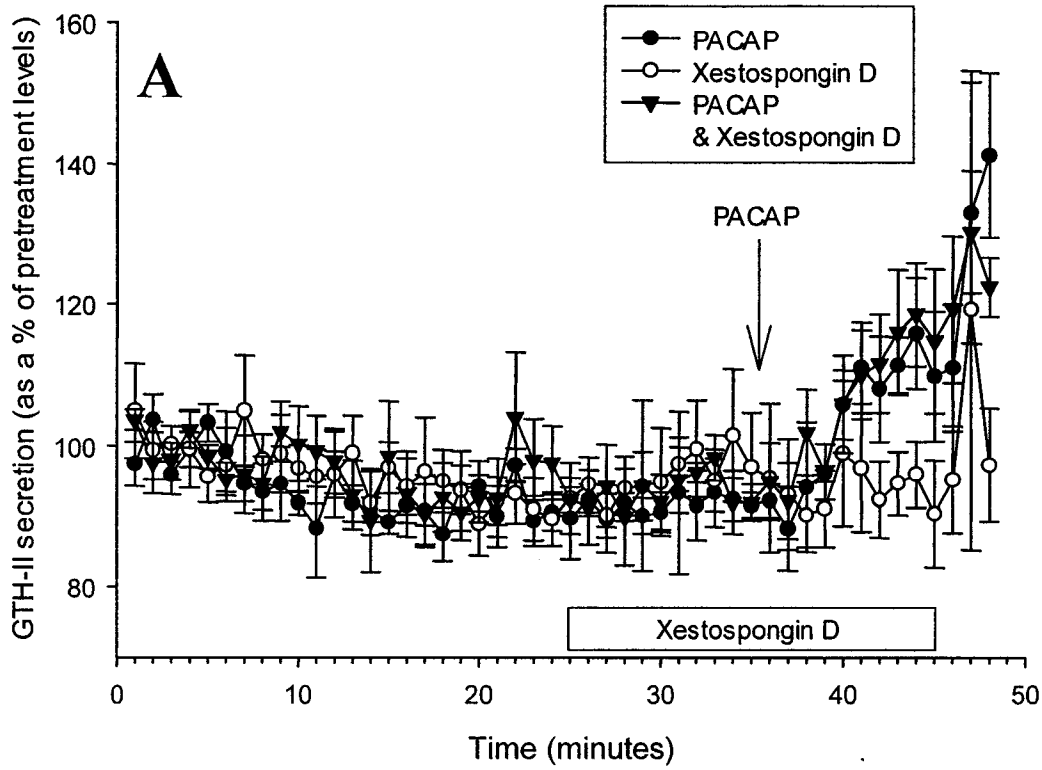
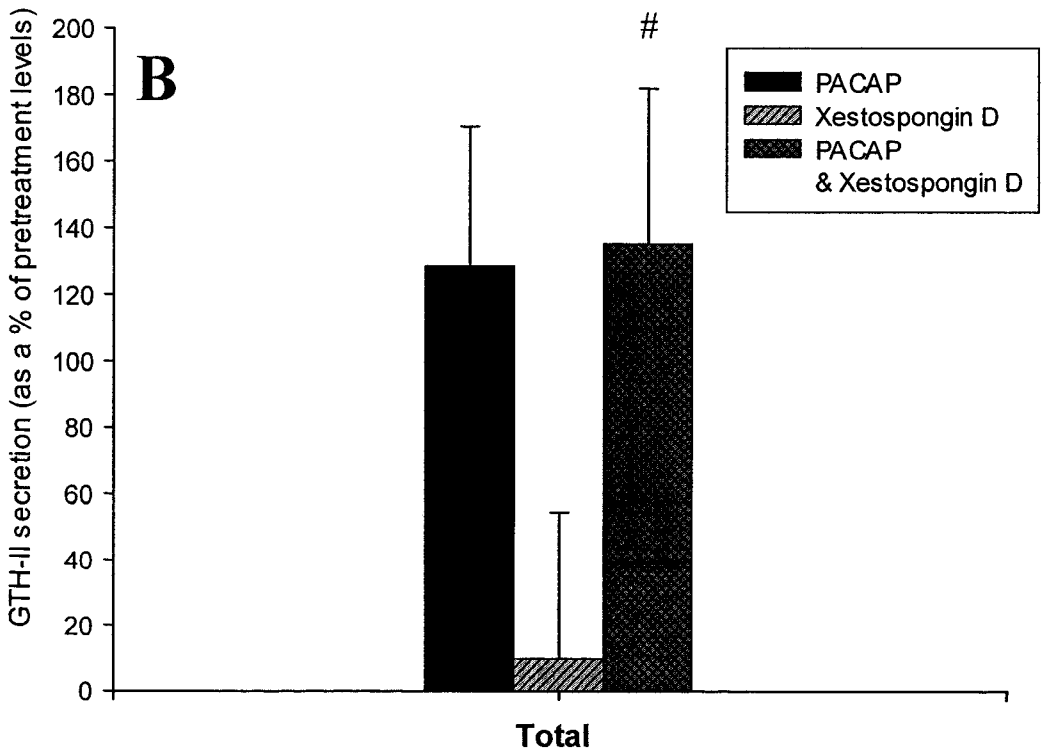



Figure 3.28. Effects of Xestospongin D on PACAP-stimulated GTH-II secretion. **A**. Temporal characteristics of the GTH-II response to PACAP (10 nM) alone or in the presence of Xestospongin D (1 μM). Arrow indicates the estimated commencement of hormone response to the 5-min PACAP pulse and the open bar indicates the duration of exposure to Xestospongin D. Pooled results (mean ± SEM; n=6 columns each) from 3 replicate experiments performed in July are presented. Average pre-treatment GTH-II values for this set of experiments are 2.6 ± 0.1 ng/ml. **B**. Quantified GTH-II responses are presented. The star indicates a significant difference ($p < 0.05$) from PACAP only group. The number sign indicates a significant difference ($p < 0.05$) from the xestospongin D only treatment group.



# 4 Discussion

The intracellular signalling mechanisms to several of the important neuroendocrine regulators of the goldfish pituitary somatotropes and gonadotropes have been examined in a fair amount of detail with the exception of PACAP. In this study, the signal transduction mechanisms mediating PACAP-induced GH and GTH-II release have been shown to involve common, as well as distinct, intracellular $Ca^{2+}$ stores (Tables 4.1 & 4.2). The role of PLC/$IP_3$ in these signalling cascades, which to now has been indeterminate and controversial, has also been investigated. Novel PLC-dependent signalling elements and effects in the regulation and modulation of PACAP action are indicated by results from this study. Also revealed, is a possible seasonal component involving ryanodine-sensitive $Ca^{2+}$ stores, as well as a seasonality in the hormone release responsiveness to PACAP. This study is the first of its kind to both examine in detail and to compare the differences in signalling mechanisms between GH and GTH-II release stimulated by PACAP. Several questions regarding this topic have been answered and the doors have been opened to many more.

*4.1 Variation in hormonal response to PACAP*

The shape of hormonal response to PACAP was generally consistent, often following a "peak and plateau" shape which has been demonstrated in previous studies (Chang et al., 2003). Although the magnitude of the GH and GTH-II responses to PACAP was variable, at no time was it unable to generate a hormonal response in experiments of this study. One possible explanation for the variations is that goldfish are seasonal spawners. Because of this, the basal and

stimulated responses of GH (Chang and Habibi, 2002) and GTH-II (Johnson and Chang, 2002) are known to fluctuate throughout the year. Similarly, the variation in hormone release observed in response to PACAP may reflect seasonal differences in the ability of PACAP to induce secretion in goldfish somatotropes and gonadotropes. In general, higher GTH-II responses to PACAP were observed in experiments using cells prepared from fish during final gonadal maturation and spawning periods (Feb through April). GH responses were greatest in experiments with cells prepared from fish during final gonadal maturation and spawning periods, as well as into early summer when somatic growth occurs (Feb through June/July). The seasonal changes in GH and GTH-II responsiveness to PACAP in perifusion observed in the present study are similar to those observed in static incubation studies reported earlier (Wirachowsky et al., 2000; Chang et al., 2001). Since GH is known to potentiate GTH-II-induced steroidogenic response in gonads (Trudeau, 1997), the co-ordinated increase in GH and GTH-II responsiveness at times of final gonadal maturation and spawning may be extremely relevant. How these seasonal differences in GH and GTH-II release responsiveness to PACAP are manifested are not known and would be an interesting topic for future studies; however, sex steroid influences likely play an important role. For example, estradiol has previously been demonstrated to greatly enhance the GH release response to sGnRH (Trudeau et al., 1992; Zou et al., 1997) and gonadal steroids alters dopamine-induced GH release (Wong et al., 1993). Likewise, testosterone affects the GTH-II responses to sGnRH and cGnRH-II (Lo and Chang, 1998). Although speculative at present,

the observed seasonal differences in GH and GTH-II responsiveness to PACAP suggest that like GnRH and dopamine, PACAP is an important neuroendocrine regulator of growth and reproduction. In support of this hypothesis, PACAP and its downstream AC/cAMP signalling elements have also been shown to enhance GnRH stimulation of GTH-II secretion (Chang et al., 2001).

*4.2 Caffeine-sensitive $Ca^{2+}$ stores*

In many systems, caffeine has been shown to participate in $Ca^{2+}$ release within the cell by actions on RyR's to cause $Ca^{2+}$-induced $Ca^{2+}$ release (Clapham, 1995); however, in goldfish, caffeine-stimulated release of GH and GTH-II is not blocked by ryanodine, indicating it acts on a separate $Ca^{2+}$ store to modulate secretion of these hormones (Wong et al., 2001; Johnson and Chang, 2000; Johnson et al., 2002). Prolonged application of caffeine is expected to keep caffeine-sensitive $Ca^{2+}$ channels in the open configuration, allowing the $Ca^{2+}$ in this store to flow down the concentration gradient into the cytosol, thereby emptying this $Ca^{2+}$ pool and rendering it unavailable to signal transduction mechanisms utilizing it. The observation that caffeine pre-treatment abolishes PACAP-elicited GH and GTH-II release, but not the ionomycin-induced hormone responses, strongly suggests that caffeine-sensitive intracellular $Ca^{2+}$ stores participate in mediating PACAP stimulation of GH and GTH-II secretion. Thus as with sGnRH- and cGnRH-II-stimulated GH release (Johnson and Chang, 2000), and sGnRH-induced GTH-II secretion (Johnson et al., 2000), PACAP appears to be a neuroendocrine factor that requires an intact caffeine-sensitive intracellular $Ca^{2+}$ store to elicit its effects in goldfish somatotropes and gonadotropes. The

mechanism(s) by which PACAP activates the caffeine-sensitive $Ca^{2+}$ store is not known but likely involves cAMP. In human chromaffin cells, PACAP mobilizes $Ca^{2+}$ from caffeine-sensitive stores via cAMP (Payet et al., 2003). Interestingly, caffeine is also a known phosphodiesterase inhibitor (Ukena et al., 1995). However, this action of caffeine would be expected to result in an increase in cAMP and thus potentiate the actions of PACAP on GH and GTH-II release, which have been shown to be dependent on cAMP (Wong et al., 1998; Wirachowsky et al., 2000; Chang et al., 2001). Since this is definitely not the case, the results with caffeine pre-treatment are more consistent with caffeine's actions on depletion of intracellular $Ca^{2+}$ stores rather than its activities on phosphodiesterase.

*4.3 Ryanodine-sensitive $Ca^{2+}$ stores*

Another important source of intracellular $Ca^{2+}$ in intracellular signalling is the ryanodine-sensitive $Ca^{2+}$ stores. Previous studies have demonstrated ryanodine treatment reduced PACAP-stimulated $Ca^{2+}$ signals in bovine and human adrenal chromaffin cells (Tanaka et al., 1998; Payet et al., 2003); thus, the dependence of PACAP action on ryanodine-sensitive stores is not without precedent and is a possibility that needs to be investigated. Within cells, $Ca^{2+}$ from ryanodine-sensitive stores can be released by cADPR (Meszaros et al., 1993; Sitsapesan et al., 1995; Lee, 1998;). In cell culture systems, micromolar concentrations of ryanodine and dantrolene block the release of $Ca^{2+}$ from these stores by binding the RyR channel presumably holding it in the closed position (Sitsapesan et al., 1995; Parness and Palnitkar, 1995); whereas 8-Br-cADPR

binds and blocks the receptor site for cADPR but does not initiate $Ca^{2+}$ release (Walseth and Lee, 1993; Iino et al., 1997). The inability of the above 3 RyR inhibitors to adversely affect GH secretion in response to PACAP indicate that ryanodine-sensitive $Ca^{2+}$ pools are not required for PACAP actions on the release of this hormone. However, these observations were derived from experiments which only covered a period from December through August and the possibility remains that these $Ca^{2+}$ stores may be seasonally responsive and active during September to November. This is a possibility that cannot be ignored given that use of these stores by cGnRH-II in stimulating GH release in goldfish is highly seasonal, being demonstrable only at times of gonadal regression (Johnson and Chang, 2002).

In the present study, ryanodine, dantrolene and 8-Br-cADPR treatments did not affect the peak phase of the GTH-II response to PACAP, suggesting that the immediate release of this hormone to PACAP stimulation is not dependent on RyR. In contrast, for the plateau phase (i.e., longer responses), both ryanodine and 8-Br-cADPR had no effect on GTH-II secretion stimulated by PACAP but dantrolene attenuated the response. Differences between signalling mechanisms mediating acute vs. prolonged LH responses to GnRH in rats are known (Chang and Jobin, 1994). Similar differences also likely exist in GTH-II release in goldfish since testosterone differentially modulates the GTH-II peak and plateau release phases in response to GnRH (Lo and Chang, 1998). So it is possible that RyR's are involved in mediating the prolonged, but not the acute, GTH-II responses to PACAP. If this is indeed the case, then why would the ability to attenuate

prolonged GTH-II responses to PACAP differ between the 3 RyR inhibitors? There are 2 possible, non-mutually exclusive explanations. First, the differences between the abilities of the three RyR inhibitors to affect the prolonged phase of GTH-II release may be due to differences in their abilities to inhibit certain isoforms of the RyR. For example, dantrolene has been shown to more selectively inhibit the RyR1 and RyR3 isoforms, but not RyR2 (Zhao et al., 2001). At present, the form(s) of RyR involved in mediating the exocytosis responses in goldfish gonadotropes are unknown. Second, the answer may involve the seasonal effects explained above. As with GH, sGnRH-induced secretion of GTH-II from goldfish pituitary cells is only sensitive to the inhibitory influence of ryanodine treatment during times of year when the gonads are regressed (Johnson and Chang, 2002). Since experiments with dantrolene were performed in the months of February and April, whereas those with 8-Br-cADPR and ryanodine were performed in July and August, and in December and January, respectively, the presence of a seasonal component in the involvement of RyRs in mediating PACAP actions on gonadotropes is still possible. Further experiments are required to dissect out the two possibilities.

*4.4 SERCA-refilled $Ca^{2+}$ stores*

Previous experiments with GnRH have revealed that goldfish pituitary cells have at least two pharmacologically distinct SERCA-refilled $Ca^{2+}$ stores; those sensitive to Tg and those sensitive to BHQ & CPA (Johnson et al., 2000). Classically, these pumps refill ER stores in which $Ca^{2+}$ can be mobilized via release through ryanodine- and $IP_3$-sensitive channels on its membrane. To

date, five SERCA isoforms have been identified in mammals, all of which are reportedly sensitive to the SERCA inhibitor, thapsigargin (Lytton et al., 1991; Inesi and Sagara, 1992; and reviewed in Martonosi and Pikula, 2003). In this study, PACAP-induced secretion of GH was differentially affected by the two SERCA inhibitors used. Tg potentiated the response while CPA had no effect. The other SERCA inhibitor, BHQ, has been shown to attenuate PACAP-induced GH secretion in previous studies (Chang et al., 2003). Differences in the ability of Tg, BHQ and CPA to modulate the GH responses are unexpected but not totally surprising. Multiple SERCA isoforms exist in vertebrate cells (reviewed in Misquitta et al., 1999; Wuytack et al., 2002) and the different SERCA isoforms have varying sensitivity to SERCA inhibitors. In particular, CPA does not broadly inhibit all SERCA's, but only specific isoforms, especially those found on the sarcoplasmic reticulum (Seidler et al., 1989), whereas Tg is reported to act on a broad range of pumps (Lytton et al., 1991). Taken together, results in the present study suggest that multiple isoforms of SERCA are present in goldfish somatotropes. While their identity and location inside goldfish somatotropes are unknown, the different SERCA's play distinct roles in regulating PACAP action. BHQ-sensitive SERCA-refilled $Ca^{2+}$ store(s) likely mediate PACAP-stimulated GH release while Tg-sensitive SERCA's modulate PACAP action on GH secretion. We hypothesize that when active, the Tg-sensitive SERCA's serve to limit the rise in $[Ca^{2+}]_c$ that is known to occur in goldfish somatotropes during PACAP stimulation and thereby attenuate the exocytosis response. Thus, inhibition of this pump enhances the GH release response to PACAP.

Alternatively, blockade of this Tg-sensitive SERCA may allow the $Ca^{2+}$ leaked passively from other stores to more easily accumulate in the PACAP-sensitive store(s) thereby enhancing the $Ca^{2+}$ release (and subsequent GH secretion) in response to PACAP receptor activation.

Since GTH-II secretion induced by PACAP is potentiated by both Tg and CPA, and GnRH-stimulated GTH-II secretion is attenuated by BHQ and CPA, it is clear that Tg-, BHQ- and/or CPA-sensitive SERCA isoforms are present in goldfish gonadotropes. Results from this study further indicate that PACAP-stimulated GTH-II secretion is modulated by Tg- and/or CPA-sensitive SERCA's in manners similar to those described for Tg-mediated enhancement of GH responses to this peptide. The enhancement of PACAP action by SERCA inhibitor treatment has also been demonstrated in other systems. For example, Taupenot et al. (1999), showed that pre-treatment of rat pheochromocytoma cells with Tg increased catecholamine secretion in response to PACAP.

*4.5 Mitochondrial $Ca^{2+}$ buffering*

Results from the present study reinforce the idea that mitochondria play a role in the regulation of hormone secretion in goldfish somatotropes and gonadotropes. Disruption of mitochondrial $Ca^{2+}$ buffering altered basal GH and GTH-II release. The less specific inhibitor CCCP produced a general increase in basal GH and GTH-II release and the more specific $Ca^{2+}$ uniport inhibitor Ru360 also caused an elevation in GTH-II secretion immediately upon application. Likewise, CCCP and ruthenium red (RR) caused increased basal GH release in previous studies (Johnson, 2000). Additionally, application of CCCP and RR

have also lead to similarly timed increases in $[Ca^{2+}]_c$ in goldfish somatotropes (Johnson, 2000). Taken together, these results indicate that mitochondrial $Ca^{2+}$ buffering and/or mitochondrial $Ca^{2+}$ as an intracellular $Ca^{2+}$ source participate in the regulation of basal GH and GTH-II secretion.

The role of mitochondrial $Ca^{2+}$ and $Ca^{2+}$-buffering in stimulated hormone release appears more controversial in the case of GH secretion evoked by PACAP. While CCCP negatively affected the peak and total GH responses to PACAP, Ru360 altered neither the peak, plateau, nor total secretion. The fact that the more specific inhibitor of the $Ca^{2+}$ uniporter, Ru360, did not affect the secretion of GH strongly implies that mitochondrial $Ca^{2+}$ does not participate in mediating PACAP-induced GH release. In such a case, what would the results with CCCP indicate? One possibility is that other non-$Ca^{2+}$-related mitochondrial function(s) required by the PACAP signalling cascade to stimulate GH secretion were affected by CCCP treatment. CCCP uncouples the $H^+$ gradient (Hehl et al., 1996; Peng, 1998; Tang and Zucker, 1997), and certain exocytotic events require ATP/GTP. The recruitment (Holz et al., 1989) and priming (Banerjee et al., 1996) of secretory vesicles have been shown to operate though an ATPase termed, N-ethylmaleimide-sensitive factor, whose activity is thought to drive the priming and fusion of the synaptic vesicle with the presynaptic plasma membrane, whereas the actual fusion of the vesicle is said to operate through a family of small GTPases termed Rab proteins (reviewed in Lin and Scheller, 2000; Zerial and McBride, 2001; Burgoyne and Morgan, 2003). Perhaps, mitochondrial energy production is required for vesicle recruitment and priming during PACAP

stimulation of GH release. Since CCCP only caused a small reduction in hormonal response, it is likely that the role mitochondria plays in the PACAP signalling cascade leading to GH release is not a crucial one or is only specific to certain aspects of the hormone release. On the other hand, PACAP-induced GTH-II secretion was not reduced by treatments that inhibit mitochondrial functions. It would seem that mitochondrial $Ca^{2+}$ and the mitochondria's energy producing role described for GH are not important factors in the release of GTH-II in response to PACAP.

Interestingly, although there was no significant difference between the quantified GTH-II responses to PACAP alone and PACAP with either CCCP or Ru360, the GTH-II release in columns treated with either CCCP or Ru360 alone was decreasing over the same time period. One might argue that the "real GTH-II responses" to PACAP in the presence of CCCP and Ru360 would be greater than that quantified and that mitochondrial $Ca^{2+}$ buffering might negatively modulate PACAP-induced GTH-II secretion under normal circumstances. Regardless of whether this is true or not, results do not support a direct role of mitochondrial $Ca^{2+}$ in mediating PACAP actions in goldfish gonadotropes.

### *4.6 PLC and $IP_3$-sensitive $Ca^{2+}$ stores*

#### *4.6.1 $IP_3$-sensitive $Ca^{2+}$ stores*

Results from the literature suggest that the PLC/$IP_3$ pathway may be an important component in mediating PACAP action. The link between PLC/$IP_3$ and PACAP action has been demonstrated in PACAP-induced release of catecholamine in adrenal medullary chromaffin cells (Isobe et al., 1993),

aldosterone secretion in adrenal cortical cells (Bodart et al., 1997) and intracellular $Ca^{2+}$ release in pancreatic acinar cells (Barnhart et al., 1997). More pertinent to this study on pituitary hormone secretion, PACAP-induced GH secretion and increases in intracellular $Ca^{2+}$ release were blocked by PLC inhibition in mammalian somatotrophs (Martinez-Fuentes et al., 1998a,b). Likewise, PACAP-stimulated elevations in $IP_3$ levels in gonadotrope-derived T3-1 cells (Schomerus et al., 1994) and PRL release were similarly abolished by PLC inhibition (Hammond et al., 1996).

The present results with two $IP_3$ receptor blockers, Xestospongin C and D, indicate that $IP_3$-sensitive $Ca^{2+}$ stores do not directly participate in PACAP stimulation of GH and GTH-II release. This is unlike the situation with sGnRH-stimulated hormone secretion where $IP_3$-sensitive $Ca^{2+}$ stores are important. sGnRH increase $IP_3$ production in goldfish pituitary cells (Chang et al., 1995) and Xestospongin C at doses used in the present study inhibited GH and GTH-II release responses to sGnRH (Johnson et al., 2000; Johnson and Chang, 2002). The observed enhancement of GH response to PACAP in the presence of Xestospongin D is a surprising finding. It is possible that the $IP_3$-sensitive $Ca^{2+}$ stores, though not directly involved in mediating PACAP-induced hormone release in somatotropes, are linked to $Ca^{2+}$ pools that are involved in PACAP action on GH release. Thus, when the $IP_3$ receptors are blocked, this creates a temporary increase in $Ca^{2+}$ availability to the store(s) used by the PACAP signalling cascade for GH secretion, this results in a greater magnitude of $Ca^{2+}$ release from this pool and a larger GH response.

*4.6.2 Phosphatidylinositol-specific PLC*

In view of the evidence for the lack of participation of PKC (Wirachowsky et al., 2000; Chang et al., 2001) and direct involvement of an $IP_3$-sensitive $Ca^{2+}$ store in PACAP stimulation of GH and GTH-II release (see Discussion section 4.6.1 above), results from experiments investigating the involvement of PLC-dependent signalling cascades in mediating GH and GTH-II secretion appear contradictory and unclear.

In the presence of the less specific PI-PLC inhibitor U73122, GH secretion induced by PACAP was not affected. In contrast, in the presence of U73122, PACAP was not able to stimulate GTH-II release above levels observed with U73122 treatment alone. Furthermore, ET-18-$OCH_3$, a more specific phosphatidylinositol-specific PLC inhibitor than U73122 (Powis et al., 1992), attenuated both the GH and GTH-II responses to PACAP. The results with ET-18-$OCH_3$ in particular would suggest that phosphatidylinositol-specific PLC is important in mediating PACAP action, which is opposite to what would be expected given that the two major metabolites of PLC action would be DAG and $IP_3$. However, multiple PKC isoforms are known to be present in goldfish pituitary cells including conventional, novel and atypical forms (Klausen, 2004). Given that 1) inhibitors of atypical PKC are not commonly available, 2) at least one of the PKC inhibitors tested (calphostin C) is unlikely to inhibit atypical PKC very effectively since atypical PKC lacks the phorbol ester/DAG binding site required for calphostin C action (Kobayashi et al., 1989; Newton AC, 2001), and 3) preliminary experiments cited in Wong et al. (2000) hinted at a role of PKC in

mediating the GH-releasing actions of PACAP, the conclusion that all isoforms of PKC do not participate in mediating PACAP-induced GH and GTH-II secretion may need to be revised pending future investigations with isoform specific PKC inhibitors when these become available.

The subsequent actions of PLC activation also need not be restricted to the classical events of PKC activation and mobilization of intracellular $Ca^{2+}$. It is possible that DAG is not causing a functional effect through activation of PKC. In addition to PKC, DAG can bind to several other substances in the cell that may be required for hormone secretion by PACAP (reviewed in Brose and Rosenmund, 2002). These include chimaerins, protein kinase D1 (PKD1), RasGRP, and Munc13; all of these are known to play a role in exocytosis.

Upon binding DAG, chimaerins translocate to phospholipid membranes (Caloca et al., 1997) and activate rac-GTPase, a member of the Rho family of proteins that have been implicated in membrane trafficking (Ridley, 2001). Both effects could possibly play a role in modulation of hormone vesicle binding with the plasma membrane.

PKD1 has been implicated in the control of several kinase signal-transduction pathways, such as activation of the p42 ERK mitogen-activated protein (MAP) kinase and inhibition of the c-Jun N-terminal kinase (JNK) signalling pathway (Van Lint et al., 2002), either one or both of which could play a role in the PACAP signalling cascade. Of particular note, PACAP has been shown to modulate FSH release via the MEK-ERK cascade (Gur et al., 2002). Supporting the notion that PACAP operates through PKD1, JNK was reported to

be inhibited by PACAP administration in neurons in the CA1 region of the rat hippocampus (Shioda et al., 1998; Dohi et al., 2002). Additionally, PKD1 has been shown to play a role in the control of the $Na^+/H^+$ exchanger (NHE; Haworth et al., 1999). NHE has been shown to participate in GnRH-stimulated GH and GTH-II secretion in goldfish (Van Goor et al., 1996; Van Goor et al., 1997) and PACAP has been reported to affect the function of NHE in human enterocyte-like cell lines (Anderson et al., 2003).

RasGRP is an important activator of the Ras/Raf/MEK/ERK signalling pathway (Brose and Rosenmund, 2002). Recent evidence has shown a link between the MEK/ERK pathway and GnRH stimulation of GH and GTH-II subunit mRNA levels in goldfish (Klausen, 2004). In terms of exocytosis, this pathway is also known to mediate interleukin-2 secretion from mouse thymocytes following T-cell receptor activation (Ebinu et al., 2000).

The final non-PKC target of DAG is Munc13, which have been reported to be essential for vesicle priming (Augustin et al., 1999). A recent study on Munc13 has shown that it potentiates insulin secretion in rats by enhancing exocytosis (Sheu et al., 2003).

As it stands now, any one or a combination of these could be targets of the DAG produced by the PACAP signalling cascade leading to GH or GTH-II release.

There is also a possibility that PLC activity itself could modify membrane phospholipid composition through cleavage of phosphatidylinositol 4,5 bisphosphate, thereby causing an effect on phospholipid-dependent signalling

pathways or hormone release (Spector and Yorek, 1985). It has been shown that alteration of membrane fluidity can affect vesicle trafficking (Giocondi et al., 1995), pituitary hormone release (Galfi et al., 2000), and NHE activity (Bookstein et al., 1997). Since modifying membrane composition also affects membrane fluidity and impacts vesicular fusion, this may be one of the reasons for the observed ability of PLC inhibitors to increase basal GH and GTH-II release in many cases when applied on their own (Figures 3.21-26 & 3.28). Additionally, phosphatidylinositol (PI) 3-kinase/PKB is an important lipid signalling cascade, the activity of which is highly dependent on the integrity and/or composition of inositol-containing phospholipids and would thus be sensitive to the effects of PLC. PI 3-kinase has been linked to the control of GH release by insulin-like growth factor-I (Fruchtman et al., 2001) and has been shown to have a role in signalling cascades initiated by PACAP in several cell types (Straub and Sharp, 1996; Nakata et al., 1999; Bhave and Hoffman, 2004).

*4.6.3 Phosphatidylcholine-specific PLC*

Evidence indicate that phosphatidylcholine-specific PLC modulates secretion events. The activity of this enzyme has been linked to inhibition of NE release in rat heart (Schafer et al., 2002), and to enhancement of mucin secretion in guinea pig tracheal epithelial cells (Fischer et al., 1999), cytokine release in alveolar macrophages (Carter et al., 1998), and AVP release in rat hypothalamic cells (Wayte et al., 1997). In this light, it is interesting that D609, a phosphatidylcholine-specific PLC inhibitor, elevates basal GTH-II secretion and further enhances the GH and GTH-II release responses to PACAP (Figures 3.25

& 3.26). The potentiating effect on PACAP-induced GH & GTH-II secretion by phosphatidylcholine-specific PLC inhibition suggests that the products of this PLC have an inhibitory effect on PACAP-induced hormone secretion. Although uncommon, enhancement of secretion responses by D609 (through phosphatidylcholine-specific PLC inhibition) has been previously reported in IL-1-induced secretion of IL-6 from osteoblast-like MC3T3-E1 cells (Kozawa et al., 1997).

To attempt to explain the ability of D609 to enhance hormone release, one needs to look at one of the products of phosphatidylcholine-specific PLC action on phosphatidylcholine, DAG. Although DAG (and PKC activation) has been assigned a stimulatory role in terms of GH and GTH-II release in goldfish based on previous hormone release studies (Chang et al., 1994; Chang et al., 1996b), it is possible that the DAG produced by phosphatidylcholine-specific PLC action differs from those generated by phosphatidylinositol-specific PLC in terms of molecular identity (i.e. variations in the fatty acid sidechains on the molecule) and target protein activation (Section 4.6.2). One of such function may result in inhibitory effects on stimulated GH and GTH-II release. This would account for the observation that blocking PLD, which also produces DAG, increases PACAP-induced hormone release.

Another explanation for the secretion inhibitory effects of phosphatidylcholine-specific PLC may lay in its relationship with sphingomyelin signalling. Sphingomyelin can be enzymatically produced by the transfer of phosphocholine from phosphatidylcholine to ceramide. One possible explanation

for the results seen may be that under normal conditions, phosphatidylcholine-specific PLC operates to degrade phosphatidylcholine and this would decrease the amount of sphingomyelin but increase the amount of ceramide. Interestingly, not only is sphingomyelin an important molecule in the sphingolipid signalling pathway (reviewed in Hannun et al., 2001), ceramide is also important in its own right particularly in terms of pituitary hormone release. In this regard, it is interesting to note that GH autofeedback inhibition of GH release is accompanied by an increase in ceramide, suggesting that ceramide plays an inhibitory role in secretion at least in rat somatotropes (Liu et al., 2002). Thus, the inhibition of phosphatidylcholine-specific PLC would produce a temporary increase in the availability of phosphatidylcholine which would allow greater conversion of the inhibitory ceramide to sphingomyelin. This would enhance PACAP-stimulated GH and GTH-II secretion. Recently, PACAP has also been shown to inhibit ceramide-induced apoptosis (Vaudry et al., 2003), providing evidence that PACAP signalling can interact with ceramide actions.

Although the same potentiation effect by D609 is observed with both GH and GTH-II release in response to PACAP, a major difference exist in terms of basal hormone secretion. D609 on its own did not affect basal GH secretion but a large rise in basal GTH-II was evident upon application of this drug. This suggests that in gonadotropes, the phosphatidylcholine-specific PLC signalling is linked to PACAP-stimulated GTH-II release, as well as basal hormone secretion; whereas in somatotropes, this enzyme system is not linked to the control of basal secretion. That basal and stimulated release can be controlled separately has

been demonstrated for GH and GTH-II release by GnRH in goldfish (Chang et al., 2000).

*4.7 Future experiments*

This study has pharmacologically established roles for various known $Ca^{2+}$ stores, $Ca^{2+}$ signalling components and PLC components in PACAP-induced GH and GTH-II signalling. Although much of the evidence is compelling, knowledge as to what is actually occurring to intracellular $Ca^{2+}$ during many of these manipulations is lacking. $Ca^{2+}$-imaging studies on identified goldfish somatotropes and gonadotropes using a $Ca^{2+}$-sensitive dye such as Fura2-AM (as in Wirachowsky et al., 2000; Chang et al., 2001) would be needed to confirm the assumptions on changes in $Ca^{2+}$ homeostasis involved in many of the pharmacological manipulations, as well as to provide further insight into the relationships between changes in $[Ca^{2+}]_c$ and secretion responses. Although precautions to minimize the inactivation by oxidation of Ru360 were made, confirmation of the "negative results" obtained with this mitochondrial inhibitor would be prudent. In this regard, the drug RR, which has been shown to affect mitochondrial $Ca^{2+}$ uniport in other systems (Moore, 1971; Ying et al., 1991; Drummond and Fay, 1996) and to affect GH release in goldfish (Johnson, 2000), may be useful. In view of the interesting results with PLC inhibitors and the implication of the multiplicity of PKC isoforms, biochemical analysis of phospholipid turnover and phospholipid metabolites during PACAP challenge and future examination of the role of specific isoforms of PKC in regulating GH and GTH-II secretion using isoform selective inhibitors would be useful. Since

inhibitors of chimaerins (N-Benzyladriamycin-14-valerate; Roaten et al., 2001) and PKD1 (resveratrol; Haworth and Avkiran, 2001) are known, it would be interesting to determine if any of these signalling molecules are involved in the PACAP-induced GH or GTH-II secretion pathway. Given the seasonal variations in GH and GTH-II responses to different neuroendocrine regulators shown here and in other studies, future experiments on signal transduction mechanisms should cover all parts of the seasonal reproductive cycle where possible.

*4.8 Summary*

Based on this information, as well as information available in the literature, working models of the signal transduction mechanisms, and especially the involvement of intracellular $Ca^{2+}$, in PACAP actions on goldfish somatotropes and gonadotropes are proposed (Figures 4.1 & 4.2).

PACAP action on GH release is mediated through activation of AC leading to increased cAMP production and PKA activity (Wirachowsky et al., 2000). Through cAMP/PKA activity, PACAP is thought to increase extracellular $Ca^{2+}$ entry via VSCC (Wirachowsky et al., 2000; Yunker and Chang, 2004). Increases in $[Ca^{2+}]_c$ subsequently stimulate GH release via CaM-dependent mechanisms. PACAP stimulation of GH secretion also requires mobilization of intracellular $Ca^{2+}$. This is in part achieved through $Ca^{2+}$ release from a caffeine-sensitive and a BHQ-sensitive SERCA-refilled $Ca^{2+}$ store, but not a ryanodine-sensitive pool. Whether the caffeine-sensitive and BHQ-sensitive stores are the same store, or are distinct, is not known; however, they are likely to be functionally linked. The $Ca^{2+}$ signal generated by release from store(s) used by the PACAP signalling

cascade is buffered by a Tg-sensitive SERCA that is not affected by CPA. Mitochondrial $Ca^{2+}$ buffering does not seem to be an important element in PACAP-induced GH release but mitochondrial participation may still be required for the energy-dependent vesicle trafficking/fusion processes. An $IP_3$-sensitive $Ca^{2+}$ store may be linked to the PACAP-sensitive $Ca^{2+}$ pool such that inhibition of $IP_3$ receptor $Ca^{2+}$ channels leads to increases in availability of $Ca^{2+}$ in the PACAP-releasable pool. A non-traditional PLC pathway is also required for PACAP-induced GH release. DAG produced by phosphatidylinositol-specific-PLC may activate an unidentified isoform of PKC or other signalling components such as chimaerins, PKD1, RasGRP, and/or Munc13 to elicit hormone secretion. Phosphatidylcholine-specific PLC actions on phosphatidylcholine either directly (via DAG production) or indirectly (via its effects on ceramide levels) negatively modulate PACAP-induced GH release (Figure 4.1).

PACAP-stimulated GTH-II release also involves activation of AC, increased cAMP production and PKA activity (Wong et al., 2000; Chang et al., 2001). PACAP action on GTH-II release does not depend on extracellular $Ca^{2+}$ entry but instead requires intracellular $Ca^{2+}$ stores. PACAP mobilizes intracellular $Ca^{2+}$ from a caffeine-sensitive $Ca^{2+}$ store and may also use a seasonally active ryanodine/dantrolene-sensitive $Ca^{2+}$ store. PACAP-induced increases in $[Ca^{2+}]_c$ is buffered by Tg- and CPA-sensitive SERCA but mitochondrial $Ca^{2+}$ buffering and/or control of vesicle trafficking by energy-dependent processes do not appear to be important to PACAP-stimulated GTH-II release. $IP_3$-induced $Ca^{2+}$ release is not important for PACAP-evoked secretion of GTH-II, but as in the

case of GH response, non-traditional PLC signalling may be involved (Figure 4.2).

This is the first study to look comprehensively at the intracellular $Ca^{2+}$ signalling mechanisms mediating and regulating the GH and GTH-II secretion responses to PACAP in goldfish. Results from the present study have illustrated some of the complexities, differences and similarities in the intracellular signal transduction cascades mediating PACAP stimulation of GH and GTH-II release. The two working models described above reveal the presence of similarity and differences between the transduction mechanisms mediating the hormone-secreting actions of the same peptide (PACAP) on two pituitary cell-types.

**Table 4.1.** Present knowledge of intracellular $Ca^{2+}$ store usage by sGnRH, cGnRH-II, DA, and PACAP in evoking GH secretion in goldfish somatotropes.

| Intracellular $Ca^{2+}$ stores & $Ca^{2+}$ modulating mechanisms | Stimulatory Regulator of GH secretion | | | |
|---|---|---|---|---|
| | sGnRH | cGnRH-II | dopamine | PACAP |
| | | | | |
| General (TMB-8-sensitive stores) | Yes | Yes | Yes | Yes |
| | | | | |
| $IP_3$ receptor | | | | |
| Xestospongin C-sensitive stores | Yes | No | ND | Mod |
| Xestospongin D-sensitive stores | ND | ND | ND | Mod |
| | | | | |
| PLC | | | | |
| U73122-sensitive PLC | ND | ND | ND | No |
| ET-18-OCH3-sensitive PLC | ND | ND | ND | Yes |
| D609-sensitive PLC | ND | ND | ND | Mod |
| | | | | |
| SERCA | | | | |
| Tg-sensitive | No | No | Mod | Mod |
| BHQ-sensitive | No | No | Yes | Yes |
| CPA-sensitive | No | No | ND | No |
| | | | | |
| Ryanodine Receptor | | | | |
| Ryanodine-sensitive stores | No | Yes | No | No |
| Dantrolene-sensitive stores | No | No | ND | No |
| 8-Br-cADPR-sensitive stores | ND | ND | ND | No |
| | | | | |
| Caffeine-sensitive stores | Yes | Yes | No | Yes |
| | | | | |
| Mitochondria | | | | |
| CCCP-sensitive stores | ND | No | ND | Yes |
| Ruthenium red-sensitive stores | Yes | No | ND | ND |
| Ru360-sensitive stores | ND | ND | ND | No |

Yes = involved in mediating action
No = not involved in mediating action
ND = not determined
Mod = not directly involved but modulates action

**Table 4.2.** Present knowledge of intracellular $Ca^{2+}$ store usage by sGnRH, cGnRH-II, DA, and PACAP in evoking GTH-II secretion in goldfish gonadotropes.

| Intracellular $Ca^{2+}$ stores & $Ca^{2+}$ modulating mechanisms | Stimulatory Regulator of GTH-II secretion | | |
|---|---|---|---|
| | sGnRH | cGnRH-II | PACAP |
| | | | |
| General (TMB-8-sensitive stores) | Yes | Yes | Yes |
| | | | |
| $IP_3$ receptor | | | |
| Xestospongin C-sensitive stores | Yes | No | No |
| Xestospongin D-sensitive stores | ND | ND | No |
| | | | |
| PLC | | | |
| U73122-sensitive PLC | ND | ND | Yes |
| ET-18-OCH3-sensitive PLC | ND | ND | Yes |
| D609-sensitive PLC | ND | ND | Mod |
| | | | |
| SERCA | | | |
| Tg-sensitive | No | No | Mod |
| BHQ-sensitive | Yes | Yes | ND |
| CPA-sensitive | Yes | Yes | Mod |
| | | | |
| Ryanodine Receptor | | | |
| Ryanodine-sensitive stores | Yes | Yes | No |
| Dantrolene-sensitive stores | No | No | Yes |
| 8-Br-cADPR-sensitive stores | ND | ND | No |
| | | | |
| Caffeine-sensitive stores | Yes | No | Yes |
| | | | |
| Mitochondria | | | |
| CCCP-sensitive stores | ND | ND | No |
| Ruthenium red-sensitive stores | ND | ND | ND |
| Ru360-sensitive stores | ND | ND | No |

Yes = involved in mediating action
No = not involved in mediating action
ND = not determined
Mod = not directly involved but modulates

Figure 4.1. A schematic of the known and proposed intracellular mechanisms used by PACAP to elicit GH secretion. Solid red lines and arrows indicate known and proposed signalling elements following PACAP activation of $PAC_1$ receptors. Solid green only lines indicate a PACAP-involved $Ca^{2+}$ signalling pathway. Green & pink coloured lines indicate a $Ca^{2+}$ refilling route. DAGa and DAGb represent proposed distinct pools of DAG formed by phosphatidylinositol-specific (PI)-PLC and phosphatidylcholine-specific (PCh)-PLC, respectively. Dark green lines indicate entry of extracellular $Ca^{2+}$ into the cell. Striped blue arrows indicate an unknown signalling mechanism leading to GH secretion. Although not part of the PACAP signalling pathway, release of $Ca^{2+}$ from $IP_3$-sensitive stores and stores refilled by Tg have also been shown to lead to GH release. Hatched arrows indicate $Ca^{2+}$ release from stores for other cellular processes. Whether the indicated pharmacologically identified $Ca^{2+}$ pools adequately represents all the TMB-8-sensitive pools is not known, thus a separate (though functionally linked) TMB-8-sensitive pool is depicted.

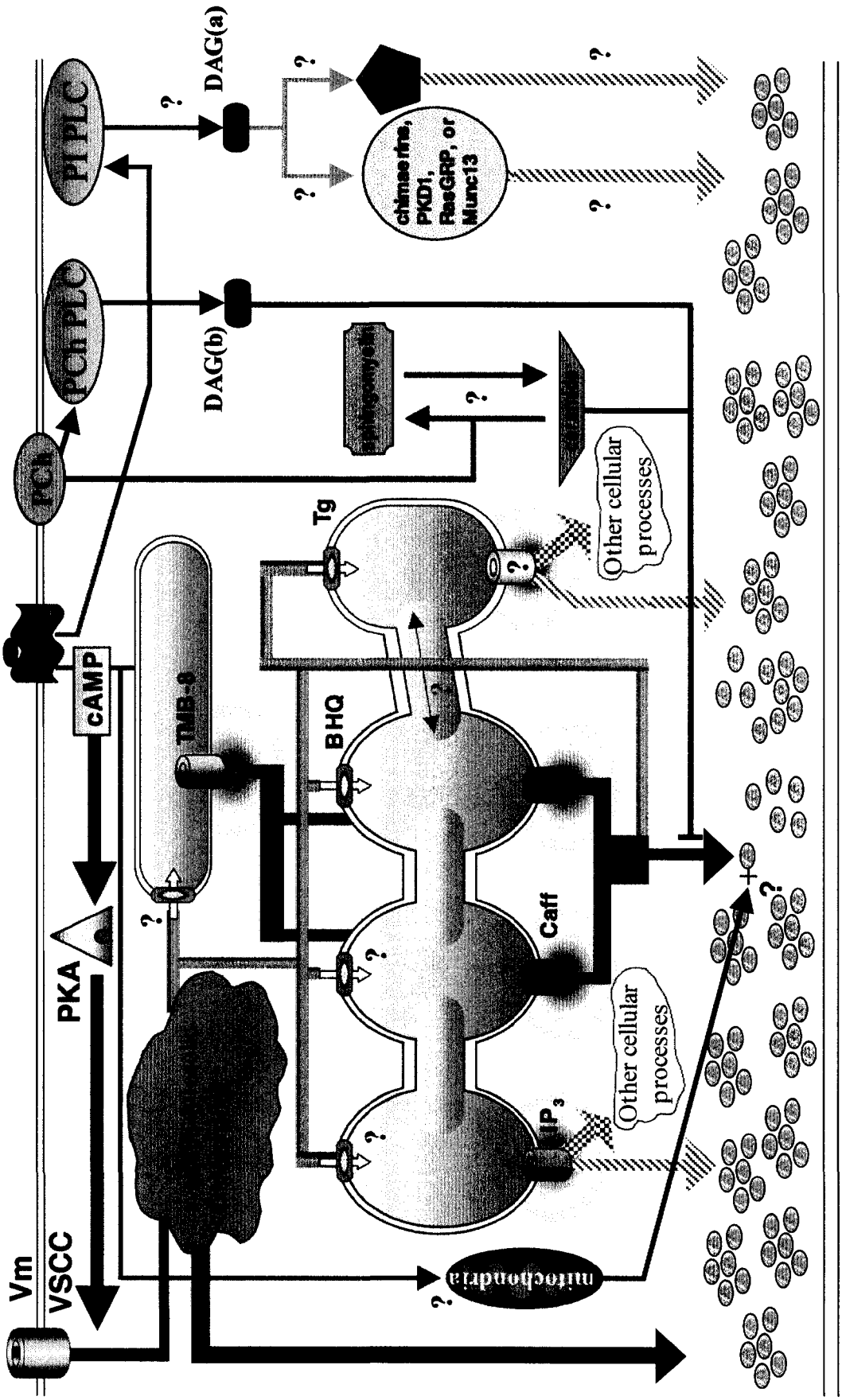
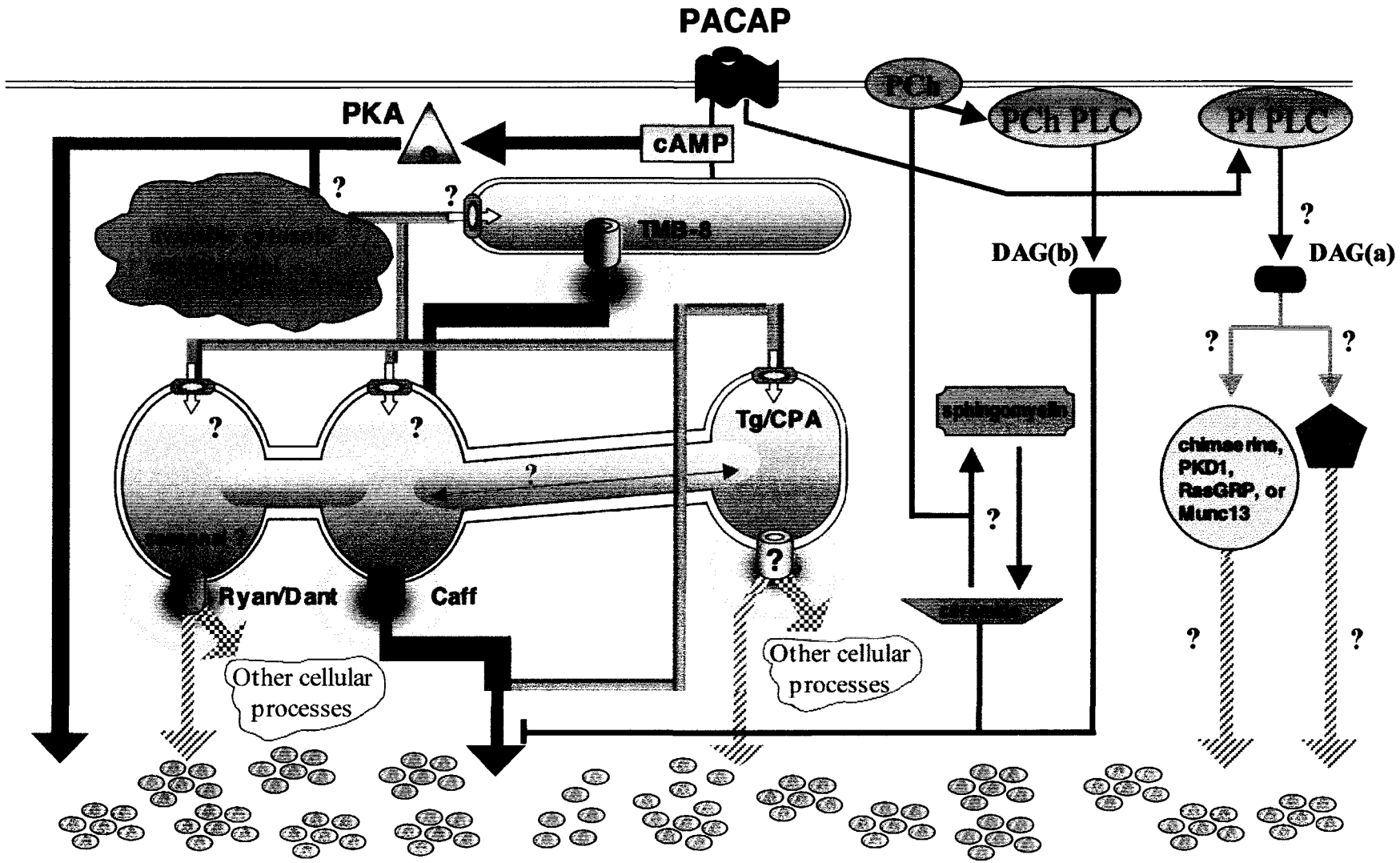


Figure 4.2. A schematic of the known and proposed intracellular mechanisms used by PACAP to elicit GTH-II secretion. Solid red arrows and lines indicate known and proposed signalling components activated following PACAP activation of $PAC_1$ receptors. Solid green only lines indicate a PACAP-involved $Ca^{2+}$ signalling pathway. Green & pink coloured lines indicate a $Ca^{2+}$ refilling route. DAGa and DAGb represent proposed distinct pools of DAG formed by phosphatidylinositiol (PI) specific-PLC and phosphatidylcholine (PCh) specific-PLC respectively. Striped blue arrows indicate an unknown signalling mechanism leading to GTH-II secretion. Although not part of the PACAP signalling pathway, release of $Ca^{2+}$ from ryanodine-sensitive stores and stores refilled by Tg have also been shown to lead to GTH-II release. Hatched arrows indicate $Ca^{2+}$ release from stores for other cellular processes. Whether the indicated pharmacologically identified $Ca^{2+}$ pools adequately represents all the TMB-8-sensitive pools is not known, thus a separate (though functionally linked) TMB-8-sensitive pool is depicted.




**GTH-II Exocytosis**

## Endnotes

Portions of the results section have been published.

**Chang JP, Wong CJ, Davis PJ, Soetaert B, Fedorow C, Sawisky G.** 2003. Role of $Ca^{2+}$ stores in dopamine- and PACAP-evoked growth hormone release in goldfish. Mol Cell Endocrinol. 206:63-74.

Portions of the results have been published in abstract format.

**Sawisky GR and Chang JP.** 2004. The 43rd annual meeting of the Canadian Society of Zoologists.

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# 6 Appendix I.

## Relationships between nitric oxide and $Ca^{2+}$ in GH release and the possible role of nitric oxide in PACAP-stimulated GH and GTH-II secretion.

## *6.1 Introduction*

### *6.1.1 Nitric Oxide discovery*

$Ca^{2+}$ is probably the best studied intracellular signalling messenger molecule but the more recently discovered molecule, nitric oxide (NO), has also been shown to be very important. NO is a diffusible gaseous signalling molecule that was first shown to have the same relaxant effects on vascular smooth muscle as the then unidentified endothelial-derived relaxation factor (EDRF) in the late 1980's (reviewed in Moncada et al., 1991). Since then, NO was shown to be the EDRF, as well as to participate in the regulation of many cellular functions.

### *6.1.2 NO production and nitric oxide synthase tissue distribution in mammals*

Bredt et al., (1990) discovered that NO is liberated through the enzymatic action of nitric oxide synthase (NOS) which converts arginine to citrulline. NOS requires the co-factors CaM, $Ca^{2+}$, and NADPH for its function. Three major NOS isoforms were subsequently identified in mammals: 1) brain or neuronal (n)NOS, which was originally thought to only function in brain (Bredt and Snyder, 1990); 2) inducible (i)NOS, which was first identified in macrophages, and its presence was induced during macrophage activation (Xie et al., 1992); and 3) endothelial (e)NOS, which was most closely associated with epithelial cells (Pollock et al., 1991). Subsequently, the tissue distribution of each of the three NOS isoforms has been shown to be very wide spread. The nNOS isoform has been detected not only in the brain, but also in skeletal muscle, lung, kidney, adrenal, and pituitary (Förstermann et al., 1998). Similarly, eNOS is also found in muscle, GI tract, brain, and gonads (Förstermann et al., 1998). The known tissue distribution

of iNOS now includes heart (Balligand et al., 1994), kidney (Kone, 1999), uterus (Chwalisz and Garfield, 1998), liver (Geller et al., 1993), and brain (Minc-Golomb et al., 1996).

### *6.1.3 Pituitary actions of NO in mammals*

Since its discovery, NO has been shown to be distributed almost ubiquitously in every tissue with just as many diverse functions. In the pituitary, NO has been found to modulate the secretion of many of the pituitary hormones. NO has been shown to 1) enhance the release of TSH by TRH (Coiro et al., 1995), 2) inhibit ACTH secretion (Giordano et al., 1996; Bazzani et al., 1997), 3) stimulate prolactin PRL release (Brunetti et al., 1995), 4) either stimulate (Gonzalez and Aguilar, 1999; McCann et al., 1998) or inhibit (Chatterjee et al., 1997) LH release, 5) inhibit AVP release (Kadowaki et al., 1994), 6) inhibit oxytocin release (Kadowaki et al., 1994), and 7) stimulate GH release (Cuttica et al., 1997; Pinilla et al., 1999).

### *6.1.4 Intracellular effects of NO in mammals*

The intracellular action of NO has been demonstrated to occur through activation of soluble guanylate cyclase (sGC); this enzyme is activated by the binding of NO to its heme group (Lewicki et al., 1982; Palacios et al., 1989). Once activated, this enzyme produces cyclic guanosine monophosphate (cGMP), which in turn activates cGMP-dependent protein kinases (PKG's). PKG has many effects, including modulation of the activities of $IP_3$ receptors, phospholamban, vimentin, phosphatase inhibitor G substrate, and subunits of

myosin light chain phosphatase (Hoffmann et al., 2000; Krumenacker et al., 2004).

Many studies have also revealed a link between NO and $[Ca^{2+}]_c$ fluctuations. Because the NOS enzyme requires $Ca^{2+}$-bound calmodulin as a cofactor, the upstream requirement is already obvious. Many other studies have also shown that NO triggers extracellular $Ca^{2+}$ entry (Alagarsamy et al., 1994; Hattori et al., 1995) and intracellular $Ca^{2+}$ release (Takasugi, 1993; Levin et al., 1997; Hutcheson and Griffith, 1997), suggesting that a downstream relationship between the two signalling molecules also exists. Although NO increases $[Ca^{2+}]_c$ in many systems examined, $[Ca^{2+}]_c$ has also been shown to be reduced by NO (Garg and Hassid, 1991; Blatter and Wier, 1994; Schlossman et al., 2003). Presumably, NO affects mobilization of $Ca^{2+}$ either directly, or indirectly through sGC activation.

Several of the targets through which NO actions modulates $[Ca^{2+}]_c$ have been identified. NO has been shown to modulate the entry of extracellular $Ca^{2+}$ through VSCC (Quignard et al., 1997). In terms of intracellular $Ca^{2+}$ stores, NO has been shown to decrease (Meszaros et al., 1996; Zahradnikova et al., 1997; Heunks et al., 2001), as well as to increase, $Ca^{2+}$ release though the RyR (Willmott et al., 1996; Stoyanovsky et al., 1997; Sun et al., 2003). NO can also prevent the RyR activator caffeine from causing an increase in $[Ca^{2+}]_c$ (Kannan et al., 1997; Prakash et al., 1997). In addition, much evidence suggests that NO stimulates $Ca^{2+}$ release from mitochondrial $Ca^{2+}$ stores (Richter et al., 1994; Horn et al., 2002; Dedkova et al., 2004). Conversely, the effects of NO on $Ca^{2+}$ release

from $IP_3$-sensitive $Ca^{2+}$ stores are largely inhibitory in nature (Lang and Lewis, 1989; Kannan et al., 1997; Tertyshnikova et al., 1998). The relationship between NO and SERCA is controversial. While NO has been shown to inhibit SERCA pumps (Pernollet et al., 1996; Ishii et al., 1998) and to prevent $Ca^{2+}$ release from SERCA-refilled stores (Yuan et al., 1997), NO action in rat pituitary cells (Duvilanski et al., 1998) and mouse and rabbit aorta (Cohen et al., 1999) depends on the $Ca^{2+}$ in these pools. Although the relationship between NO and $Ca^{2+}$ is far from clear, the conclusion one can make from the available evidence is that the actions of these two signalling molecules are closely dependant on each other.

*6.1.5 PACAP and NO*

PACAP and the NOS enzyme have been shown to coexist in many tissues (Okamura et al., 1994; Ny et al., 1994; Radziszewski et al., 1996; Elsas et al., 1997; Edvinsson et al., 2001). This peptide has been shown to modulate NO synthesis to produce a variety of cellular effects (Cardell et al., 1997; Delgado et al., 1999; Fox-Threlkeld et al., 1999; Sekiya et al., 2000; Onoue et al., 2002). Conversely, several actions of PACAP are also regulated by NO (Chakder and Rattan, 1998; Fujimiya et al., 1998).

*6.1.6 NO in goldfish*

Although not as well studied as in mammals, several studies have looked at the presence and action of NO in goldfish. The NOS enzyme has been detected in the retina (Liepe et al., 1994), CNS (Bruning et al., 1995), PNS

(Bruning et al., 1996), macrophages (Laing et al., 1996), as well as in somatotropes and gonadotropes of this species (Uretsky and Chang, 2000; Uretsky, 2001). NO production has also been detected in goldfish brain (Villani and Guarnieri, 1995) and retina (Villani and Guarnieri, 1996).

A handful of reports on the actions of NO in goldfish exist. It has been shown to play a role in goldfish learning (Li et al., 1995), to enhance $K_{ATP}$ channels in goldfish heart (Cameron et al., 2003), to stimulate the release of GH and GTH-II, and to be an important intracellular component mediating GnRH-stimulated, as well as dopamine D1 receptor-mediated, GH secretion (Uretsky and Chang, 2001; Uretsky et al., 2003, Uretsky, 2001). In terms of NO stimulation of GH release, NO downstream signalling has been shown to involve activation of GC and the production of cGMP (Uretsky et al., 2003). In this system, application of 8-bromo-cGMP stimulates GH secretion while the GH response to NO is accompanied by elevations in cellular cGMP levels and is blocked by inhibitors of GC.

*6.1.7 NO and PACAP in goldfish*

Although PACAP and NO have been co-localized in a variety of tissues in mammals (rat hypothalamus, Okamura et al., 1994; cat esophagus, Ny et al., 1994; rat urethra, Radziszewski et al., 1996; rat ciliary ganglion, Elsas et al., 1997; rat brain, Edvinsson et al., 2001) and in fish (gut of the Atlantic cod and spiny dogfish; Olsson and Karila, 1995); similar data are not available for goldfish. However, the reported ability of PACAP and NO to operate together to inhibit contractions in the proximal intestine of the Atlantic cod suggests that

PACAP and NO also interacts functionally with one another in fish (Olsson and Holmgren, 2000). Pertaining to pituitary hormone release in goldfish, preliminary results showed that PACAP stimulation of GH was reduced by an inhibitor of sGC, LY 83583 (Uretsky, 2001). Since sGC is a downstream element in NOS/NO signalling, this observation suggests that the signalling pathways mediating PACAP action on GH release may have an NO component. Interestingly, in the same study, NOS inhibitors did not attenuate PACAP-induced GH production in static incubation experiments; thus, the role of NO in mediating PACAP actions on pituitary hormone release in goldfish is still unclear.

### *6.1.8 Proposed research*

There are two primary goals in this study.

#### *6.1.8.1 Role of $Ca^{2+}$ in NO-induced GH release in goldfish*

GnRH and dopamine stimulation of GH release in goldfish are dependent on extracellular and intracellular $Ca^{2+}$, as well as NOS/NO signalling (Chang et al., 1994, 2000, 2003; Uretsky and Chang, 2000; Uretsky et al., 2003; Section 1.1.1.1). In mammals, many of the effects of NO are mediated by $Ca^{2+}$ and $Ca^{2+}$ may also exert its effects through NO synthesis (Section 6.1.4). Thus, it is likely that NO and $Ca^{2+}$ interact in mediating the control of GH release in goldfish; however, nothing is known regarding these possible interactions in this model system. As an initial attempt to understand such relationships, the dependence of NO-induced GH release on extracellular and intracellular $Ca^{2+}$ were examined. Increases in cellular NO were achieved by treatment with sodium nitroprusside

(SNP), an NO donor (Oh and McCaslin, 1995). The dependence on extracellular $Ca^{2+}$ entry through VSCC was examined using an inorganic inhibitor of $Ca^{2+}$ entry, $CdCl_2$. The dependence on intracellular $Ca^{2+}$ was examined using TMB-8, ryanodine, caffeine, Tg, and BHQ. Since the caffeine-sensitive intracellular $Ca^{2+}$ store represents a major intracellular $Ca^{2+}$ pool for several important GH neuroendocrine regulators, such as sGnRH and cGnRH-II (Johnson and Chang, 2000), as well as PACAP (thesis section 3.2, 4.2), the effects of a NO scavenger on caffeine-induced GH release was also examined to investigate the possible participation of NOS/NO in mediating GH responses to mobilization of intracellular $Ca^{2+}$.

*6.1.8.2 Role of NO in PACAP-induced GH and GTH-II release*

As described in Section 6.1.7, the link between PACAP-stimulated GH secretion and NO is inconclusive and warrants further investigation. Whether the GTH-II response to PACAP is mediated by NO is also unknown. In this study, the possible participation of NOS/NO in mediating PACAP action on GH and GTH-II secretion in goldfish was further examined using two NO scavengers and three NOS inhibitors.

*6.1.8.3 Experimental system*

In the study by Uretsky (2001), static incubation experiments were used to test the effects of NOS inhibitors on PACAP action. However, with static incubation studies, the effects of pharmacological agents alone on basal hormone secretion can be a confounding issue as small hormone responses

often cannot be easily distinguished from changing basal release. On the other hand, cell column perifusion experiments often allow a small hormone release response to be distinguished from changes in basal release (Chang et al., 1990). Thus, a perifusion protocol was chosen for all experiments in this study.

### *6.2 Materials and Methods*

#### *6.2.1 Animals*

All fish maintenance and experimental protocols used have been approved by the animal care committee of the University of Alberta in accordance with national guidelines. Common goldfish (*Carassius auratus*; 8 – 13 cm in length) were purchased from Aquatic Imports (Calgary, AB) and maintained under conditions described in Section 2.1. Post-pubertal male and female goldfish were used in all experiments. All experiments were performed between January and September with most experiments being performed between April and September. In order to minimize possible seasonal effects on the results, replicates experiment were generally done within a short time period when possible. Nonetheless, to facilitate future interpretation of possible seasonal effects, the times of year when the different sets of experiments were performed were indicated in the appropriate figure legends.

#### *6.2.2 Drugs and reagents*

Most chemicals were obtained from Sigma (St. Louis, MO) unless otherwise stated. Stock solutions of PACAP (Peninsula Laboratories, Belmont, CA), 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO; Calbiochem,

La Jolla, CA), $CdCl_2$, TMB-8 (Calbiochem), sodium nitroprusside (SNP; Calbiochem) and aminoguanidine hemisulfate (AGH; Calbiochem) were all dissolved in distilled deionized water. Caffeine was prepared directly in testing medium. Rutin hydrate (Calbiochem), BHQ (Calbiochem), high purity ryanodine (99.5%; Calbiochem), 7-nitroindazole (7Ni), N-(3-aminomethyl) benzylacetamidine dihydrochloride (1400W; Calbiochem), and Tg were all dissolved in DMSO. Aliquots of concentrated stock solutions were stored at –20°C, the exception being ryanodine, which was kept at 4°C,. Final concentrations were achieved by dilution in testing medium. At the concentrations used in these studies, the final concentrations of DMSO (0.1%) had no effect on basal hormone release or goldfish pituitary cell membrane ion currents (Van Goor et al., 1997).

*6.2.3 Pituitary cell dispersion*

As described in Section 2.3, dispersed pituitary cells were prepared from both male and female goldfish using a trypsin/DNase treatment procedure (Chang et al., 1990). Following dispersion, cells were re-suspended in plating medium.

*6.2.4 Column perifusion experiments*

Cell column perifusion studies were performed as previously described (Chang, et al., 1990; Section 2.4). Perifusates were collected as 5-min fractions and stored at –20°C until being sampled for GH or GTH-II content by specific radioimmunoassays (Marchant et al., 1987; Peter et al., 1984).

Generally, PACAP and SNP were applied as a 5-min pulse at 45 min into an experiment. The dose of PACAP used was 10 nM, which has previously been shown to be maximally effective in stimulating GH and GTH-II release in this system (Wirachowsky et al., 2000; Chang et al., 2001). The dose of SNP used was 100 μM and has also been shown to be maximum effective in stimulating GH secretion in previous studies (Uretsky et al., 2000). Inhibitors or other pharmacological agents were applied from 25 min to 85 min. The flow rate and dead volume of the system is such that the hormone release response to a treatment will commence in the fraction collected roughly 5-6 min after the beginning of drug application (Chang et al., 1990).

Hormone responses from individual columns were expressed as a percentage of basal release, which was the average of the first 5 fractions collected at the beginning of an experiment. Net hormone response to PACAP treatment (base-line corrected) was quantified as area under the curve (Wong et al., 1992) and separated into peak, plateau, and total responses as described in Section 2.4. The hormone responses to 5-min applications of SNP and caffeine were presented as total responses; these were quantified by integrating the area under the curve for duration of the response, which was observed to be 25 and 20 min, respectively (i.e., the general duration of these responses in the present study). 10 nM PACAP and 100 μM SNP, which had been shown to be maximally effective in stimulating pituitary hormone release from dispersed goldfish pituitary cells in previous studies (Wirachowsky et al., 2000; Chang et al., 2001; Uretsky et al., 2000: Uretsky 2001), were effective in stimulating GH and/or GTH-II

release in all experiments in this study. Experiments were usually repeated a minimum of three times using separate cell preparations. Pooled data were used for statistical analysis. Statistical analyses were performed by ANOVA followed by a Fisher's PLSD test. Differences were considered significant when $p < 0.05$. All data are expressed as mean ± SEM.

*6.3 Results*

*6.3.1 $Ca^{2+}$ and NO-induced GH release*

*6.3.1.1 The role of extracellular $Ca^{2+}$ entry through VSCC in NO-induced GH release*

Extracellular $Ca^{2+}$ availability and its entry through L-type VSCC have been shown to be important for the GH release responses to GnRH (Jobin and Chang, 1992), dopamine (Wong et al., 2001) and PACAP (Wirachowsky et al., 2000). To examine the importance of extracellular $Ca^{2+}$ entry through VSCC in NO-induced GH secretion, we tested the effects of 50 μM $CdCl_2$ on the GH response to 100 μM SNP. Previous studies have shown 50 μM $CdCl_2$ to be an effective blocker of $Ca^{2+}$ entry through VSCC in goldfish pituitary cells (Wong et al., 2001; Van Goor et al., 1998). Presumably, $Cd^{2+}$ acts to block the $Ca^{2+}$ channel pore on the plasma membrane, thereby preventing the inward flow of $Ca^{2+}$ through VSCC (Miller, 1987). It is hypothesized that if extracellular $Ca^{2+}$ entry through VSCC is required for SNP-stimulated GH production, then treatment with this blocker should attenuate the release of this hormone.

Application of 50 μM $CdCl_2$ resulted in a small reduction of basal GH secretion that lasted the length of the treatment (Figure 6.1A). 100 μM SNP

significantly increased GH release and the GH response to SNP was not affected by $CdCl_2$ (Figure 6.1 A & B). These results indicate that extracellular $Ca^{2+}$ entry through VSCC is not important in mediating SNP-stimulated GH secretion.

*6.3.1.2 Involvement of intracellular $Ca^{2+}$ in mediating NO-stimulated GH secretion*

The release of $Ca^{2+}$ from intracellular stores has previously been shown to be important in mediating the GH-release responses to GnRH (Johnson and Chang, 2000), dopamine (Chang et al., 2003) and PACAP (Chang et al., 2003). Next, the possible participation of intracellular $Ca^{2+}$ release in mediating NO-stimulated GH release was investigated using a broad spectrum inhibitor of intracellular $Ca^{2+}$ release channels, TMB-8 (Malgodi and Chiou, 1974; Chini and Dousa, 1996). At a dose of 100 μM, TMB-8, has previously been shown to be effective in goldfish pituitary cells (Chang et al., 2003). Treatment with TMB-8 abolished the GH response to SNP (Figure 6.2A). The GH response to SNP in the presence of TMB-8 was not significantly different from that to TMB-8 alone over the same time period and both responses were significantly smaller than that to SNP alone (Figure 6.2B). Given these findings, it seems that release of $Ca^{2+}$ from intracellular sources plays a large role in SNP/NO-induced GH secretion in goldfish.

*6.3.1.3 The specific intracellular $Ca^{2+}$ stores used in NO-induced GH secretion*

Since intracellular $Ca^{2+}$ stores seems to be important in mediating NO-induced GH secretion, the next objective was to find out which of the known pharmacologically identifiable pools in goldfish somatotropes are essential to this

signalling mechanism. To this end, the effects of caffeine, ryanodine, Tg, and BHQ on SNP-stimulated GH release were examined.

*6.3.1.3.1 Role of caffeine-sensitive intracellular $Ca^{2+}$ stores in NO-stimulated GH release*

It is hypothesized that if caffeine-sensitive $Ca^{2+}$ stores are involved in mediating NO action, then pre-incubation with caffeine should adversely affect SNP-stimulated hormone secretion. In this set of studies, treatment with 10 mM caffeine alone produced a large initial secretion response in GH, which returned to near basal levels after about 20 min (Figure 6.3A). This is consistent with previous observations (Section 3.2; Johnson and Chang, 2000). A 5-min pulse of 100 μM SNP elicited a significant GH response; however, the GH secretion initiated by SNP was eliminated by caffeine treatment (Figure 6.3A & B).These results suggest that NO-stimulated GH release involves an intracellular $Ca^{2+}$ store that is sensitive to caffeine.

*6.3.1.3.2 Role of ryanodine-sensitive $Ca^{2+}$ stores in NO-stimulated GH release*

In goldfish, ryanodine-sensitive $Ca^{2+}$ stores are important in GnRH- and PACAP-stimulated GH secretion in goldfish (Johnson and Chang, 2002; Chang et al., 2003; Section 4.3). To examine the role these stores play in NO-induced GH release, 10 μM ryanodine (Johnson and Chang, 2002) was used to block the release of $Ca^{2+}$ from these pools. If ryanodine-sensitive $Ca^{2+}$ stores are involved in NO-stimulated GH release, inhibition of $Ca^{2+}$ release through these channels should reduce hormone secretion.

Ryanodine on its own produced a slight depression in basal GH secretion but did not affect SNP-induced GH secretion (Figure 6.5A & B). From these results, it appears that ryanodine-sensitive $Ca^{2+}$ stores are not involved in mediating the GH secretion response induced by SNP.

*6.3.1.3.3 SERCA-refilled stores function in NO-stimulated GH secretion*

SERCA's on the ER membrane have long been known to play a role in the refilling of $Ca^{2+}$ stores in this organelle (Carafoli, 2003). Several adenosine triphosphatase (ATPase) inhibitors are known to block the action of these pumps, including Tg and BHQ (Lytton et al., 1991; Treiman et al., 1998). Inhibition of SERCA prevents the refilling of these stores, which eventually empty due to a slow leakage of $Ca^{2+}$ across the ER membrane into the cytosol. SERCA also buffers changes in $[Ca^{2+}]_c$ (Yoshikawa et al., 1996; Hill et al., 2003; Chang et al., 2003). Thus the use of SERCA inhibitors such as Tg and BHQ can reveal the role of these $Ca^{2+}$ ATPases and/or SERCA-refilled $Ca^{2+}$ stores in mediating and/or regulating hormone secretion responses.

Application of 2 μM Tg, which has previously been shown to be effective in goldfish pituitary cells (Johnson and Chang, 2000), induced a slow rise in GH secretion that eventually reached a plateau after 15 min (Figure 6.5A). Such changes in basal GH in response to Tg application has previously been observed (Chang et al., 2003). Treatment with 100 μM SNP increased GH secretion (Figure 6.5A). However in the presence of Tg, SNP was unable to elicit an increase in GH release above levels observed with Tg alone (Figure 6.5A & B).

Treatment with 10 μM BHQ, a dose that has been shown to be effective in goldfish pituitary cells (Chang et al., 2003), evoked a slow steady decline in basal GH secretion that recovered slightly upon cessation of treatment (Figure 6.6A). Application of 100 μM increased GH release as expected (Figure 6.6A). However, when SNP was applied during BHQ treatment, no stimulated GH response was observed (Figure 6.6A & B).

When the results of Tg and BHQ treatments were viewed together, it appears there NO-stimulated GH release has a dependence on BHQ- and Tg-sensitive SERCA-refilled $Ca^{2+}$ store(s).

*6.3.1.3.4 Role of NO in caffeine-stimulated GH release*

It is known that caffeine-induced GH release is in part mediated by $Ca^{2+}$ (Johnson et al., 2000; Wong et al., 2001) and that GnRH-stimulated GH release is mediated by caffeine-sensitive $Ca^{2+}$ stores, as well as NOS/NO (Johnson and Chang, 2000; Uretsky et al., 2003). When viewed together with the known interdependence of NO and $Ca^{2+}$ in intracellular signalling (Section 6.1.4), it is possible that caffeine stimulation of GH release has an NO-dependent component. To examine this possibility, the NO scavenger PTIO (Hogg et al., 1995) was used to limit the NO response potentially produced by caffeine administration. In goldfish, the effective concentration of PTIO has been shown to be 10 μM (Uretsky et al., 2000). If caffeine-evoked GH release is mediated by NO generation, then 10 μM PTIO should attenuate the GH response. Treatment with 10 mM caffeine alone produced a significant increase in GH secretion (Figure 6.7A). By itself, 10 μM PTIO caused a minor decrease in basal GH

secretion (Fig 6.7A). Surprisingly, the caffeine-stimulated GH secretion was significantly potentiated by the NO scavenger (Figure 6.7A & B). This suggests that NO production does not mediate the caffeine-stimulated GH response; however, these two signalling pathways appear to interact in the control of GH release.

*6.3.2 Possible involvement of NO in PACAP-induced GH and GTH-II release*

The hypothesis that NO mediates PACAP action on GH and GTH-II release was tested using NO scavengers, PTIO and rutin hydrate, as well as NOS inhibitors, 7Ni, AGH and 1400W. PTIO scavenges NO in a stiochiometric fashion (Hogg et al., 1995); whereas rutin hydrate acts as an antioxidant, pushing the $NO^-/NO•/NO^+$ equilibrium to the relatively unreactive $NO^-$ (Korkina and Afanas'ev, 1997). However, because of its antioxidant activity, rutin hydrate may also inhibit NOS since it affects the oxidation of nicotinamide adenine dinucleotide (NAD)H to $NAD^+$. In mammals, 7Ni is reported to selectively affect nNOS (Mayer et al., 1994) and eNOS (Wolff et al., 1994) while 1400W has a preference for iNOS (Garvey et al., 1997). On the other hand, AGH is reported to be a broad spectrum NOS inhibitor. Since goldfish pituitary cells have been shown to contain NOS isoforms with immunological properties of mammalian iNOS and nNOS (Uretsky, et al., 2003), the use of these three NOS inhibitor was deemed appropriate.

*6.3.2.1 NO scavengers and PACAP-stimulated GH and GTH-II secretion*

Previous studies have demonstrated the effective NO scavenging doses in goldfish of PTIO and rutin hydrate to be 10 μM (Uretsky et al., 2003). Administration of PTIO by itself produced no change in basal GH secretion and did not affect the GH secretion induced by 10 nM PACAP (Figure 6.8A & B). Rutin hydrate also had minimal effect on basal GH secretion but it potentiated the GH response to PACAP (Figure 6.9A & B). The peak, plateau and total GH responses to PACAP plus rutin hydrate were significantly greater than those to PACAP treatment alone (Figure 6.9A & B).

Basal GTH-II secretion was slightly reduced upon PTIO application (Figure 6.10A). Application of PACAP stimulated GTH-II release and this response was not affected by PTIO treatment (Figure 6.10A & B). Rutin hydrate did not alter basal GTH-II release (Figure 6.11A). Although the GTH-II responses to PACAP were slightly greater in the presence than in the absence of rutin hydrate, the differences were not significant (Figure 6.11A & B).

The inability of PTIO and rutin hydrate to attenuate the hormone release responses to PACAP suggests that NO does not mediate PACAP-stimulated GH and GTH-II secretion.. However, these results do not rule out the possibility that NO signalling and its effects on GH secretion influences the ability of PACAP to induce GH secretion in goldfish.

*6.3.2.2 Effects of NOS inhibitors on PACAP-induced GH and GTH-II release*

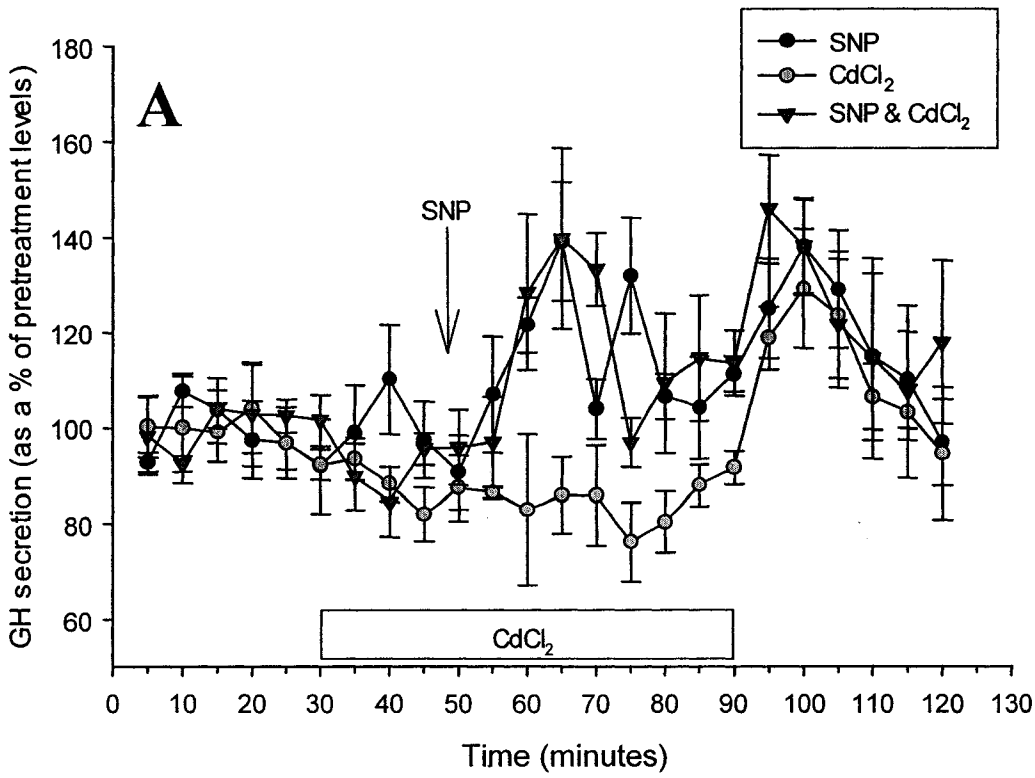
The influence of NOS inhibitors on PACAP-elicited GH and GTH-II release was examined to investigate, in further detail, the role of NO in the PACAP

signalling cascades in goldfish somatotropes and gonadotropes. In goldfish, the optimal dose for AGH has been shown to be 1 mM (Uretsky and Chang, 2000), whereas 7-Ni and 1400W have been determined to be effective at concentrations of 1 μM (Uretsky et al., 2003). Treatment with 1 mM AGH did not affect basal GH secretion and did not significantly alter the GH response to PACAP (Figure 6.12A & B). As with AGH treatment, 1 μM 1400W did not alter basal GH secretion (Figure 6.13A). Although the magnitudes of the peak, plateau and total GH responses to PACAP were smaller in the presence of 1400 W as compared to PACAP treatment alone, the differences were not significant (Figure 6.13B). Treatment with 1 μM 7Ni also did not alter basal GH secretion; however, it significantly enhanced the magnitude of the plateau and total GH secretion responses to PACAP (Figure 6.14A & B).

Treatment with AGH, 1400W and 7Ni had no major effects on basal GTH-II secretion (Figures 6.15A, 6.16A, & 6.17A). On the other hand, in the presence of AGH, the plateau and total GTH-II secretion responses to PACAP were significantly increased above those observed with PACAP alone (Figure 6.15A & B). In contrast, neither 7Ni treatment nor 1400W application significantly altered the peak, plateau and total GTH-II secretion responses to PACAP (Figures 6.16 & 6.17).

These results indicate that activation of NOS is not part of the signal transduction mechanisms mediating PACAP-induced GH and GTH-II secretion. However, the possibility that NOS/NO signalling affects the actions of PACAP on GH and GTH-II secretion is suggested.

Figure 6.1. Effects of $CdCl_2$ on SNP-stimulated GH secretion. **A**. Temporal characteristics of the GH response to SNP (100 μM), either alone or in the presence of $CdCl_2$ (50 μM). The arrow indicates the estimated commencement of hormone release response to the 5-min SNP pulse and the open bar indicates the duration of exposure to $CdCl_2$. Pooled results (mean ± SEM; n=4 columns each) from 2 replicate experiments performed August are presented. Average pre-treatment GH values for this set of experiments are 44.0 ± 1.7 ng/ml. **B**. Quantified GH responses are presented. A number sign indicates a significant difference ($p < 0.05$) from the $CdCl_2$ only group.



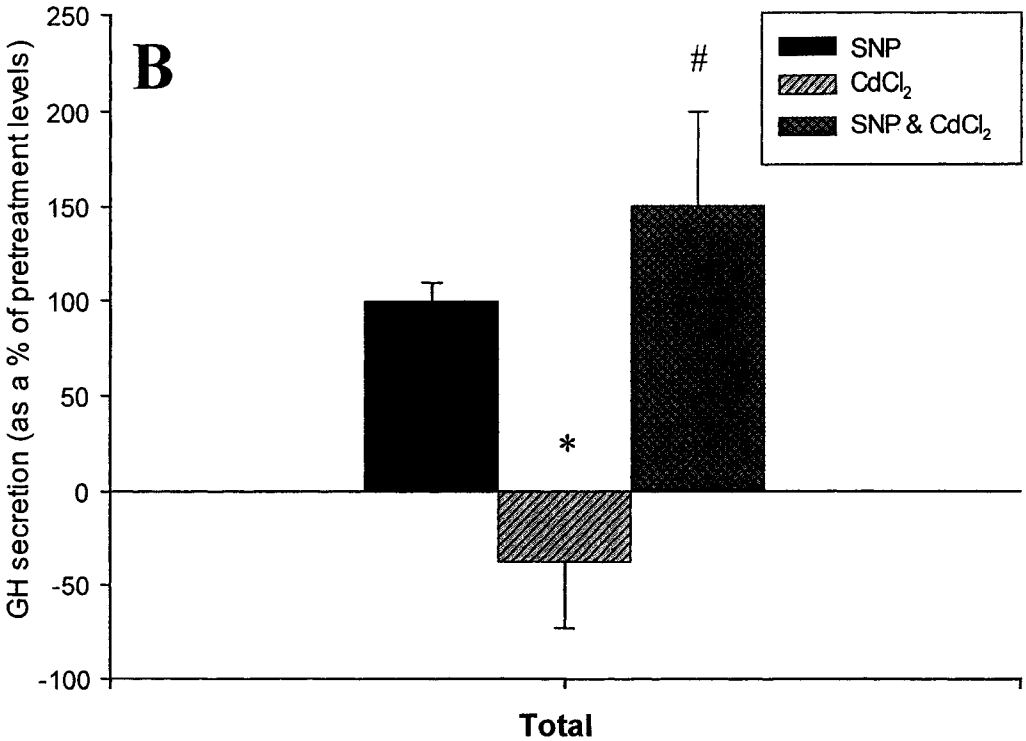
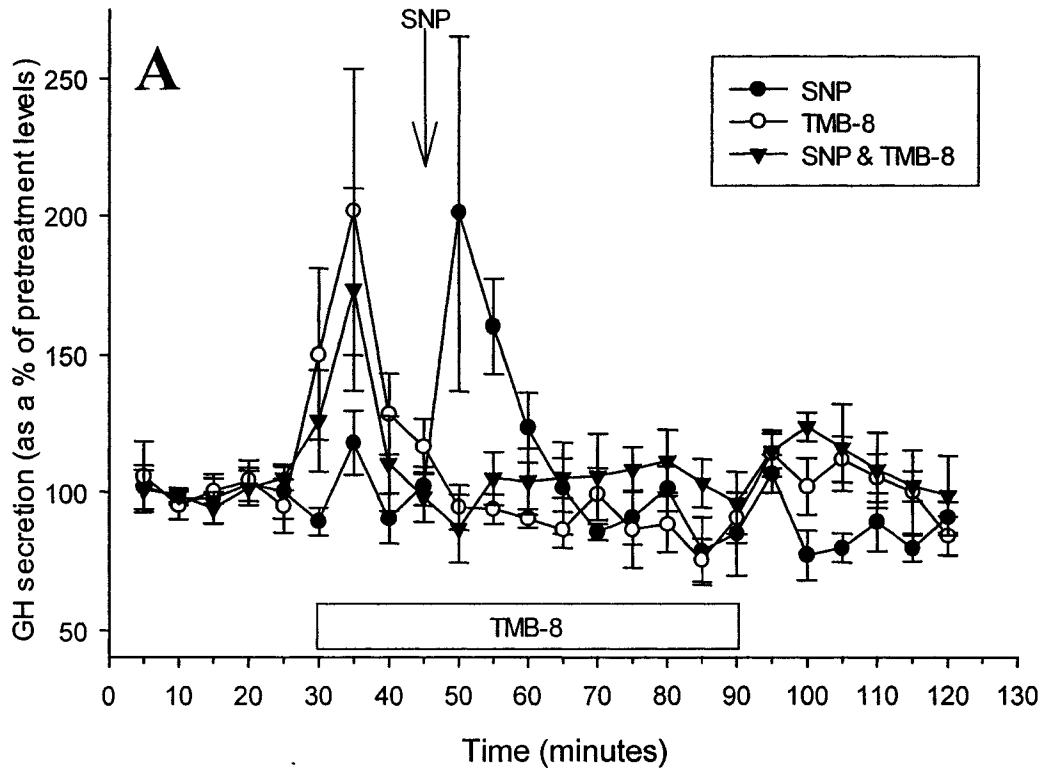



Figure 6.2. Effects of TMB-8 on SNP-stimulated GH secretion. **A**. Temporal characteristics of the GH response to SNP (100 μM), either alone or in the presence of TMB-8 (100 μM). The arrow indicates the estimated commencement of hormone release response to the 5-min SNP pulse and the open bar indicates the duration of exposure to TMB-8. Pooled results (mean ± SEM; n=6 columns each) from 3 replicate experiments (performed once in July and twice in August) are presented. Average pre-treatment GH values for this set of experiments are 38.7 ± 1.8 ng/ml. **B**. Quantified GH responses are presented. A star indicates a significant difference ($p < 0.05$) from the SNP only group.



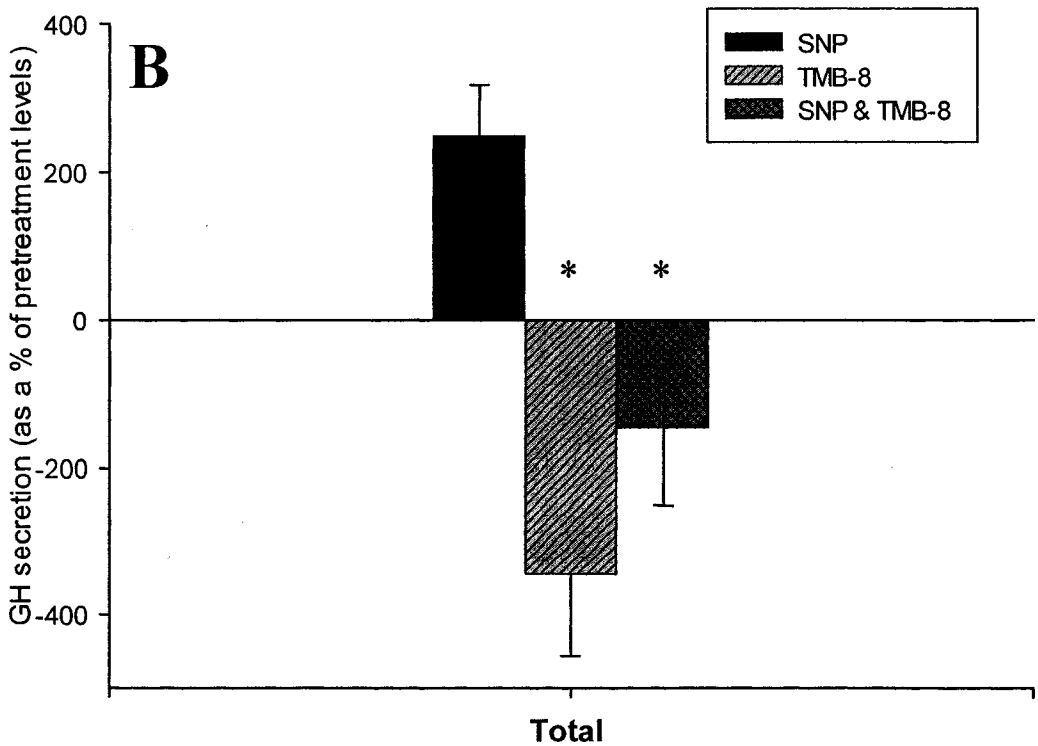
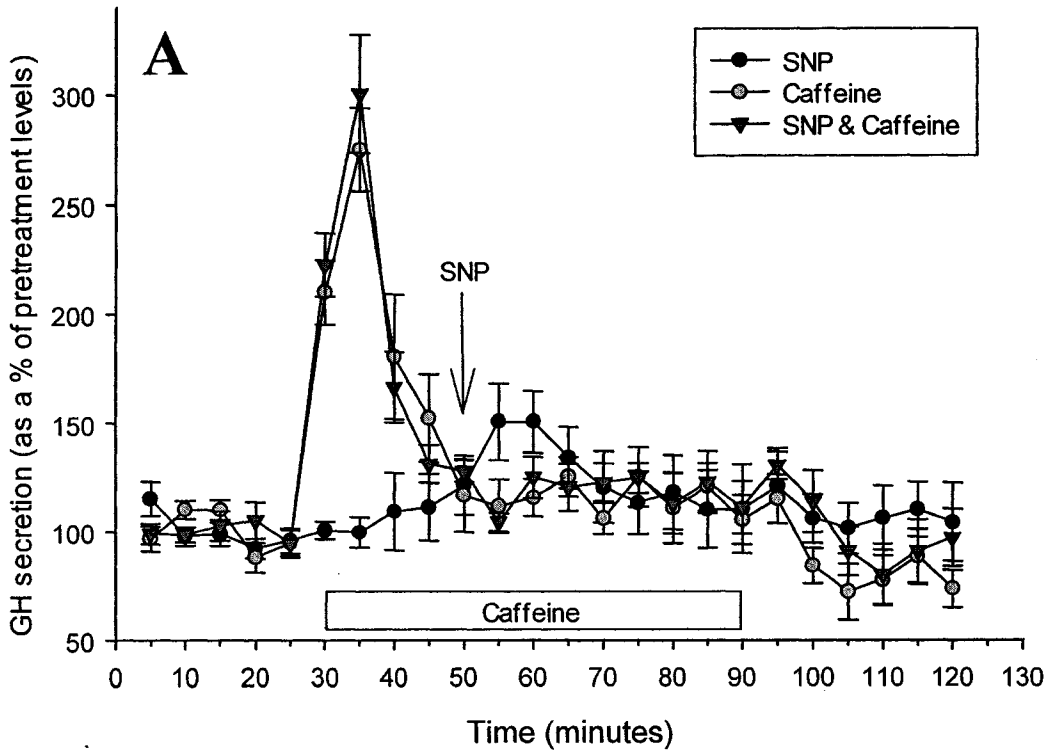



Figure 6.3. Effects of caffeine on SNP-stimulated GH secretion. **A**. Temporal characteristics of the GH response to SNP (100 μM), either alone or in the presence of caffeine (10 mM). The arrow indicates the estimated commencement of hormone release response to the 5-min SNP pulse and the open bar indicates the duration of exposure to caffeine. Pooled results (mean ± SEM; n=8 columns each) from 4 replicate experiments (performed twice in February, and once each in April and June) are presented. Average pre-treatment GH values for this set of experiments are 38.7 ± 1.8 ng/ml. **B**. Quantified GH responses are presented. A star indicates a significant difference ($p < 0.05$) from the SNP only group.



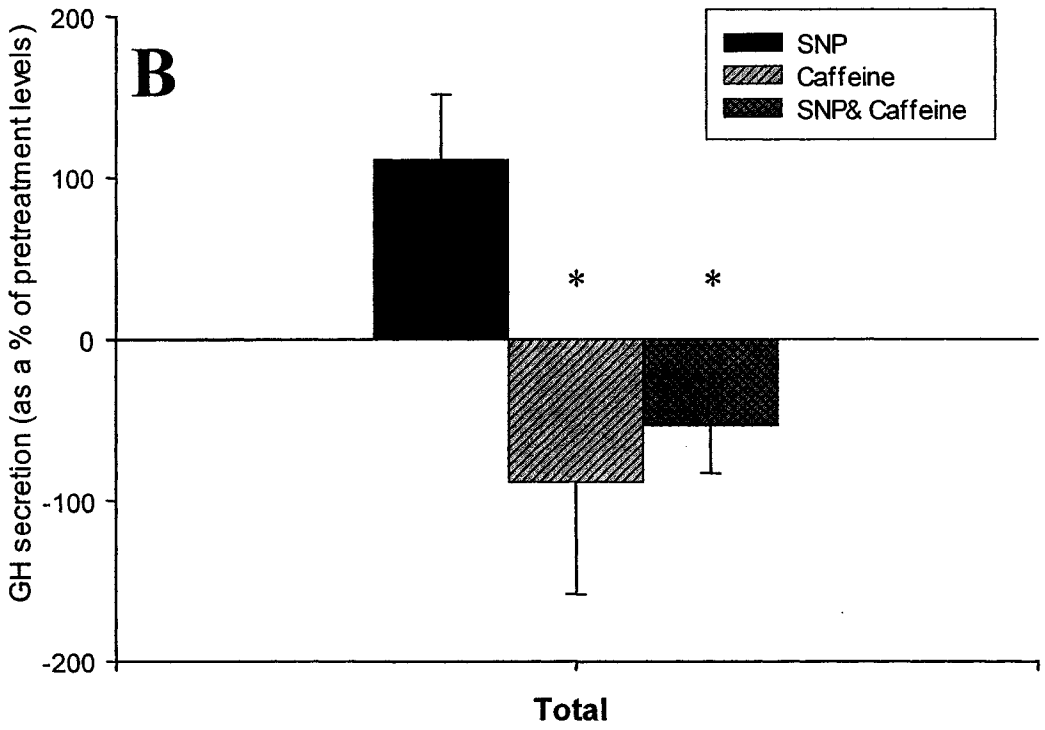
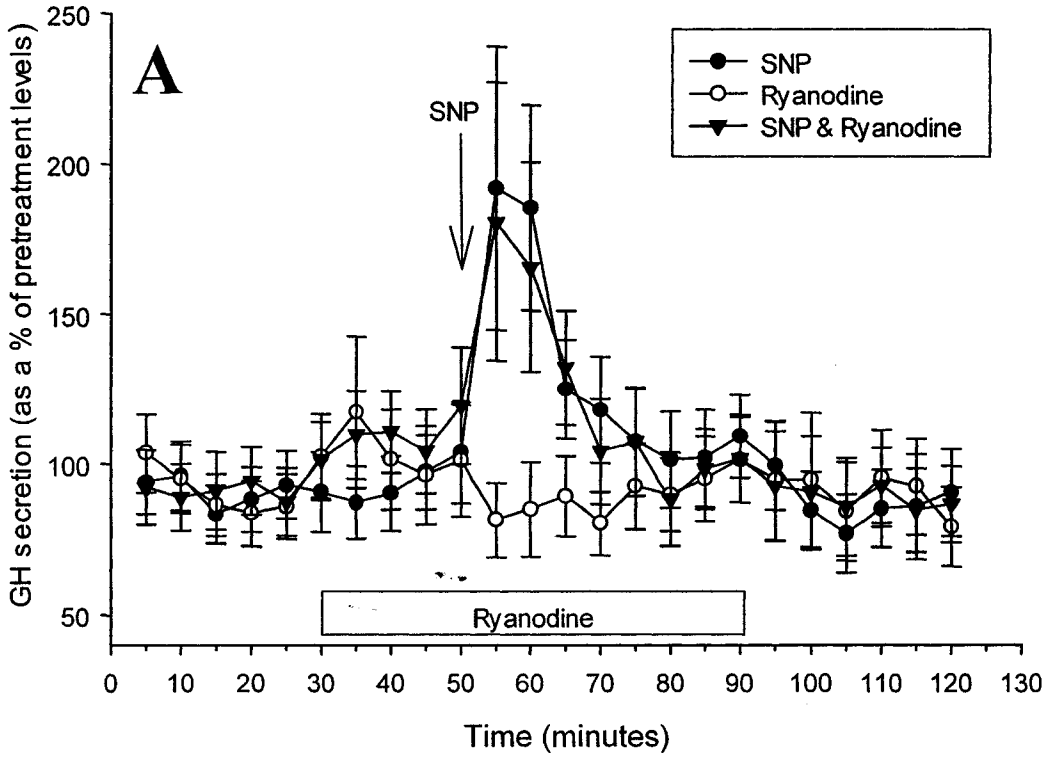



Figure 6.4. Effects of ryanodine on SNP-stimulated GH secretion. **A**. Temporal characteristics of the GH response to SNP (100 μM), either alone or in the presence of ryanodine (10 μM). The arrow indicates the estimated commencement of hormone release response to the 5-min SNP pulse and the open bar indicates the duration of exposure to ryanodine. Pooled results (mean ± SEM; n=10 columns each) from 5 replicate experiments (performed twice in May and three times in June) are presented. Average pre-treatment GH values for this set of experiments are 25.7 ± 3.0 ng/ml. **B**. Quantified GH responses are presented. A star indicates a significant difference ($p < 0.05$) from the SNP only group. A number sign indicates a significant difference ($p < 0.05$) from the ryanodine only group.



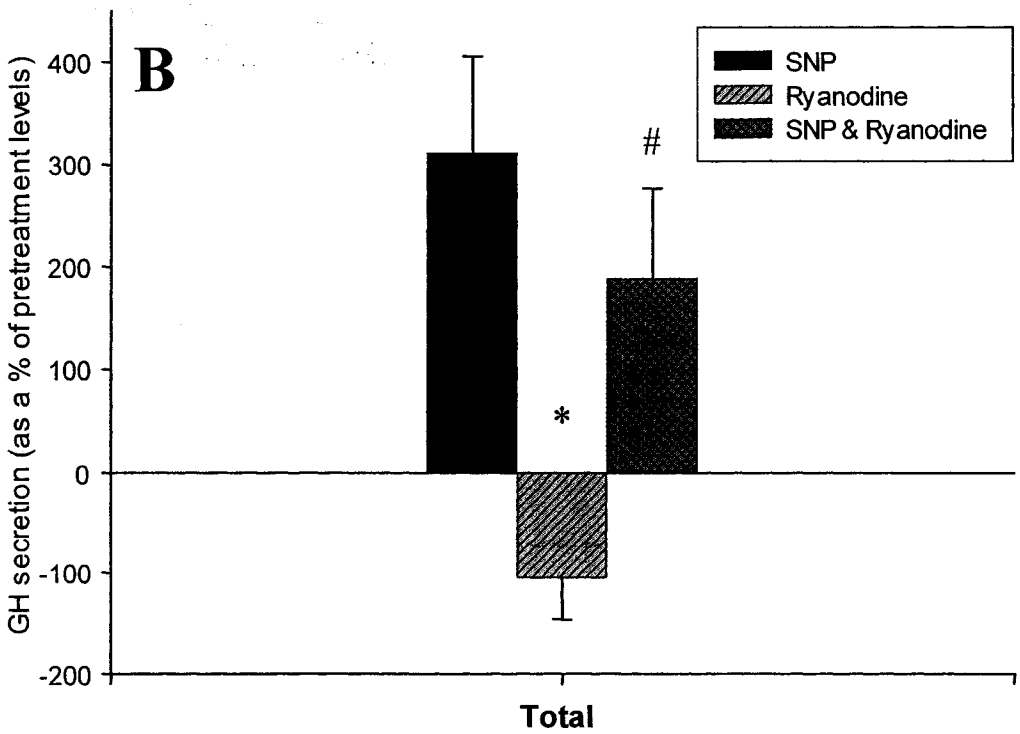
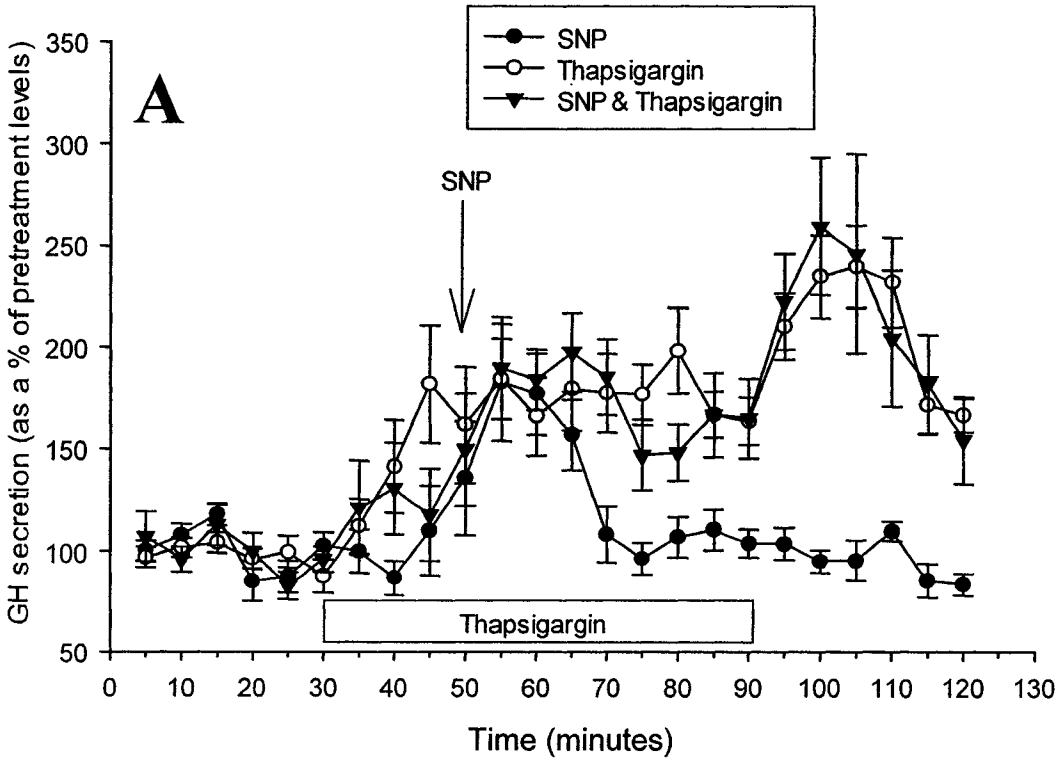



Figure 6.5. Effects of thapsigargin on SNP-stimulated GH secretion. **A**. Temporal characteristics of the GH response to SNP (100 μM), either alone or in the presence of thapsigargin (2 μM). The arrow indicates the estimated commencement of hormone release response to the 5-min SNP pulse and the open bar indicates the duration of exposure to thapsigargin. Pooled results (mean ± SEM; n=8 columns each) from 4 replicate experiments (performed twice in April and twice in May) are presented. Average pre-treatment GH values for this set of experiments are 28.0 ± 2.5 ng/ml. **B**. Quantified GH responses are presented.



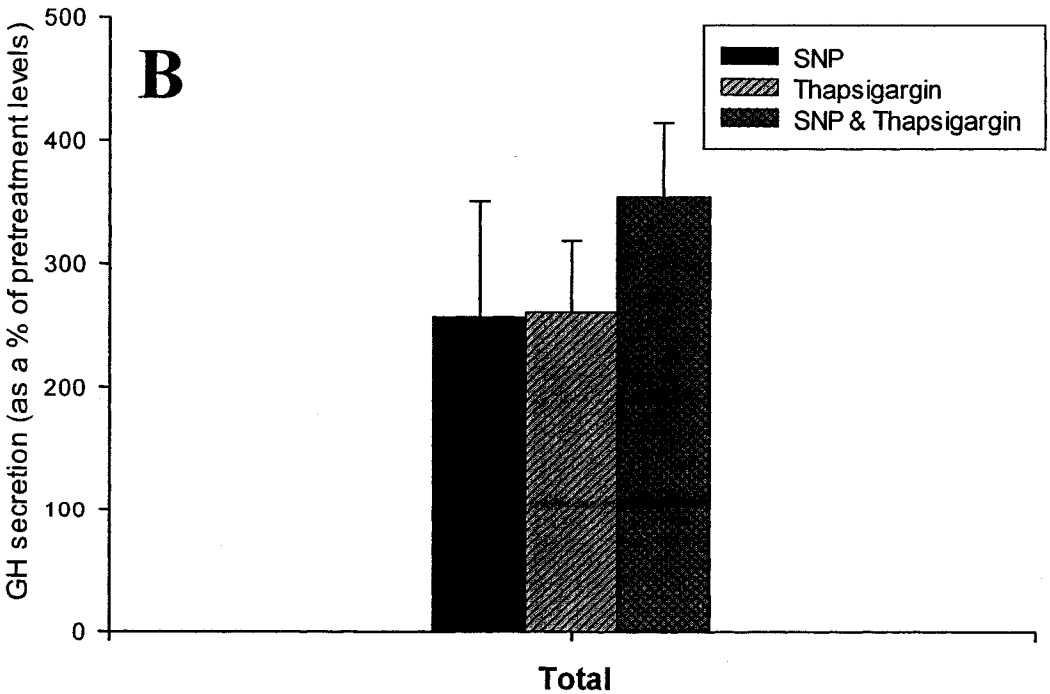
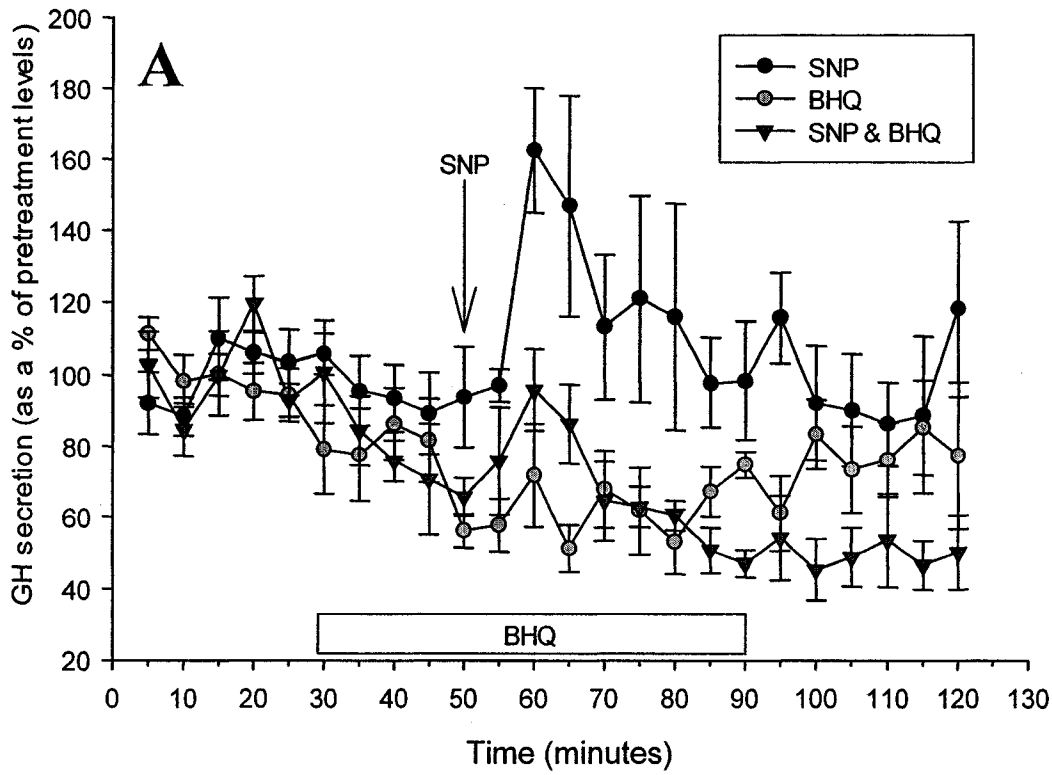



Figure 6.6. Effects of BHQ on SNP-stimulated GH secretion. **A**. Temporal characteristics of the GH response to SNP (100 μM), either alone or in the presence of BHQ (10 μM). The arrow indicates the estimated commencement of hormone release response to the 5-min SNP pulse and the open bar indicates the duration of exposure to BHQ. Pooled results (mean ± SEM; n=4 columns each) from 2 replicate experiments performed in September are presented. Average pre-treatment GH values for this set of experiments are 31.0 ± 1.2 ng/ml. **B**. Quantified GH responses are presented. A star indicates a significant difference ($p < 0.05$) from the SNP only group.



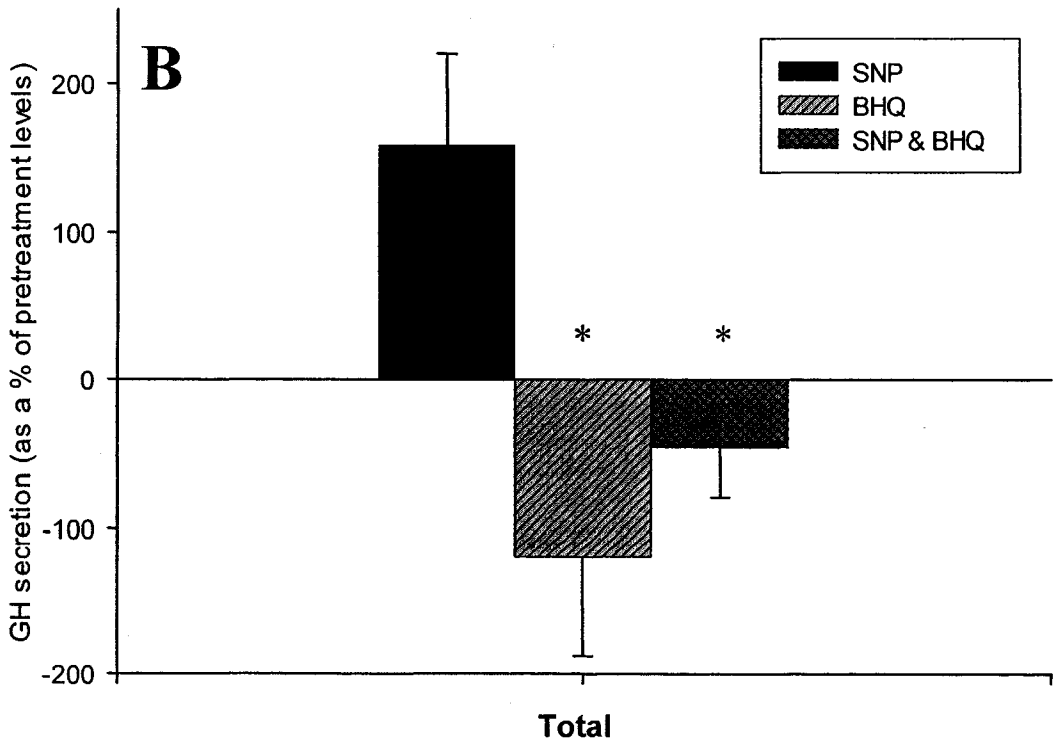
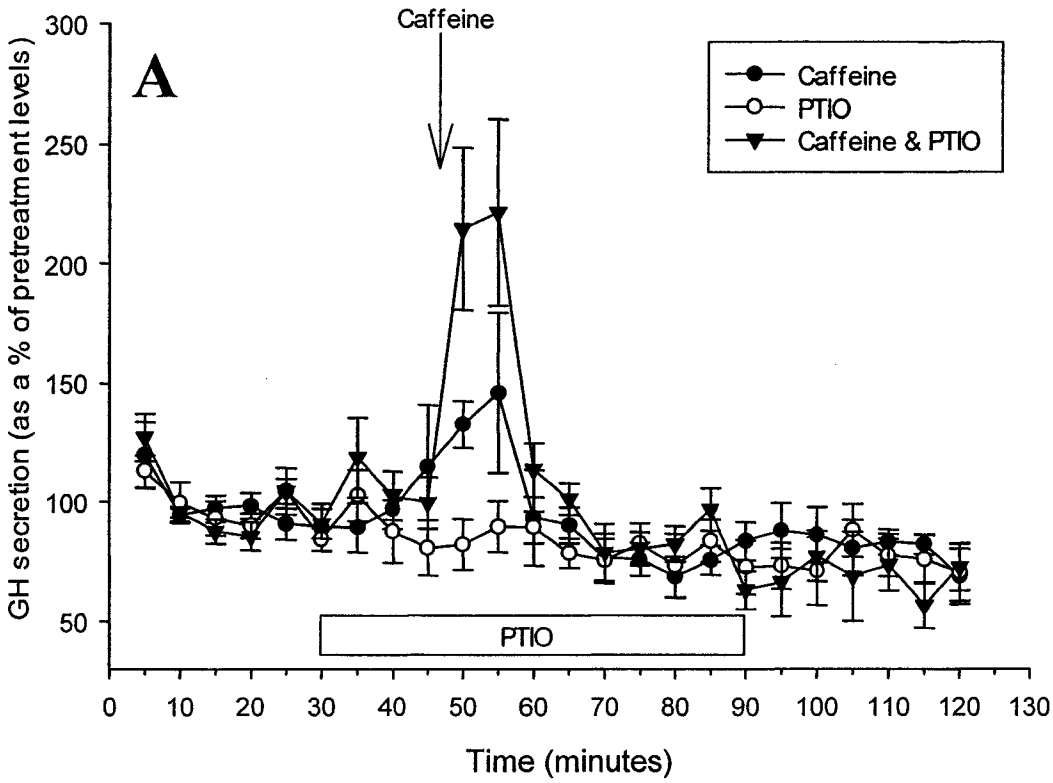



Figure 6.7. Effects of PTIO on caffeine-stimulated GH secretion. **A**. Temporal characteristics of the GH response to caffeine (10 mM), either alone or in the presence of PTIO (10 μM). The arrow indicates the estimated commencement of hormone release response to the 5-min caffeine pulse and the open bar indicates the duration of exposure to PTIO. Pooled results (mean ± SEM; n=6 columns each) from 3 replicate experiments (performed once in July and twice in August) are presented. Average pre-treatment GH values for this set of experiments are 37.5 ± 1.7 ng/ml. **B**. Quantified GH responses are presented. A star indicates a significant difference ($p < 0.05$) from the caffeine only group. A number sign indicates a significant difference ($p < 0.05$) from the PTIO only group.



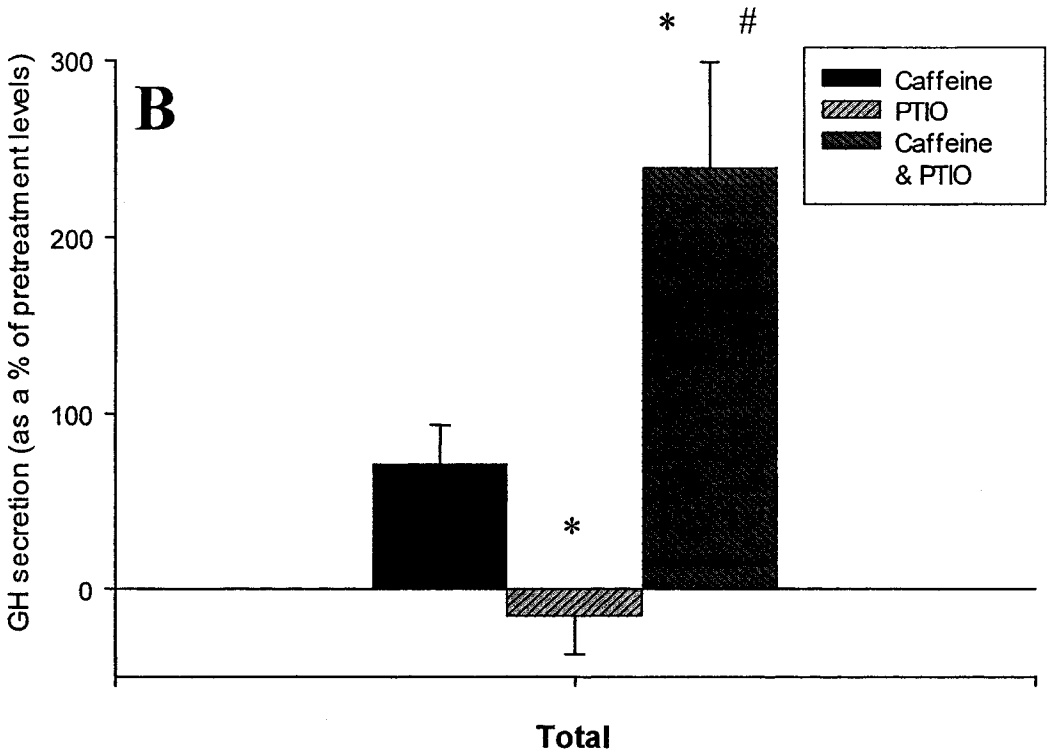
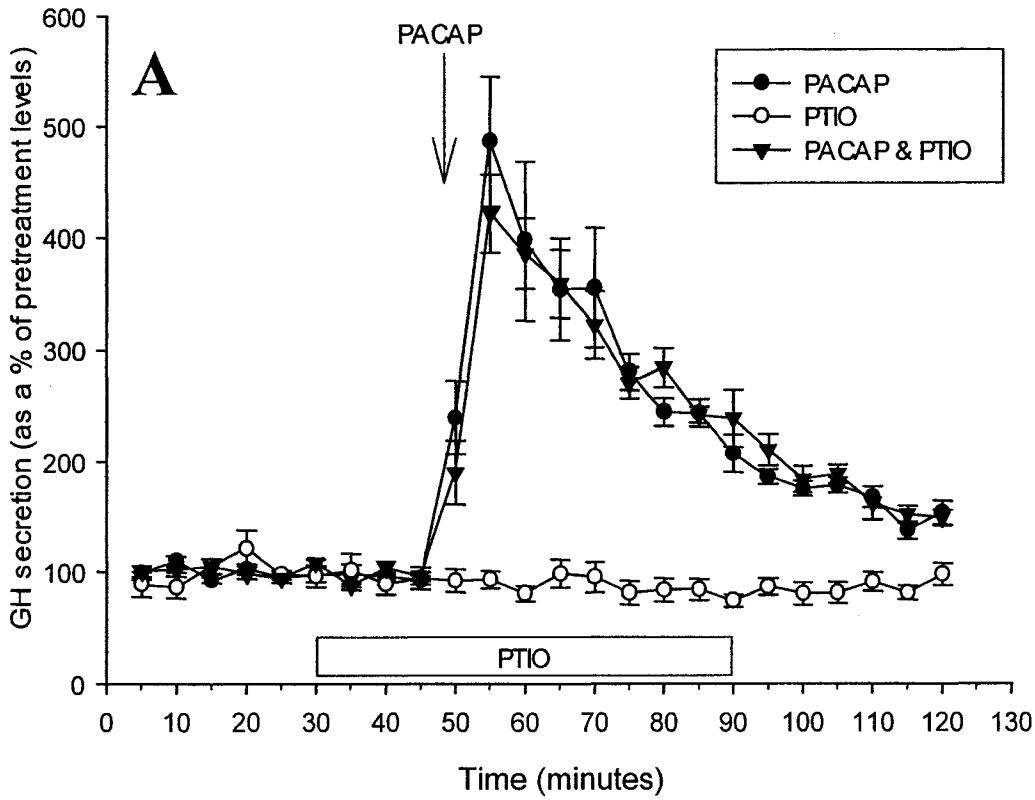



Figure 6.8. Effects of PTIO on PACAP-stimulated GH secretion. **A**. Temporal characteristics of the GH response to PACAP (10 μM), either alone or in the presence of PTIO (10 μM). The arrow indicates the estimated commencement of hormone release response to the 5-min PACAP pulse and the open bar indicates the duration of exposure to PTIO. Pooled results (mean ± SEM; n=6 columns each) from 3 replicate experiments performed in May are presented. Average pre-treatment GH values for this set of experiments are 27.3 ± 1.0 ng/ml. **B**. Quantified GH responses are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only group. A number sign indicates a significant difference ($p < 0.05$) from the PTIO.



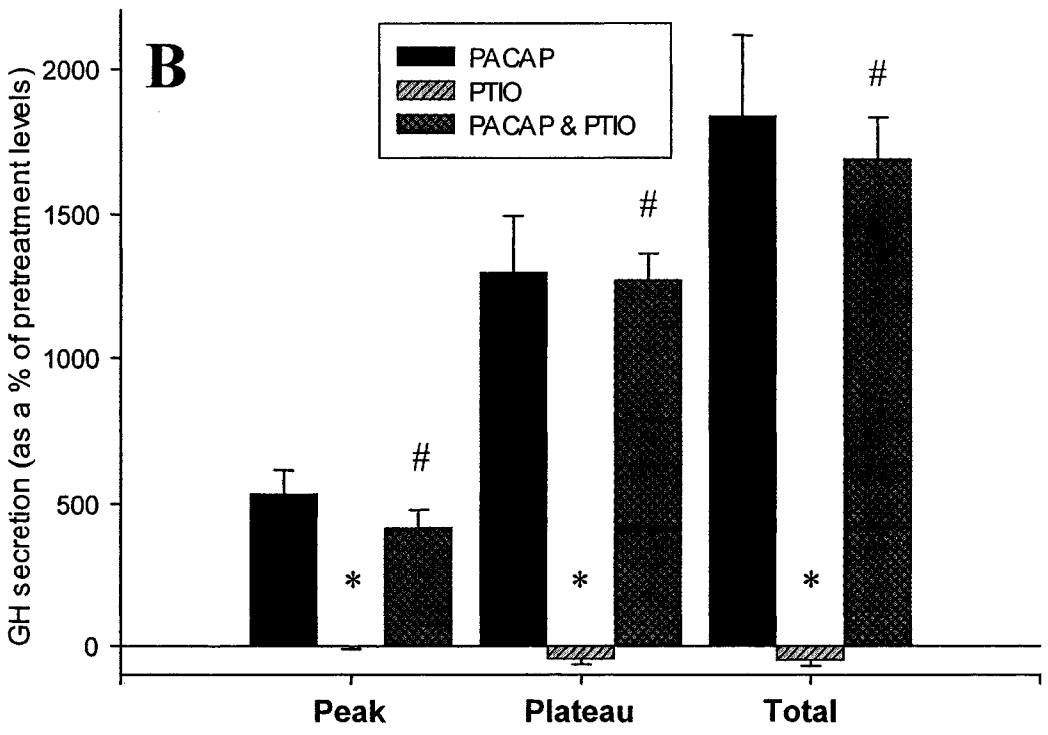
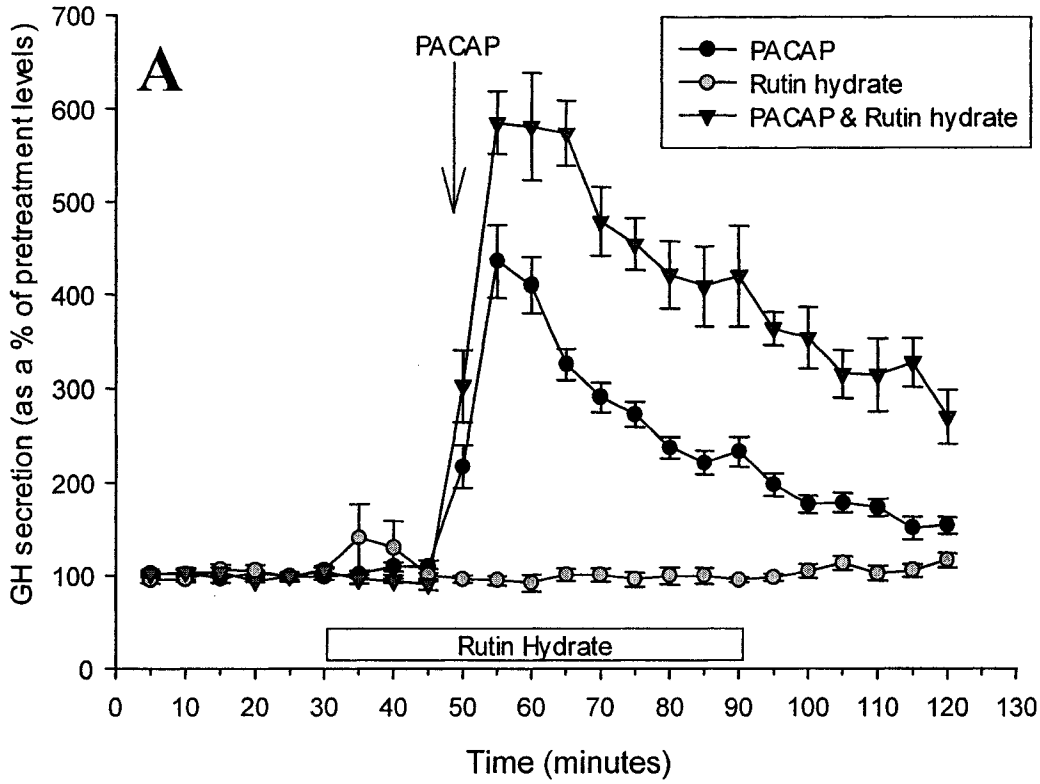



Figure 6.9. Effects of rutin hydrate on PACAP-stimulated GH secretion. **A**. Temporal characteristics of the GH response to PACAP (10 μM), either alone or in the presence of rutin hydrate (10 μM). The arrow indicates the estimated commencement of hormone release response to the 5-min PACAP pulse and the open bar indicates the duration of exposure to rutin hydrate. Pooled results (mean ± SEM; n=6 columns each) from 3 replicate experiments (performed twice in January and once in April) are presented. Average pre-treatment GH values for this set of experiments are 33.4 ± 1.7 ng/ml. **B**. Quantified GH responses are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only group. A number sign indicates a significant difference ($p < 0.05$) from the rutin hydrate only treatment group.



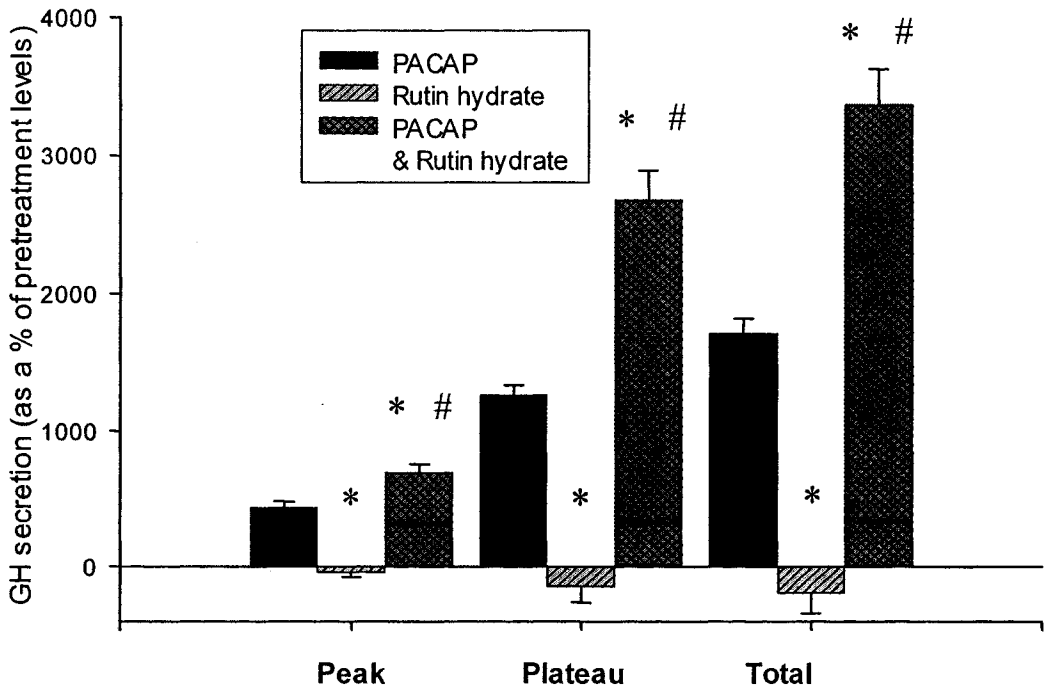
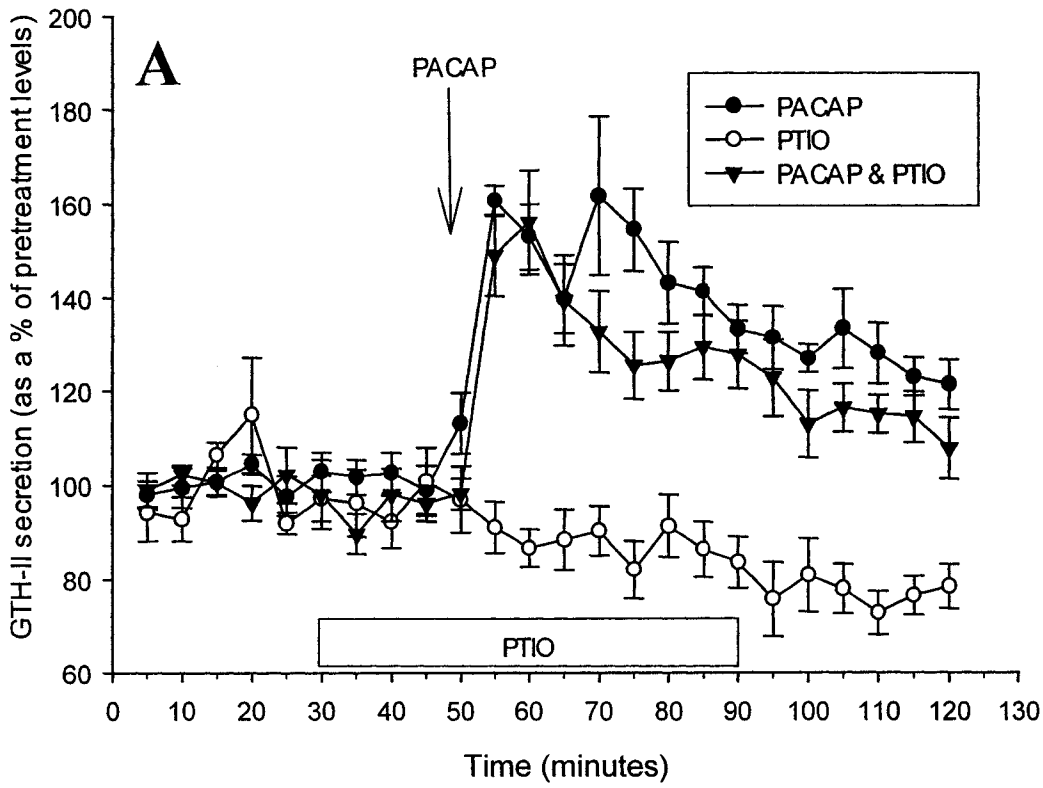



Figure 6.10. Effects of PTIO on PACAP-stimulated GTH-II secretion. **A.** Temporal characteristics of the GTH-II response to PACAP (10 μM), either alone or in the presence of PTIO (10 μM). The arrow indicates the estimated commencement of hormone release response to the 5-min PACAP pulse and the open bar indicates the duration of exposure to PTIO. Pooled results (mean ± SEM; n=6 columns each) from 3 replicate experiments performed in May are presented. Average pre-treatment GTH-II values for this set of experiments are 1.4 ± 0.1 ng/ml. **B**. Quantified GTH-II responses are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only group. A number sign indicates a significant difference ($p < 0.05$) from the PTIO.



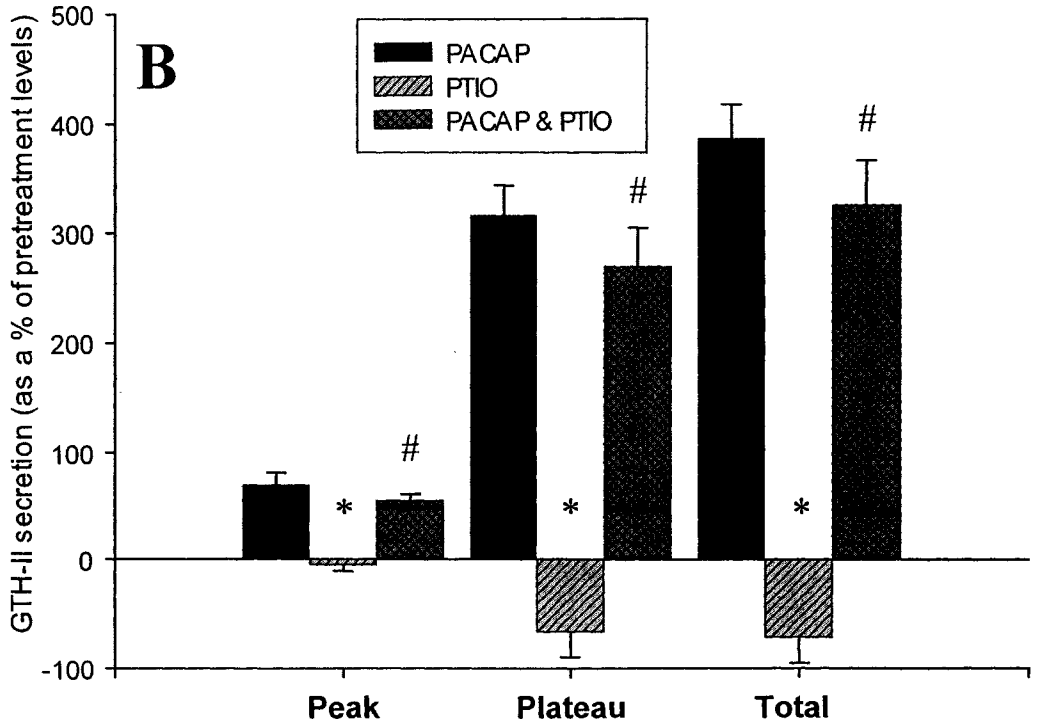
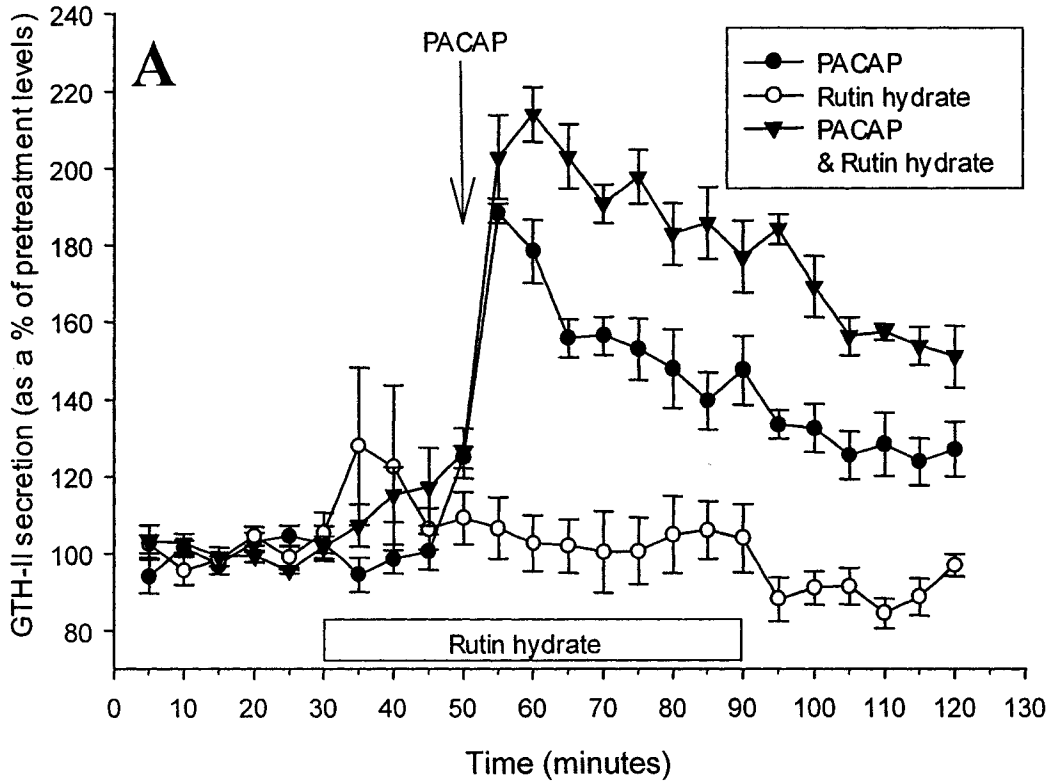



Figure 6.11. Effects of rutin hydrate on PACAP-stimulated GTH-II secretion. **A.** Temporal characteristics of the GTH-II response to PACAP (10 μM), either alone or in the presence of rutin hydrate (10 μM). The arrow indicates the estimated commencement of hormone release response to the 5-min PACAP pulse and the open bar indicates the duration of exposure to rutin hydrate. Pooled results (mean ± SEM; n=6 columns each) from 3 replicate experiments (performed twice in January and once in Apr) are presented. Average pre-treatment GTH-II values for this set of experiments are 1.5 ± 0.1 ng/ml. **B.** Quantified GH responses are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only group. A number sign indicates a significant difference ($p < 0.05$) from the rutin hydrate only treatment group.



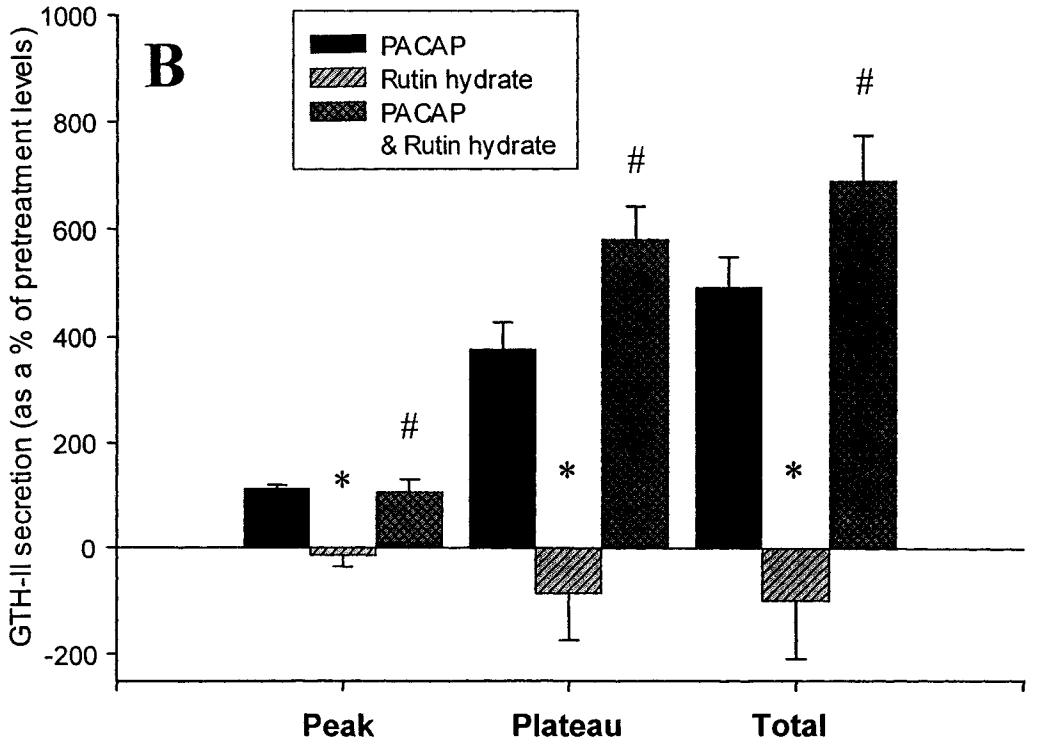
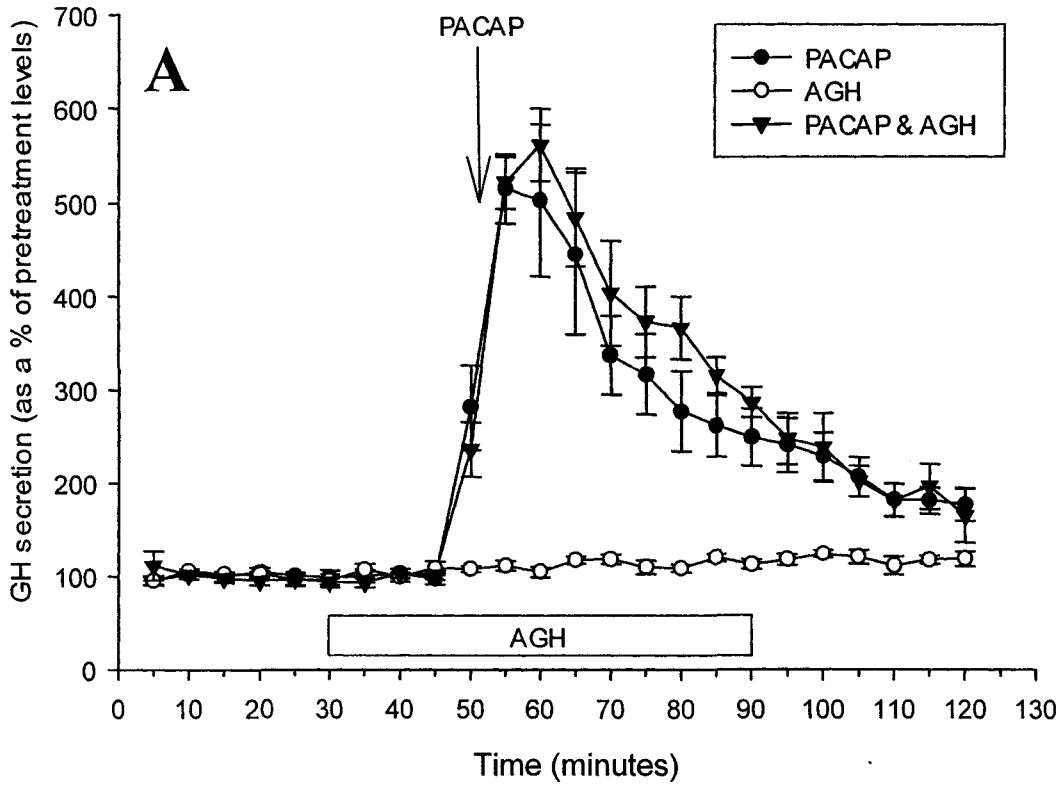



Figure 6.12. Effects of AGH on PACAP-stimulated GH secretion. **A**. Temporal characteristics of the GH response to PACAP (10 μM), either alone or in the presence of AGH (1 mM). The arrow indicates the estimated commencement of hormone release response to the 5-min PACAP pulse and the open bar indicates the duration of exposure to AGH. Pooled results (mean ± SEM; n=6 columns each) from 3 replicate experiments performed in May are presented. Average pre-treatment GH values for this set of experiments are 31.9 ± 2.1 ng/ml. **B**. Quantified GH responses are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only group. A number sign indicates a significant difference ($p < 0.05$) from the AGH only treatment group.



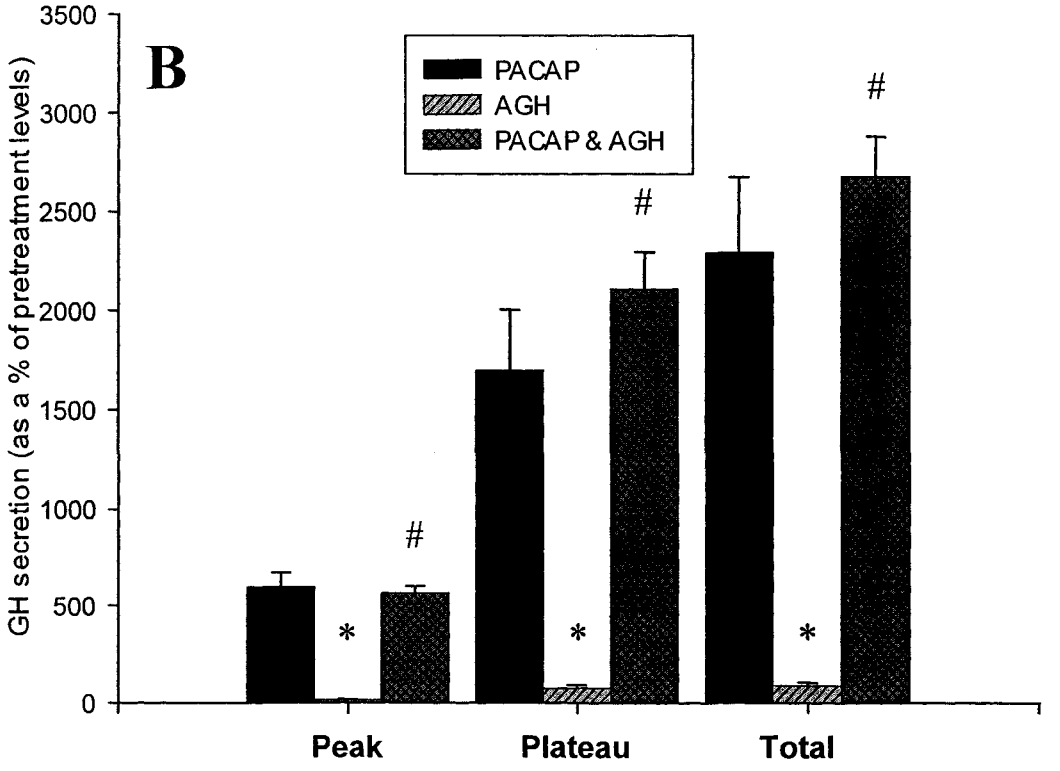
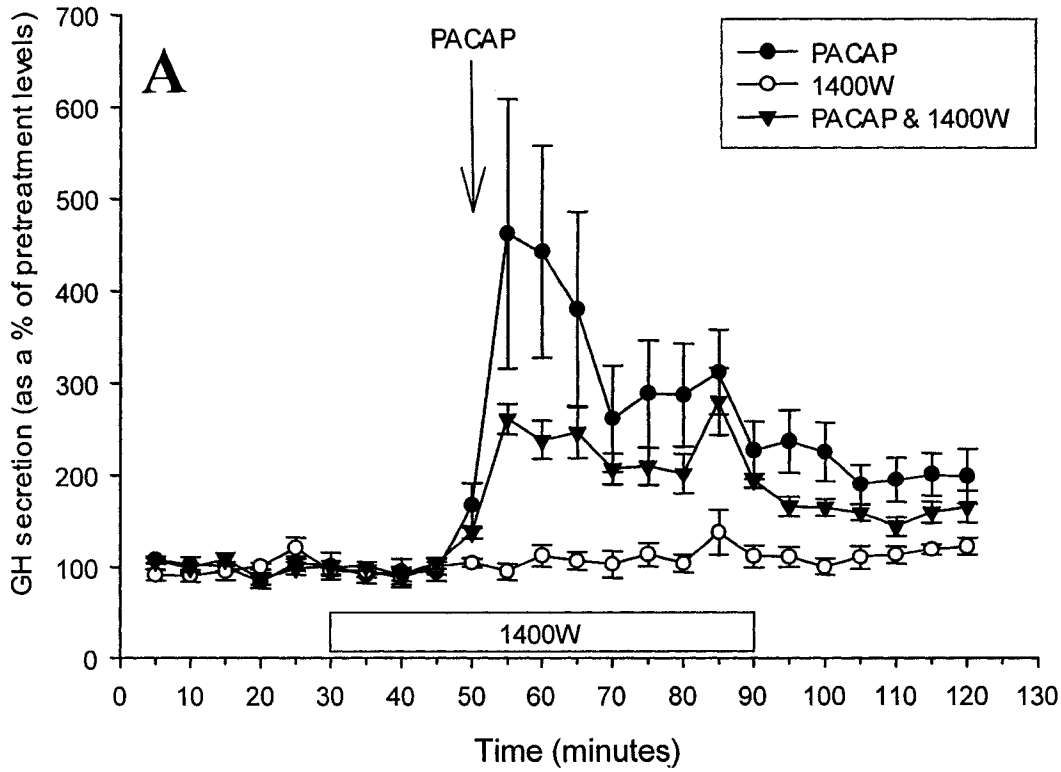



Figure 6.13. Effects of 1400W on PACAP-stimulated GH secretion. **A**. Temporal characteristics of the GH response to PACAP (10 μM), either alone or in the presence of 1400W (1 μM). The arrow indicates the estimated commencement of hormone release response to the 5-min PACAP pulse and the open bar indicates the duration of exposure to 1400W. Pooled results (mean ± SEM; n=6 columns each) from 3 replicate experiments (performed twice in June and once in July) are presented. Average pre-treatment GH values for this set of experiments are 29.1 ± 1.3 ng/ml. **B**. Quantified GH responses are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only group.



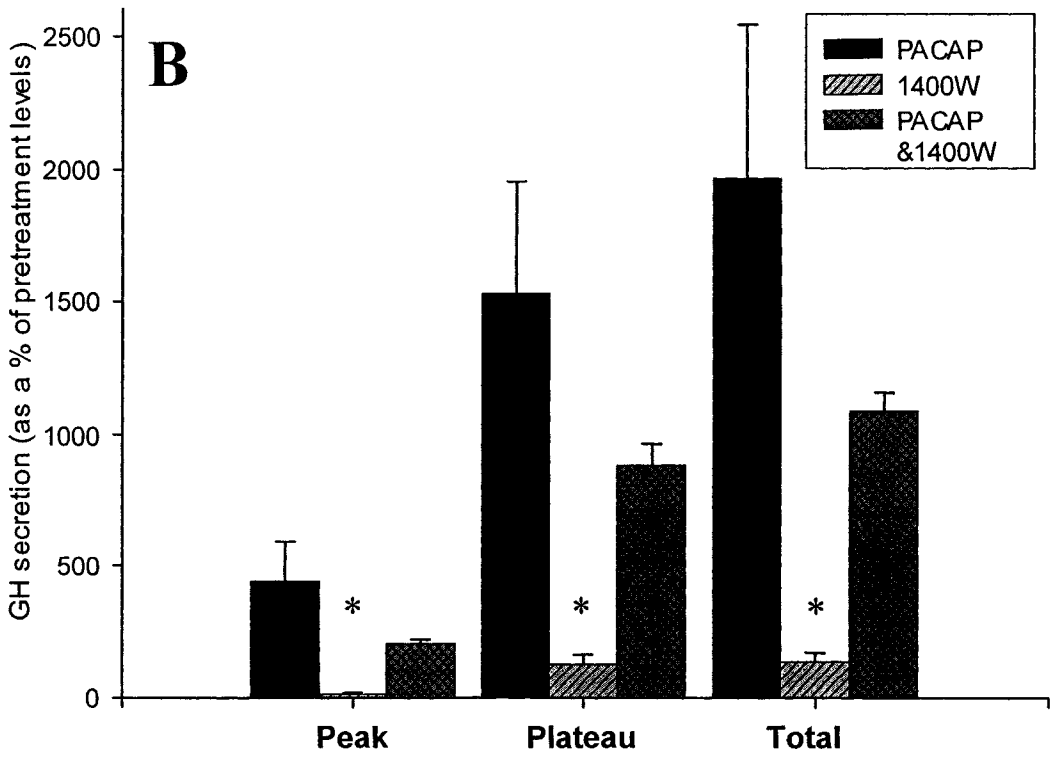
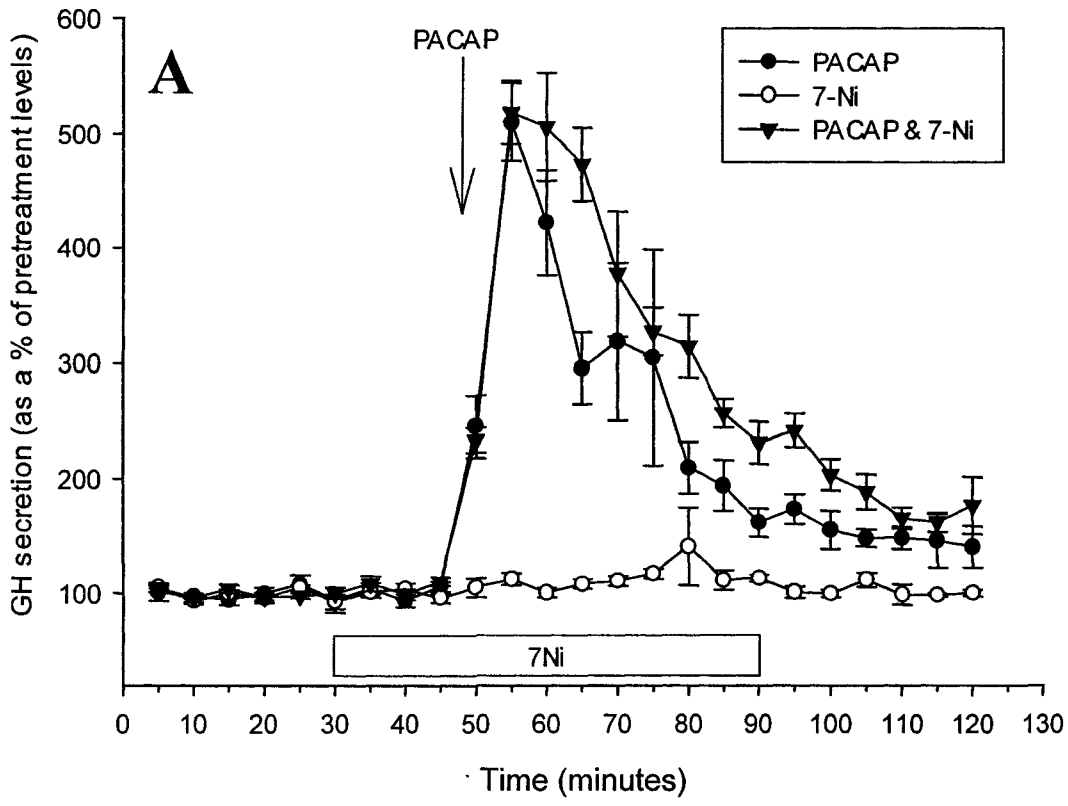



Figure 6.14. Effects of 7-Ni on PACAP-stimulated GH secretion. **A**. Temporal characteristics of the GH response to PACAP (10 μM), either alone or in the presence of 7-Ni (1 μM). The arrow indicates the estimated commencement of hormone release response to the 5-min PACAP pulse and the open bar indicates the duration of exposure to 7-Ni. Pooled results (mean ± SEM; n=6 columns each) from 3 replicate experiments performed in May are presented. Average pre-treatment GH values for this set of experiments are 27.8 ± 0.8 ng/ml. **B**. Quantified GH responses are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only group. A number sign indicates a significant difference ($p < 0.05$) from the 7-Ni only treatment group.



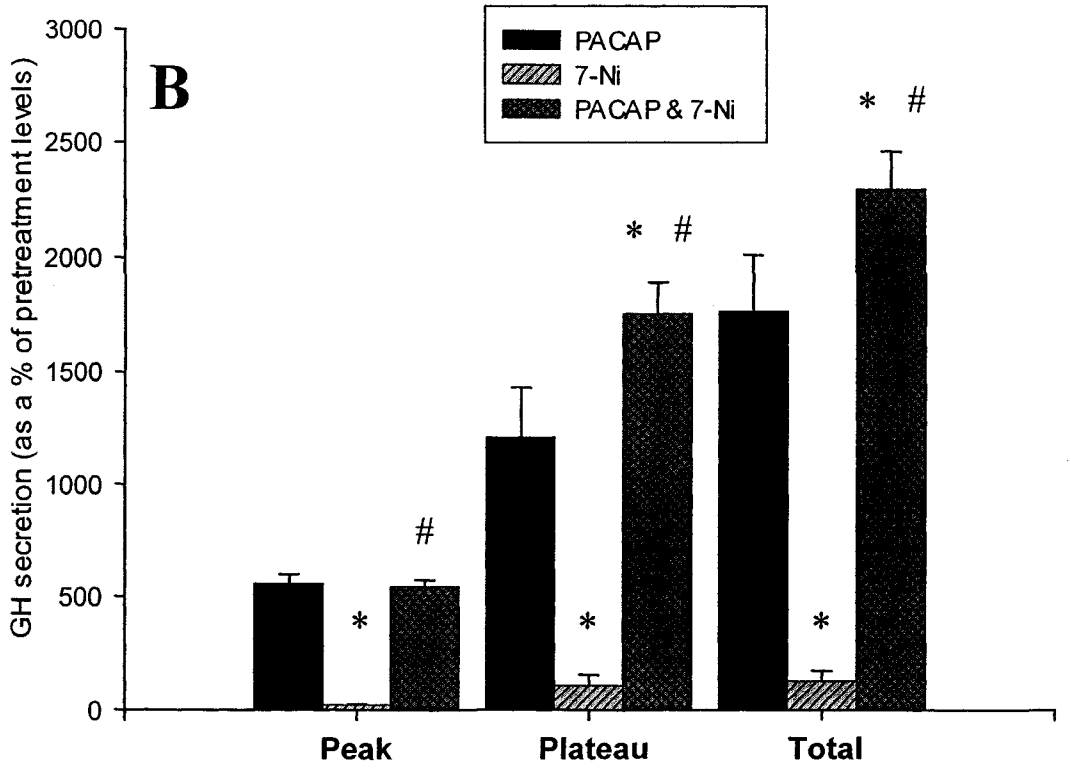
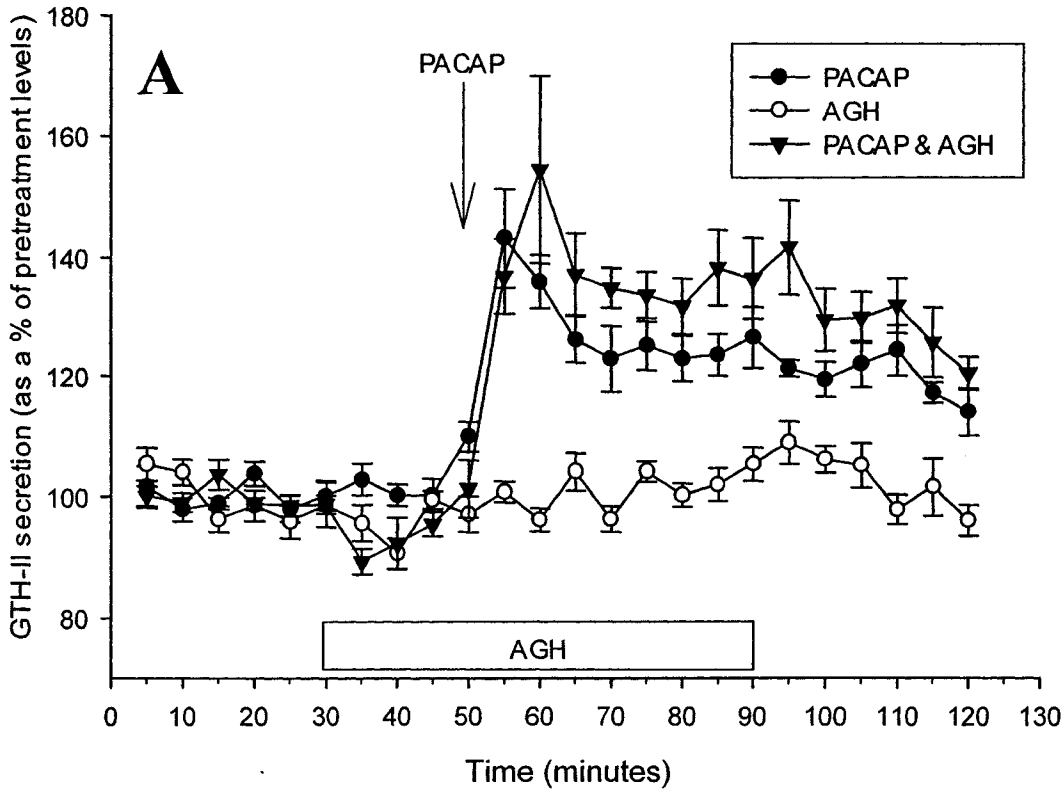



Figure 6.15. Effects of AGH on PACAP-stimulated GTH-II secretion. **A**. Temporal characteristics of the GTH-II response to PACAP (10 μM), either alone or in the presence of AGH (1 mM). The arrow indicates the estimated commencement of hormone release response to the 5-min PACAP pulse and the open bar indicates the duration of exposure to AGH. Pooled results (mean ± SEM; n=6 columns each) from 3 replicate experiments performed in May are presented. Average pre-treatment GTH-II values for this set of experiments are 3.1 ± 0.1 ng/ml. **B**. Quantified GTH-II responses are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only group. A number sign indicates a significant difference ($p < 0.05$) from the AGH only treatment group.



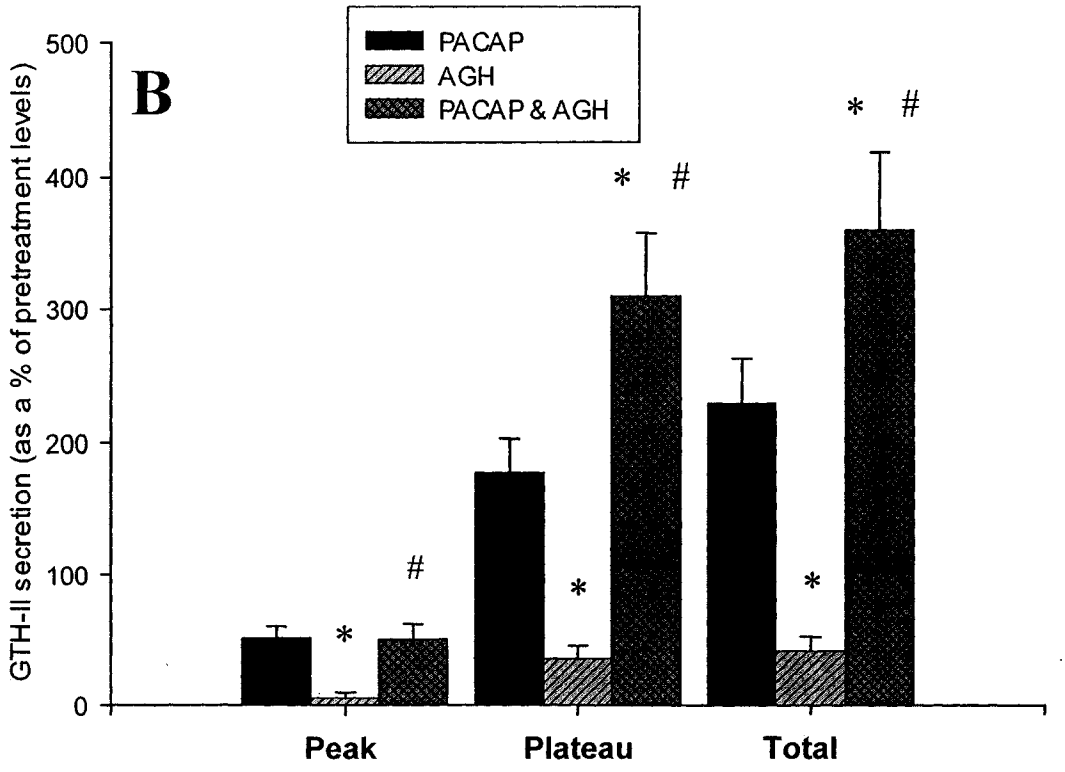
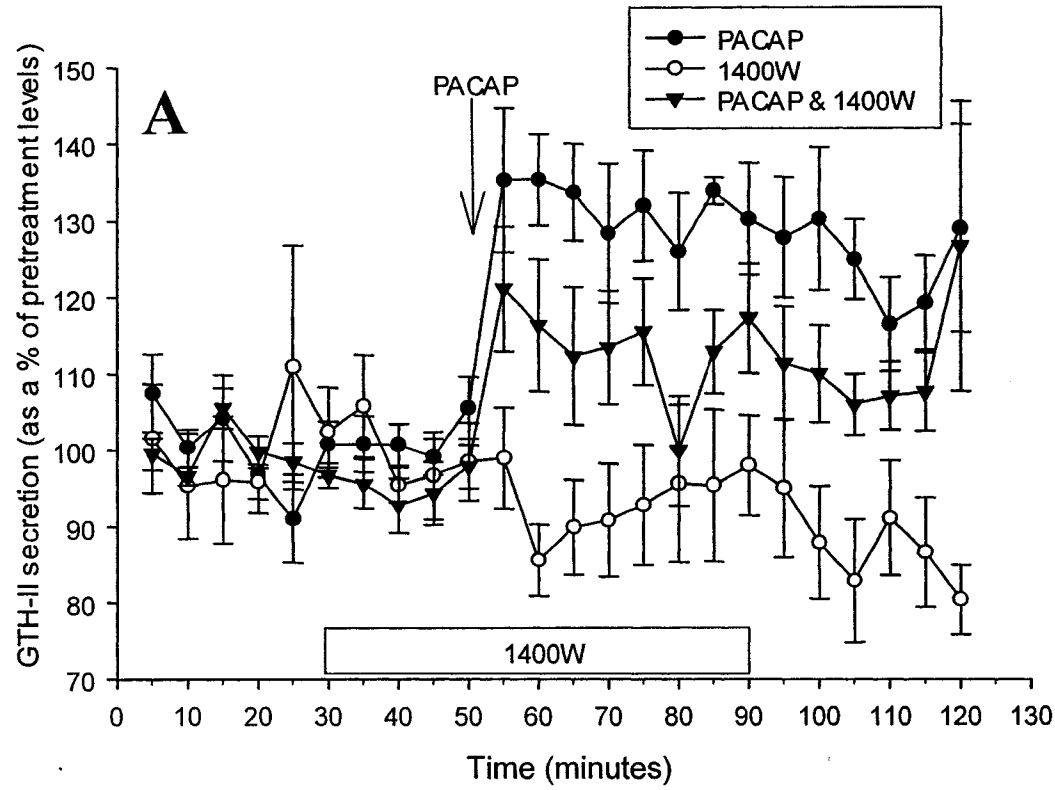



Figure 6.16. Effects of 1400W on PACAP-stimulated GTH-II secretion. **A**. Temporal characteristics of the GTH-II response to PACAP (10 μM), either alone or in the presence of 1400W (1 μM). The arrow indicates the estimated commencement of hormone release response to the 5-min PACAP pulse and the open bar indicates the duration of exposure to 1400W. Pooled results (mean ± SEM; n=6 columns each) from 3 replicate experiments (performed twice in June and once in July) are presented. Average pre-treatment GTH-II values for this set of experiments are 3.1 ± 0.1 ng/ml. **B**. Quantified GTH-II responses are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only group. A number sign indicates a significant difference ($p < 0.05$) from the 1400W only treatment group.



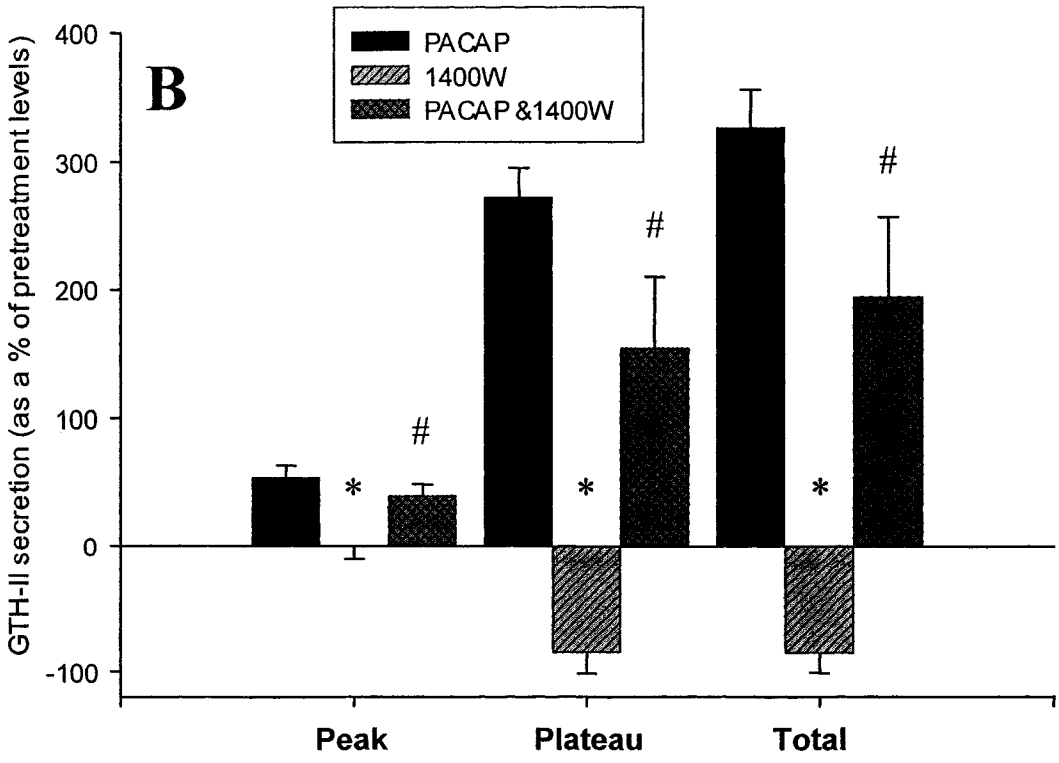
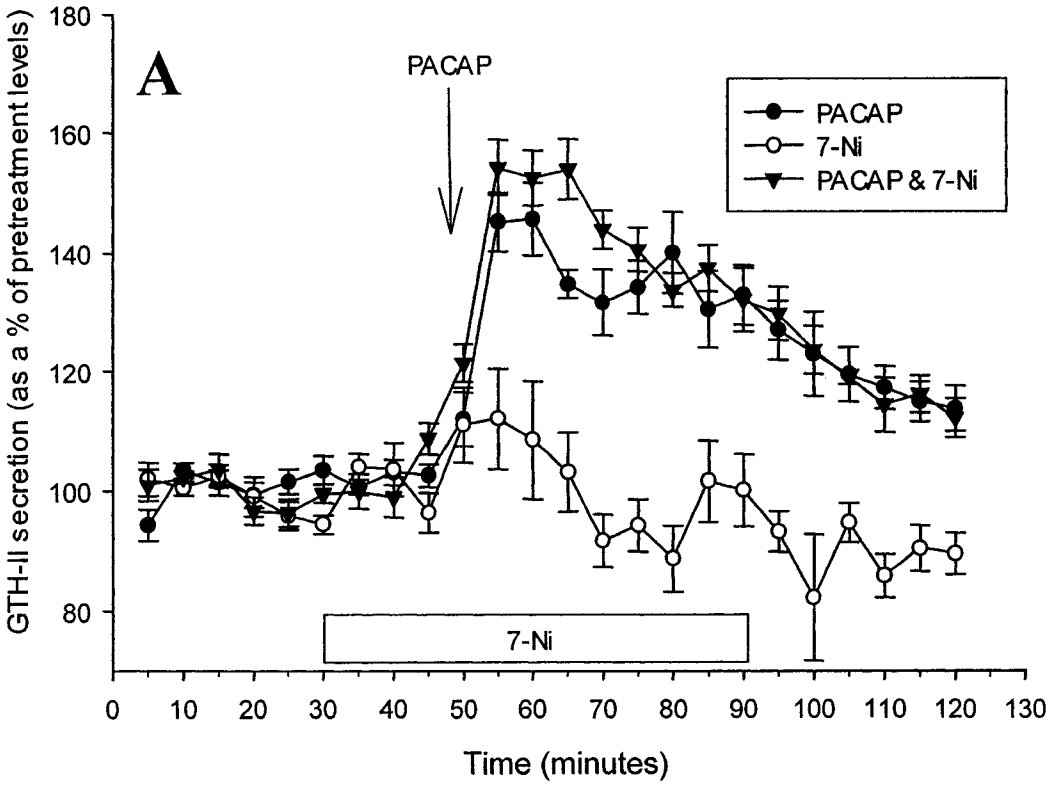
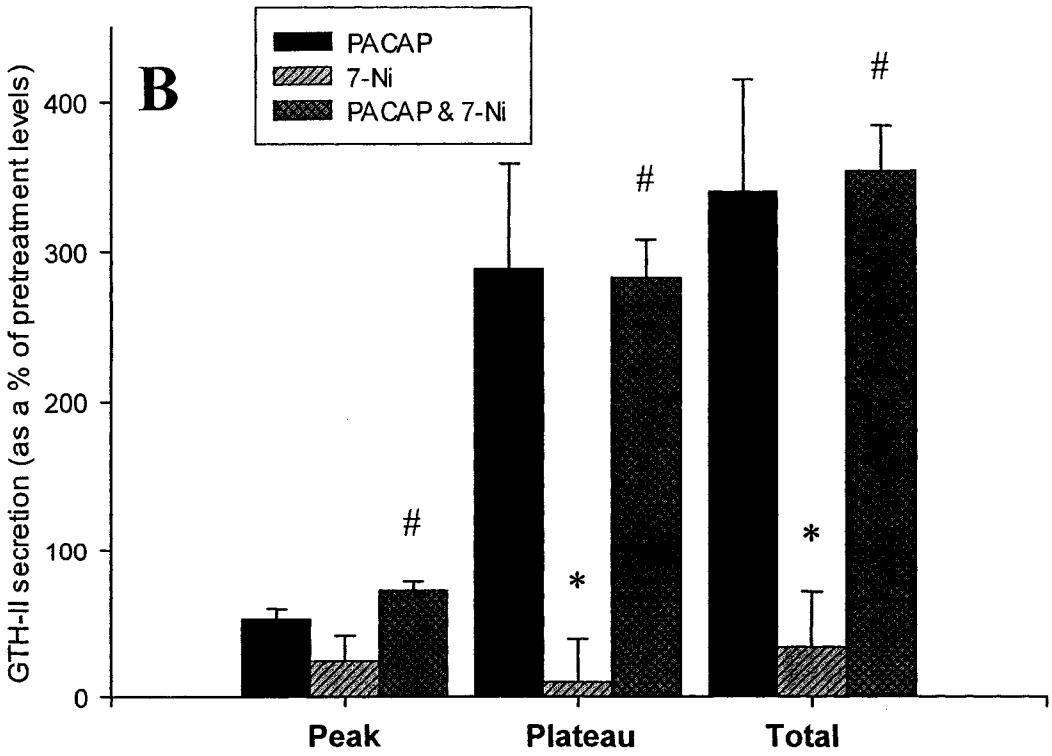



Figure 6.17. Effects of 7-Ni on PACAP-stimulated GTH-II secretion. **A**. Temporal characteristics of the GTH-II response to PACAP (10 μM), either alone or in the presence of 7-Ni (1 μM). The arrow indicates the estimated commencement of hormone release response to the 5-min PACAP pulse and the open bar indicates the duration of exposure to 7-Ni. Pooled results (mean ± SEM; n=6 columns each) from 3 replicate experiments performed in May are presented. Average pre-treatment GTH-II values for this set of experiments are 3.1 ± 0.1 ng/ml. **B**. Quantified GTH-II responses are presented. A star indicates a significant difference ($p < 0.05$) from the PACAP only group. A number sign indicates a significant difference ($p < 0.05$) from the 7-Ni only treatment group.



*6.4 Discussion*

NO has previously been shown to be an effective stimulator of GH release in goldfish (Uretsky et al., 2001). Consistent with such findings, application of SNP reliably stimulated GH secretion in the present study. Although SNP-stimulated GH release has been shown to involve a downstream GC/cGMP signalling component (Uretsky, 2001), the role of $Ca^{2+}$ in mediating the GH response to NO has not been investigated. In addition, while the involvement of NO in GnRH action on GH release in goldfish is relatively well studied, its role in mediating the hypophysiotropic actions of other neuroendocrine systems is largely unknown or unclear in this animal model system. In this study, the role of extracellular and intracellular $Ca^{2+}$ in NO-stimulated GH release were investigated as was the role of NO in mediating the GH and GTH-II release responses elicited by PACAP. It was found that NO requires intracellular $Ca^{2+}$ to elicit GH release and the NOS/NO system may play a role in modulating PACAP-evoked GH and GTH-II release in goldfish.

*6.4.1 Extracellular $Ca^{2+}$ involvement in NO action*

At the concentration used (50 μM), $Cd^{2+}$ is a highly effective blocker of VSCC (Van Goor et al., 1996; Wong et al., 2001). The inability of $Cd^{2+}$ to attenuate GH secretion by SNP indicates that extracellular $Ca^{2+}$ entry via VSCC is not an important element in NO-induced GH release. Although it is tempting to conclude that extracellular $Ca^{2+}$ does not play a role in mediating NO action on GH secretion, this may be premature. It is known that other channels also allow $Ca^{2+}$ entry, such as the capacitive $Ca^{2+}$ entry channels (Birnbaumer et al., 2000)

and the non-specific ion-passing noncapacitative $Ca^{2+}$ entry channels, TRPC6 (Taylor, 2003). Thus, one cannot rule out that other means of extracellular $Ca^{2+}$ entry may be required by NO to elicit GH secretion. The observation that another, more general, blocker of extracellular $Ca^{2+}$ entry, $Co^{2+}$, abolished GH secretion by SNP in static incubation experiments in preliminary studies (data not shown) is consistent with such a hypothesis. Thus, the possibility that in this system, extracellular $Ca^{2+}$ is required but is not entering the cell through VSCC should be tested in future studies.

### *6.4.2 Intracellular $Ca^{2+}$ in NO-evoked GH secretion*

Results presented in this study strongly indicate that intracellular $Ca^{2+}$ stores participate in mediating NO-evoked GH release. The observation that TMB-8 abolished the GH secretion response to SNP suggests an important role of $Ca^{2+}$ release from intracellular stores in this signalling cascade. In mammalian studies, NO has been shown to mobilize $Ca^{2+}$ from intracellular stores leading to GH (Pinilla et al., 1999), and LH and FSH release (Pinilla et al., 1998); thus, the above conclusion is not without support. However, given that TMB-8 completely abolished the response to SNP, it might be argued that this drug was preventing secretion per se, and not just the release of intracellular $Ca^{2+}$. In previous experiments, TMB-8 treatment did not reduce GH secretion responses to ionomycin and to a PKC-activating phorbol ester (Johnson and Chang, 2000). Thus, the action of TMB-8 is likely specific to the inhibition of intracellular $Ca^{2+}$ release channels and is not acting on downstream exocytosis elements in goldfish somatotropes.

Previous studies in goldfish somatotropes have revealed that, in addition to the mitochondria, at least three pharmacologically distinct intracellular $Ca^{2+}$ stores participate in the control of hormone secretion; these are the caffeine-sensitive, ryanodine-sensitive and $IP_3$-sensitive stores (Section 1.1.1.1). This study also provides insight into the identity of the intracellular $Ca^{2+}$ store involved in NO-induced GH release. The ability of pre-treatment with caffeine, but not with ryanodine, to inhibit SNP-stimulated GH release indicates that NO action in goldfish somatotropes is mediated by the caffeine-sensitive $Ca^{2+}$ store and not by the ryanodine-sensitive pool. In support of this conclusion, the participation of a caffeine-sensitive store in mediating stimulatory NO actions on GH and LH release in mammals has also been reported (Pinella et al., 1998, 1999). On the other hand, the involvement of ryanodine-sensitive $Ca^{2+}$ stores in GH release in goldfish is dependent on the seasonal reproductive conditions of the animal (Johnson and Chang, 2002; Section 4.3). The experiments with ryanodine were all performed with primary pituitary cell cultures prepared from fish late in the spawning season (May and June), a time when ryanodine-sensitive stores have been shown to be inactive in mediating cGnRH-II-stimulated GH release (Johnson and Chang, 2002). Thus it would be prudent to only conclude that NO does not require the use of ryanodine-sensitive $Ca^{2+}$ stores in goldfish during the spawning season. Whether ryanodine-sensitive $Ca^{2+}$ stores plays a role in NO action at other stages of the sexual reproductive cycle remains to be determined. Likewise, clarification of the role of $IP_3$-sensitive $Ca^{2+}$ stores and mitochondria in mediating NO stimulation of goldfish GH release awaits future studies.

The inability of SNP to cause an increase in GH release in the presence of Tg and BHQ strongly suggests that NO activates a $Ca^{2+}$ store(s) that is refilled by Tg- and BHQ-sensitive SERCA's. Although several studies point to an inhibitory influence of NO on the release of $Ca^{2+}$ from SERCA-refilled stores (Pernollet et al., 1996; Yuan et al., 1997; Ishii et al., 1998) evidence also exists for its ability to mobilize release from these stores (Willmott et al., 1995; Volk et al., 1997). These latter findings support our results.

Whether the caffeine-sensitive pool utilized by NO is identical to these SERCA-refilled pools has not been directly evaluated in the present series of experiments but some speculations can be made. Previous studies have demonstrated that Tg- and BHQ-sensitive SERCA's do not play the same role in modulating PACAP-stimulated, $Ca^{2+}$-dependent GH release (Chang et al., 2003; Sections 1.1.1.1 and 4.4). Furthermore, sGnRH, cGnRH-II and PACAP all utilize caffeine-sensitive $Ca^{2+}$ stores in their GH-releasing actions but only the PACAP effects are attenuated by BHQ (Chang et al., 2003). Thus, if PACAP and the two GnRHs target the same population of somatotropes, then it is tempting to hypothesize that the caffeine-, Tg- and BHQ-sensitive pools involved in NO action may not be one and the same store. Regardless, it is likely that these stores are linked or interrelated in terms of $Ca^{2+}$ flow. Whether mitochondrial $Ca^{2+}$ stores participate in NO-stimulated GH release has not been examined in this study but is a topic that needs to be investigated. The mitochondrial $Ca^{2+}$ store and mitochondrial $Ca^{2+}$ buffering have been shown to be important in downstream NO actions (Section 6.1.4), as well as in the NO-dependent sGnRH

action on GH secretion (Johnson, 2000; Uretsky et al., 2003). Thus, it is likely that NO action in goldfish somatotropes also involves interaction(s) with this $Ca^{2+}$ buffering and storage organelle.

*6.4.3 Interactions between NO and caffeine in GH release*

Caffeine is an effective mobilizer of intracellular $Ca^{2+}$ and secretagogue in goldfish somatotropes (Johnson and Chang, 2002). Despite evidence in the literature suggesting that $Ca^{2+}$ signalling may also activate downstream NOS/NO components, data from the present study suggest that caffeine-induced GH release do not utilize a NOS/NO pathway. NO scavenging by PTIO did not inhibit caffeine-stimulated release. Surprisingly, PTIO increased the magnitude of the GH release response to caffeine. This suggests that NO has stimulatory, as well as potentially inhibitory, influences on goldfish somatotropes. An inhibitory action of NO on caffeine-induced $Ca^{2+}$ release has been reported (Li et al., 2000). Furthermore, an AC isoform that is inhibited by NO has also been discovered recently (Freeman and MacNaughton, 2004). Caffeine has been shown to have a dual function within the cell, one as a PD inhibitor and the other to mobilize intracellular $Ca^{2+}$ (Wong et al., 2001). Thus if there is an endogenous level of NOS activity and NO production present, removal of NO by PTIO would remove the above described negative influences exerted by NO on caffeine, enhancing caffeine's action on either one or both of these signalling pathways leading to GH release. The source of this "negatively regulating" NO is not known.

The relationship between caffeine and NO appears to be complex. NO may stimulate $Ca^{2+}$ release from a caffeine-sensitive store to promote GH release but NO may act to attenuate caffeine-stimulated GH secretion.

*6.4.4 PACAP-induced GH and GTH-II secretion and the involvement of NO*

Previous studies showed that the use of NOS inhibitors in static incubation experiments did not affect PACAP-stimulated GH release. In the present series of cell column perifusion experiments, application of two NO scavengers, PTIO and rutin hydrate, as well as three NOS inhibitors, AGH, 7Ni and 1400W, did not inhibit the GH response to PACAP. These results confirm that the signal transduction pathway utilized by PACAP to stimulate GH release does not involve NOS/NO. Similarly, PTIO, rutin hydrate, AGH, 7Ni and 1400W did not reduce the GTH-II response to PACAP, implying that NO also does not play a role in the PACAP signalling cascade leading to hormone release in gonadotropes.

Interestingly, rutin hydrate induced a significant potentiation of the GH response to PACAP and a similar tendency was also observed for the GTH-II response. Likewise, PACAP-elicited GH and GTH-II release were significantly increased in the presence of 7Ni and AGH, respectively. These results resemble the findings that NO scavenging enhanced caffeine-induced GH release (Figure 6.4; Section 6.4.3). Since PACAP has also been shown to act through activation of AC (Wong et al., 2000) and a caffeine-sensitive $Ca^{2+}$ pool to increase GH and GTH-II release (Section 4.2), these findings are consistent with the presence of a negatively modulating NO element that can interact with AC/cAMP-dependent

transduction elements and caffeine-sensitive $Ca^{2+}$ signalling components to affect hormone release responses in goldfish somatotropes and gonadotropes.

An interesting observation with the potentiation effects of NOS inhibitors and NO scavengers is that these agents generally did not affect the peak hormone responses but only the plateau phase responses to PACAP. It may be that the NOS enzyme(s) responsible for this effect only becomes active after a certain intracellular component reaches a high enough concentration. The most likely candidate for such an intracellular component is $Ca^{2+}$ since it is required for both NOS activation and PACAP-induced GH and GTH-II release. Alternatively, the inhibitory NO influence may involve paracrine actions of PACAP (and caffeine) on other cell-types which then generate the "inhibitory NO" and/or another inhibitory signalling molecule(s) via NO-dependent mechanisms to exert negative modulation on the somatotropes and gonadotropes. Regardless of how the mechanisms by which the "inhibitory NO" signal is generated, activation of such an "inhibitory NOS/NO" element may function in limiting the total magnitude of a prolonged response to continual stimulation. It is of note that the profile of NOS inhibitors and/or NO scavengers that were able to cause this potentiating effect on hormone responses to caffeine and PACAP are not identical. Basal GH and GTH-II secretion are also generally not drastically affected by treatments with these NOS inhibitors and NO scavengers. Thus it is unlikely that there is a common and highly constitutively active NOS that is responsible for generating this inhibitory NO signal. Given that the nNOS- and eNOS-selective inhibitor 7Ni, but not the iNOS-selective inhibitor 1400W, potentiated the GH response to

PACAP, the "inhibitory NO" influence responsible for regulating GH responses was probably generated through a n/eNOS-like activity. On the other hand, only the broad spectrum NOS inhibitor AGH similarly affected the PACAP-induced GTH-II response; thus, the possible identity of the NOS involved in the generation of the inhibitory NO influence on gonadotropes is indeterminant.

*6.4.5 Summary*

This study has revealed the relationships between NO and $Ca^{2+}$ in regulating GH secretion in goldfish. It also provided information on the role of NO in PACAP-induced GH and GTH-II secretion. Schematics of the GH and GTH-II signalling mechanisms involving NO, as revealed in this study, are presented in Figures 6.18 and 6.19, and briefly described below.

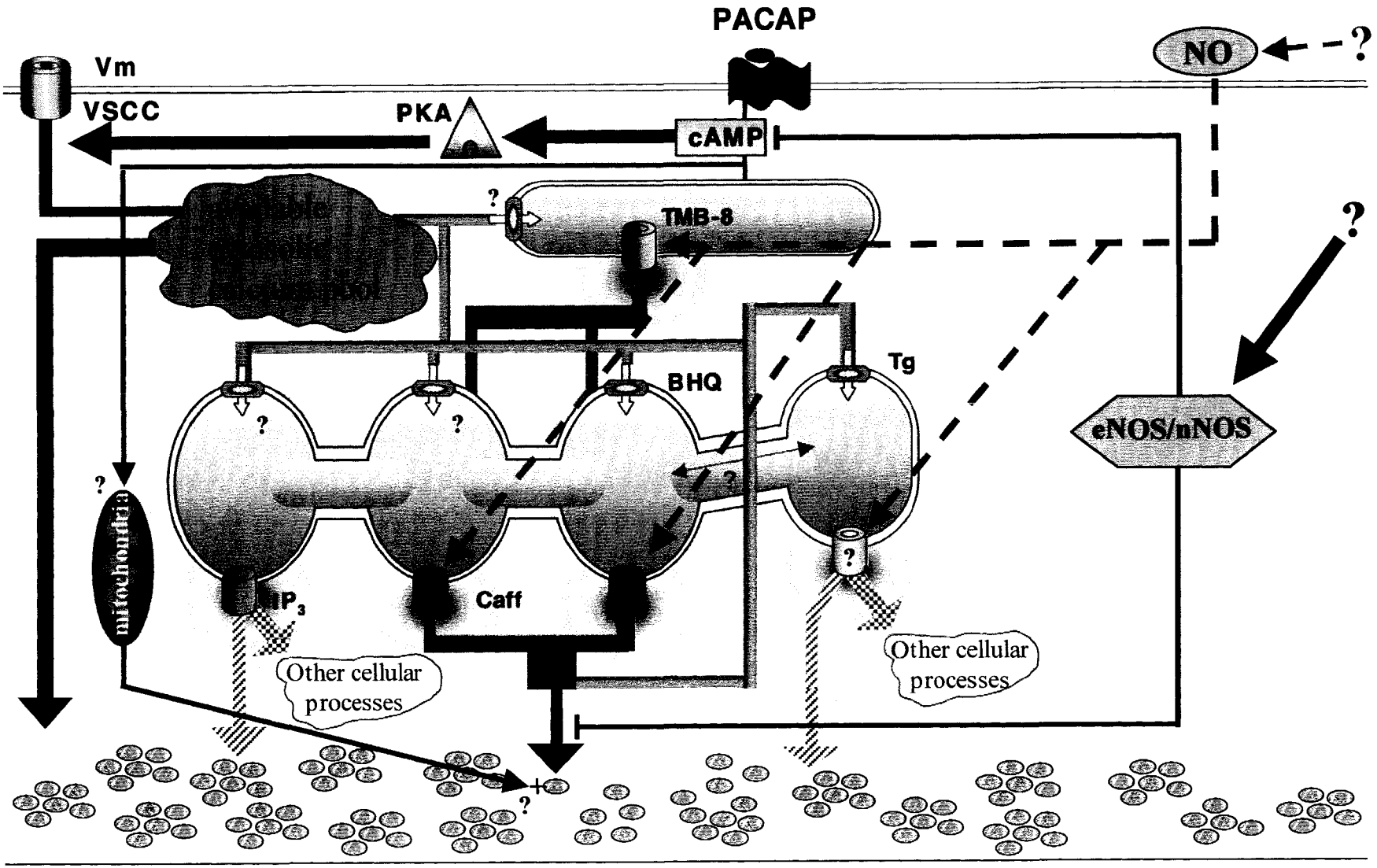
SNP-stimulated GH release appears to be independent of extracellular $Ca^{2+}$ entry through VSCC but is dependent on intracellular $Ca^{2+}$ stores. The specific stores involved are sensitive to release by caffeine and refilled by Tg- and BHQ-sensitive SERCA. However, ryanodine-sensitive $Ca^{2+}$ stores do not appear to be involved, at least during times of spawning. Interestingly, NO is also able to inhibit caffeine-sensitive GH release; this is in addition to the stimulatory function already attributed to this molecule. The inhibitory NO influence may be acting in a paracrine fashion.

PACAP-stimulated GH and GTH-II release are not mediated by NOS/NO; however the hormone responses can be attenuated by an inhibitory NO action. The results presented herein suggest that the isoform involved in the inhibition of

GH release is an eNOS or iNOS whereas the one involved in GTH-II inhibition is inconclusive.

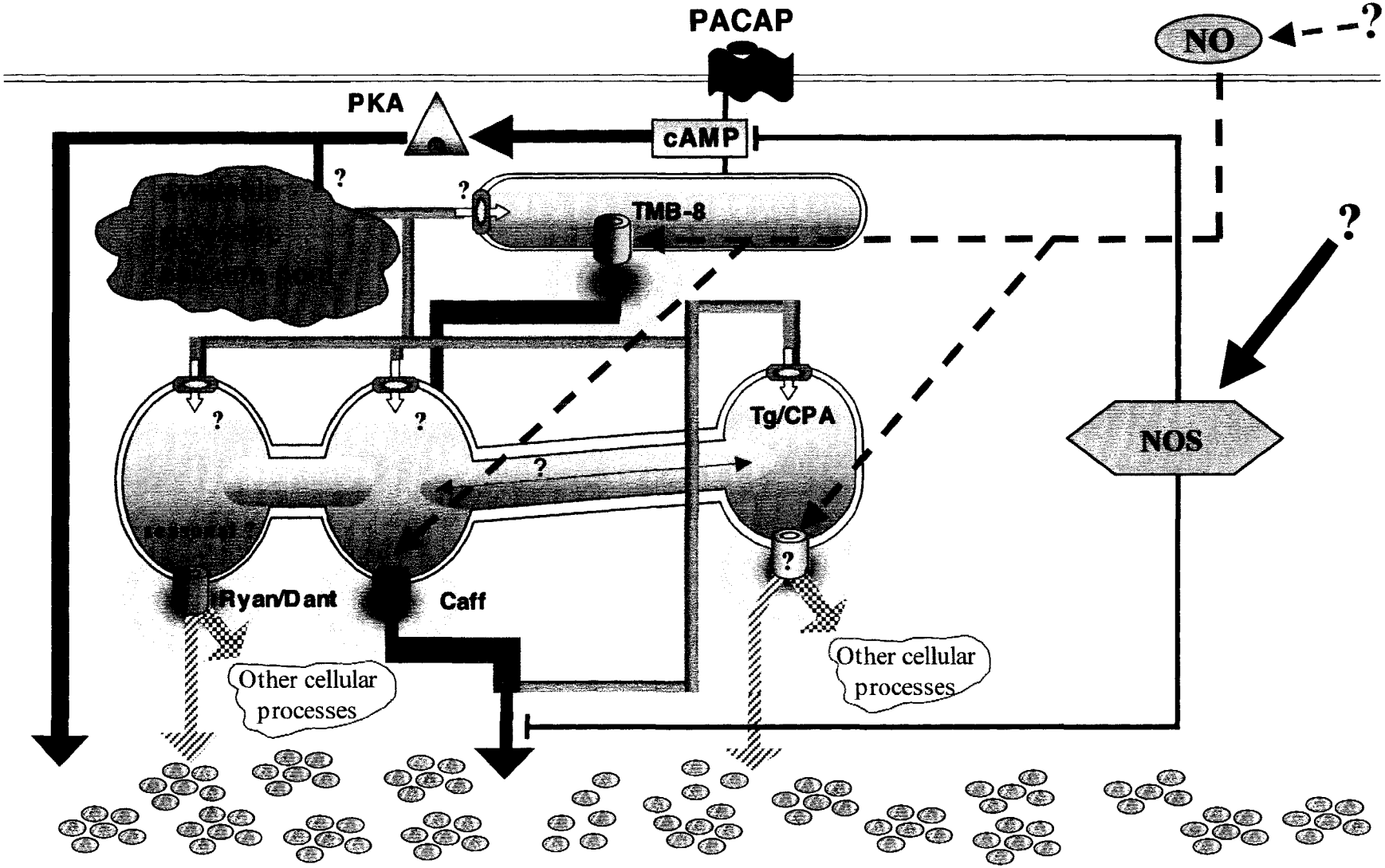
This study is one of a just a few that have looked at the relationship between $Ca^{2+}$ and NO in GH secretion. It also provides novel insights into the interactions between NO and PACAP-stimulated GH and GTH-II secretion. Just as importantly, it illustrates the complexity in signal transduction cascades and the importance of their interactions in the control of pituitary hormone release.

Figure 6.18. A schematic of the known and proposed intracellular $Ca^{2+}$ stores used by NO and PACAP to elicit GH secretion. Dashed purple lines depict NO signalling pathways leading to hormone release. Solid red lines indicate PACAP activation of AC/cAMP/PKA and their targets. Solid green only lines indicate the $Ca^{2+}$ signalling pathway involved in PACAP action on hormone release. Green & pink coloured lines indicate a $Ca^{2+}$ refilling route. Thick blue arrows indicate an unknown signalling mechanism leading to GH secretion. Dark green lines indicate entry of extracellular $Ca^{2+}$ into the cell. Blue striped arrows depict mechanisms not present in the pathway leading to PACAP-induced GH release. Green hatched arrows indicate $Ca^{2+}$ release involved in other cellular processes. The solid blunt-ended line depict "inhibitory" NO influence on PACAP- and caffeine-induced GH secretion. Whether the indicated pharmacologically identified $Ca^{2+}$ pools adequately represents all the TMB-8-sensitive pools is not known, thus a separate (though functionally linked) TMB-8-sensitive pool is depicted.




GH Exocytosis

Figure 6.19. A schematic of the known and proposed intracellular $Ca^{2+}$ stores used by NO and PACAP to elicit GTH-II secretion. Dashed purple lines depict NO signalling pathways leading to hormone release. Solid red lines indicate PACAP activation of AC/cAMP/PKA and their targets. Solid green only lines indicate $Ca^{2+}$ signalling pathways involved in PACAP action on hormone release. Green & pink coloured lines indicate a $Ca^{2+}$ refilling route. Thick blue arrows indicate an unknown signalling mechanism leading to GTH-II secretion. Blue striped arrows depict mechanisms not present in the pathway leading to PACAP-induced GH release. Green hatched arrows indicate $Ca^{2+}$ release involved in other cellular processes. The solid blunt-ended line depict "inhibitory" NO influence on PACAP- and caffeine-induced GTH-II secretion. Whether the indicated pharmacologically identified $Ca^{2+}$ pools adequately represents all the TMB-8-sensitive pools is not known, thus a separate (though functionally linked) TMB-8-sensitive pool is depicted.

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