REVIEW / SYNTHÈSE

Signal transduction mechanisms mediating secretion in goldfish gonadotropes and somatotropes

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Abstract: The intracellular signal transduction mechanisms mediating maturational gonadotropin and somatotropin secretion in goldfish are reviewed. Several major signaling mechanisms, including changes in intracellular [Ca^{2+}], arachidonic acid cascades, protein kinase C, cyclic AMP/protein kinase A, calmodulin, nitric oxide, and Na^+/H^+ antiport, are functional in both cell types. However, their relative importance in mediating basal secretion and neuroendocrine-factor-regulated hormone release differs according to cell type. Similarly, agonist- and cell-type-specificity are also present in the transduction pathways leading to neuroendocrine factor-modulated maturational gonadotropin and somatotropin release. Specificity is present not only in the actions of different regulators within the same cell type and with the same ligand in the two cell types, but this also exists between isoforms of the same neuroendocrine factor within a single cell type. Other evidence suggests that function-selectivity of signaling may also result from differential modulation of Ca^{2+} fluxes from different sources. The interaction of different second messenger systems provide the basis by which regulation of maturational gonadotropin and somatotropin release by multiple neuroendocrine factors can be integrated at the target cell level.

Key words: Ca^{2+} signaling, cAMP, PKC, arachidonic acid, NO.

Résumé : Cet article passe en revue les mécanismes de transduction de signaux intracellulaires qui interviennent dans la sécrétion de l'hormone somatotrope et de la gonadotrophine de maturation chez le cyprin doré. Plusieurs mécanismes de signalisation importants, tels les changements de la concentration intracellulaire du Ca^{2+}, les cascades de l'acide arachidonique, la protéine-kinase C, l'AMP cyclique et la protéine-kinase A, la calmoduline, l'oxyde nitrique et l'antiport Na^+/H^+, sont fonctionnels dans les cellules somatotropes et les cellules gonadotropes. Cependant, leur importance relative dans la sécrétion basale et la sécrétion réglée par un facteur neuroendocrinien diffère selon le type cellulaire. De même, les voies de transduction entraînant la sécrétion de l'hormone somatotrope et de la gonadotrophine de maturation réglée par des facteurs neuroendocriniens diffèrent selon l'agoniste et le type cellulaire. Non seulement les effets de différents régulateurs dans un type cellulaire et les effets d'un ligand dans les deux types de cellules diffèrent-ils, mais les isoformes d'un facteur neuroendocrinien ont des effets différents dans un même type de cellules. D'autres résultats suggèrent que la sélectivité de fonction de la signalisation pourrait également être attribuable à une modulation différentielle des flux de Ca^{2+} de différentes sources. L'interaction de différents systèmes à second messager assure la coordination de la régulation de la sécrétion de l'hormone somatotrope et de la

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Abbreviations: AA, arachidonic acid; BHQ, 2,5-di(t-butyl)-1,4-hydroquinone; CaM, calmodulin; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; DA, dopamine; DAG, diacylglycerol; GH, growth hormone; GnRH, gonadotropin-releasing hormone; GTH-II, maturational gonadotropin; ICa, calcium current; InsP, inositol phosphate; NE, norepinephrine; NOS, nitric oxide syntase; PACAP, pituitary adenylate cyclase activating-polypeptide; PKA, protein kinase A; PKC, protein kinase C; PLA_2, phospholipase A2; PLC, phospholipase C; SKF83566, 7-bromo-8-hydroxy-3-methyl-e-phenyl-2,3,4,5-tetrahydro-1H-3-benazepine; SRIF, somatostatin; VSCC, voltage-sensitive Ca^{2+} channels.

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gonadotrophine de maturation par plusieurs facteurs neuroendocriniens dans les cellules cibles.

Mots clés : signalisation par le Ca^{2+}, AMPc, protéine-kinase C, PKC, acide arachidonique, oxyde nitrique, NO.

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Introduction

In teleosts, as in other vertebrates, anterior pituitary hormone secretion is controlled by multiple neuroendocrine factors. However, a classical median eminence-portal system is absent in teleosts; instead, hypophysiotropic neurons terminate within the pars distalis of the adenohypophysis. Among the many teleost systems in which neuroendocrine regulation of pars distalis hormone release have been studied, control of the luteinizing-hormone-like maturational gonadotropin (GTH-II) and somatotropin (GH) release in goldfish (*Carassius auratus*) are some of the best characterized. In this species, GTH-II release is directly stimulated by two gonadoropin-releasing hormones (salmon (s)GnRH and chicken (c)GnRH-II), norepinephrine (NE), serotonin (5HT), neuropeptide Y (NPY), and activin/inhibin; inhibitory control is provided by dopamine (DA) via D2 receptors (Van Der Kraak et al. 1997; Peter and Yu 1997). Factors directly stimulating GH release include the two GnRHs, DA (via D1 receptors), GH-releasing hormone, and NPY; direct inhibitory influence on GH secretion is provided by somatostatin (SRIF), NE, and 5HT (Peng and Peter 1997; Peter and Chang 1997, 1999). Interestingly, the source of 5HT may be paracrine (from pituitary cells) rather than neuronal (from hypothalamic neurons) in nature (reviewed in Peter and Chang 1997, 1999); similarly, activin-like molecules may be produced in pituitary somatotropes and exert paracrine and autocrine influences on hormone release (Ge and Peter 1994; Yam et al. 1999). However, how the local release of 5HT and activin-like substances are regulated at the cellular and intracellular level by neuroendocrine factors is not well understood. Recently, pituitary adenylate cyclase-activating polypeptide (PACAP) has also been shown to directly stimulate GTH-II (J.P. Chang and A.O.L. Wong, unpublished) and GH secretion (Wong et al. 1998). How these neuroendocrine signals are mediated and integrated at the level of the pituitary cells is important for understanding the regulation of GTH-II and GH secretion. The presence of common neuroendocrine factors in the regulation of GTH-II and GH secretion also provides a model for examining how the bioactivity of an individual factor is transduced in two different cell types. In addition, the ability of two native GnRH peptides to stimulate GTH-II and GH secretion allows for the comparison of how two closely related peptides may exert their action at the intracellular level within a single cell type, as well as between two cell types.

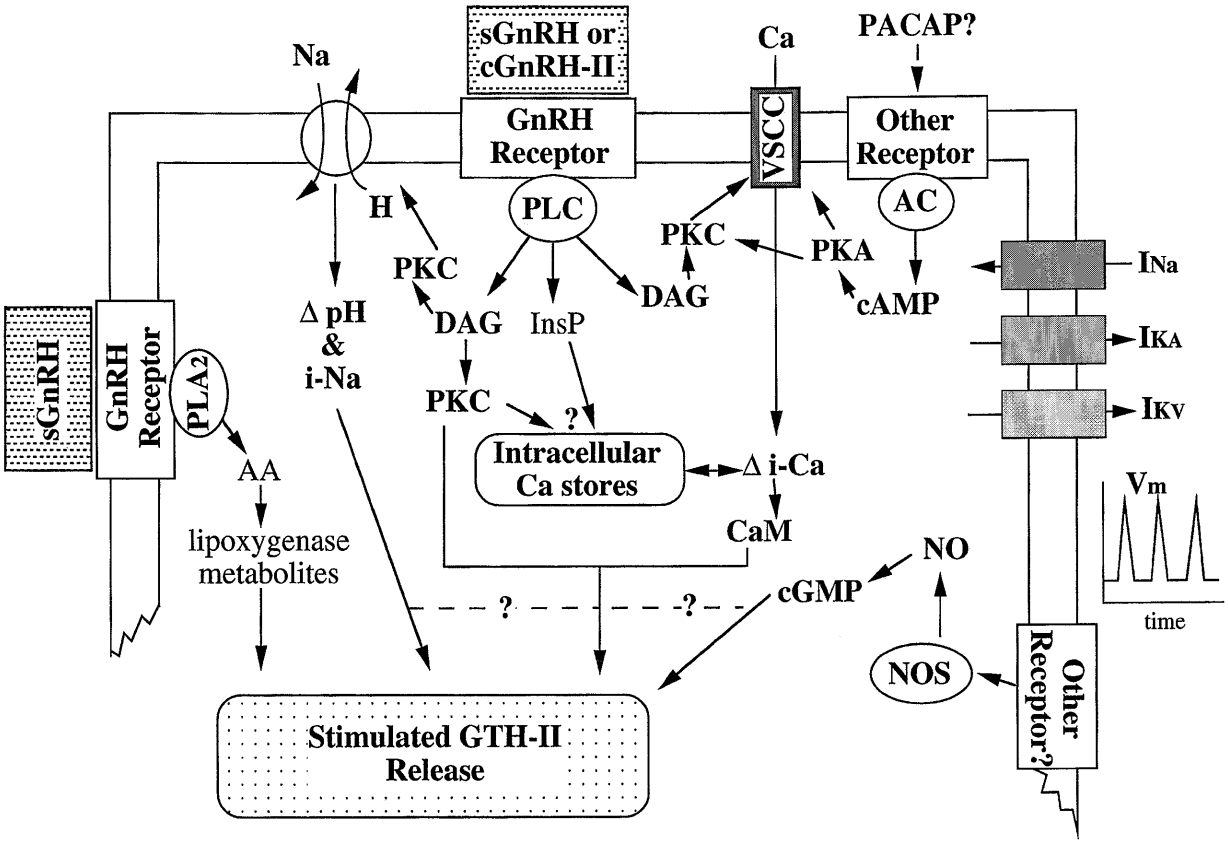
In this article, the signaling mechanisms of sGnRH, cGnRH-II, and DA on GTH-II and GH secretion in goldfish pituitary cells are first compared. Emphasis is placed on more current developments in these areas since these topics were last reviewed (Chang and Jobin 1994; Chang et al. 1996*b*, 1997). Recent results on the signal transduction mechanisms mediating the effects of SRIF, NE, and PACAP on GH secretion are also presented and reviewed. Where possible, the functional roles of some of the second messenger systems involved are discussed. In the summary section, the mechanisms by which multiple neuroendocrine regulators and multiple intracellular signalling pathways participate in the coordinated and physiological control of GTH-II and GH release, especially at various stages of gonadal maturation, are hypothesized and discussed.

sGnRH and cGnRH-II action on GTH-II and GH release

GnRH peptides and receptors

Although the presence of multiple GnRH forms in one species has been demonstrated in representative species in all vertebrate classes, including several teleost species, the general belief is that only one GnRH form acts as a hypophysiotropic factor (reviewed in Millar et al. 1997). However, in goldfish, sGnRH and cGnRH-II peptides, as well as their respective mRNAs, are present in the pituitary, and both GnRHs are released from nerve terminals within the pituitary. Results from studies on the brain distribution of GnRH protein and mRNA also support the view that both peptides act as hypophysiotropic neurohormones in goldfish (reviewed in Yu et al. 1998). The presence of GnRH receptors on both GTH-II and GH cells, the ability of sGnRH and cGnRH-II to stimulate GTH-II and GH release from pituitary cell preparations, as well as correlative changes in brain GnRH content during physiological modulations of serum GTH-II and GH levels, are consistent with a role of both sGnRH and cGnRH-II as neuroendocrine regulators of gonadotrope and somatotrope functions (reviewed in Chang and Jobin 1994; Chang et al. 1996*b*; Peng and Peter 1997). It has been suggested that GTH-II and GH cells possess different GnRH receptors, or suites of receptors (reviewed in Chang et al. 1996*b*; Illing et al. 1999). Recently, cDNAs encoding two goldfish GnRH receptors have been cloned, and their presence in the pituitary confirmed (Yu et al. 1998; Illing et al. 1999). These receptors share greater than 70% identity with one another, and both are seven-transmembrane-domain-containing, G-protein coupled receptors. These receptors also show differential selectivity for a number of synthetic artifical GnRH analogues in expression systems (Illing et al. 1999), suggesting that these receptors may be potentially functionally distinct. However, the two cloned goldfish GnRH receptors do not differentiate between sGnRH and cGnRH-II in functional analyses of phospholipase (PL)C coupling, inositol (Ins)P production, and intracellular $[Ca^{2+}]$ responses in mammalian cell expression systems, suggesting that the two cloned receptors recognize both native GnRHs equally (He et al. 1997*a*, 1997*b*; Illing et al. 1999). Similarly, radio-ligand binding studies with goldfish pituitary membrane preparations and hormone release experiments using GnRH analogs have been unable to reveal the presence of receptors selective for only one of the two native GnRHs (reviewed in

Fig. 1. Diagrammatic summary of the signal transduction pathways in the stimulatory regulation of GTH-II release in goldfish gonadotropes. The sGnRH and cGnRH-II receptor-mediated actions involve PLC, PKC, Ca^{2+} mobilization, VSCC, CaM, and Na^+/H^+ antiport; sGnRH utilizes an additional PLA_2/AA mechanism. Activation of adenylate cyclase/cAMP/PKA mechanisms positively modulates PKC- and VSCC-dependent GTH-II release, and is a possible means by which other neuroendocrine factors (perhaps PACAP) can enhance GnRH action or independently stimulate GTH-II secretion. A NO/cGMP stimulatory mechanism is also present but the neuroendocrine factor(s) utilizing this pathway has not been identified. Voltage-sensitive Na^+ and K^+ currents are present, and some gonadotropes exhibit spontaneous action potential firing, but the roles that these events play remain to be determined.



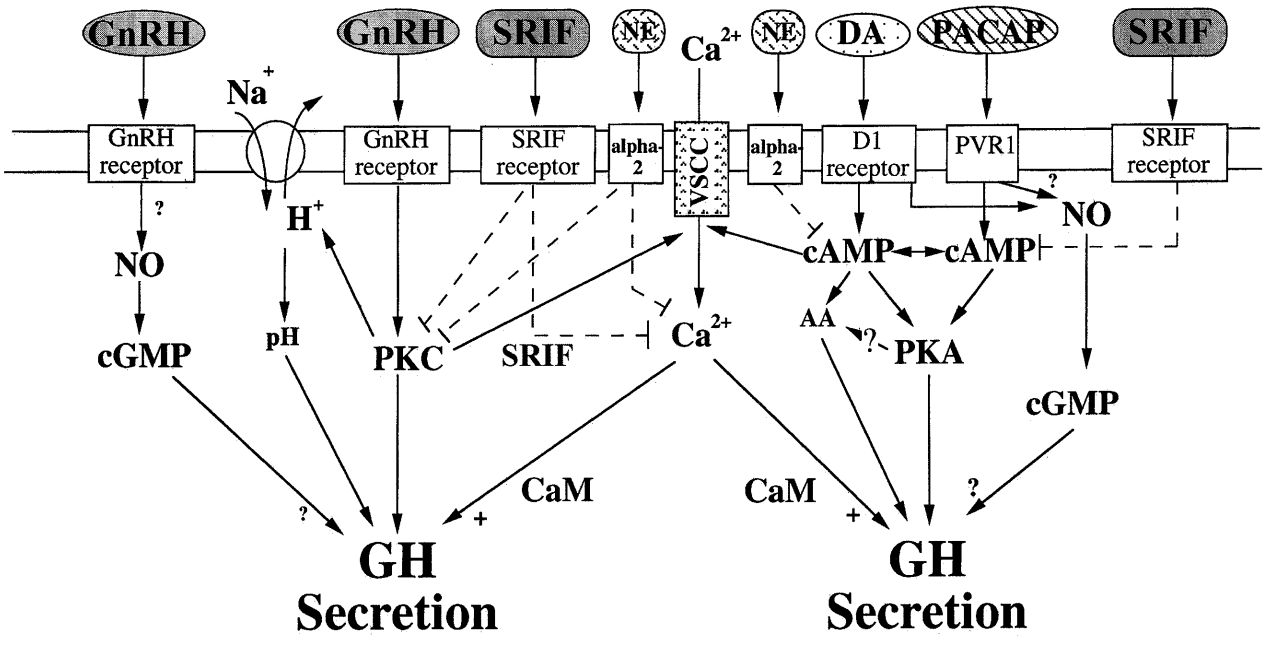
Chang and Jobin 1994). On the other hand, differential activity of sGnRH and cGnRH-II on GTH-II subunit mRNA synthesis (Khakoo et al. 1994) and dissimilar signal transduction activation in GTH-II release (reviewed below and summarized in Fig. 1) have been reported. These results strongly indicate that differential receptor-signal transduction-coupling must occur in the goldfish pituitary GnRH receptor systems, but how this is achieved is not known. At present, there is insufficient evidence (especially from results using native test systems) to assign the two cloned goldfish GnRH receptors into functional subtypes.

Calcium involvement

GTH-II release

Pharmacological data indicate that the availability of extracellular Ca^{2+} and its entry through membrane L-type voltage-sensitive Ca^{2+} channels (VSCC) are important for both the acute and prolonged GTH-II response to sGnRH and cGnRH-II (Chang et al. 1996*b*, 1997; Fig. 1). The presence of L-type VSCC (Van Goor et al. 1996*a*) and the ability of both sGnRH and cGnRH-II to increase intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in morphologically identified goldfish gonadotropes have been confirmed (Johnson et al. 1999*b*). The $[Ca^{2+}]_i$ response to GnRH has also been shown to be receptor mediated (Johnson et al. 1999*b*). Where examined, both sGnRH and cGnRH-II can induce elevations in $[Ca^{2+}]_i$ in the same gonadotrope (Johnson et al. 1999*b*), suggesting that individual gonadotropes respond to both GnRH peptides. Whether all gonadotropes have this property is not known. Results from recent hormone-release experiments with cells pretreated with BAPTA-AM substantiate the idea that increase in $[Ca^{2+}]_i$ is a vital component in the transduction pathway, leading to the secretion response to sGnRH and cGnRH-II (J.D. Johnson and J.P. Chang, unpublished). $[Ca^{2+}]_i$ measurement studies with mixed populations of dispersed goldfish pituitary cells (Chang et al. 1996*b*) and identified gonadotropes (Mollard and Kah 1996) reveal that the GnRH-induced increase in $[Ca^{2+}]_i$ can be reduced by pretreatment with VSCC inhibitors and Ca^{2+}-depleted medium. Although these results indicate that GnRH stimulates Ca^{2+} entry through VSCC in gonadotropes, direct evidence for GnRH activation of VSCC is lacking. Application of GnRH does not appear to increase spontaneous action potential firing and the magnitude of ionic current-flow through VSCC in identified gonadotropes in patch-clamp recording experiments (Van Goor 1997; C.J.H. Wong and J.P. Chang, unpublished.) In addition, Ca^{2+}-sensitive K^+ chan-

Fig. 2. Diagrammatic summary of the signal transduction pathways mediating the multifactorial neuroendocrine regulation of GH secretion by GnRH, DA, PACAP, SRIF, and NE in goldfish somatotropes. PKC- and cAMP-dependent pathways apparently access distinct releasable GH pools. PKC mediates GnRH action whereas cAMP participates in DA/D1- and PACAP/PVR1-stimulated GH secretion. Activation of PKC and cAMP also lead to increases in Ca^{2+} influx through VSCC and CaM-dependent hormone release. In addition, PKC activates a Na^+/H^+ antiport system, whereas cAMP-dependent mechanisms leads to mobilization of AA. Evidence suggests that a NO/cGMP cascade also participates in GnRH, D1, and PACAP stimulation of GH release, but how this pathway interacts with the other signaling pathway has not been determined. SRIF and NE (via alpha-2 receptors) inhibit GH responses by actions on PKC-, cAMP-, and Ca^{2+}-dependent GH release. Inhibitory actions are denoted by the blunt-ended dashed lines.



nels, an important element in the GnRH-induced activation of VSCC and extracellular Ca^{2+} entry in the rat gonadotrope model (reviewed in Stojilkovic and Catt 1995; Vergara et al. 1997), cannot be detected in goldfish gonadotropes (Van Goor et al. 1996*a*). Furthermore, apamin, an inhibitor of one class of Ca^{2+}-dependent K^+ channels, also does not alter the GTH-II response to sGnRH (P. Kwong and J.P. Chang, unpublished) and cGnRH-II (Van Goor et al. 1996*a*). Whether other K^+ currents present on gonadotropes (Van Goor et al. 1996*a*) are involved in mediating or modulating GnRH action, has not been determined (Fig. 1). How GnRH activates Ca^{2+} flux through VSCC in goldfish gonadotropes is not known.

Despite the fact that the GTH-II-releasing action of both GnRHs involves Ca^{2+}, the relative importance of different Ca^{2+} sources in sGnRH and cGnRH-II effects are not identical. The long-term GTH-II response to cGnRH-II is more sensitive to (and often abolished by) the reduction of $[Ca^{2+}]$ in the incubation medium and treatments with VSCC inhibitors, than that elicited by sGnRH (reviewed in Chang et al. 1996*b*; 1997). These results suggest that prolonged cGnRH-II-induced GTH-II release is more dependent on the availability of extracellular Ca^{2+}. Differential involvement of Ca^{2+} in short-term sGnRH and cGnRH-II action also appears to exist. Although both GnRH forms activate PLC and increase total InsP formation in mammalian cells expressing cloned goldfish GnRH receptors, and in mixed populations of dispersed goldfish pituitary cells, only sGnRH significantly elevates $InsP_3$ levels in the native test system (Chang et al. 1995; He et al. 1997*a*, 1997*b*; Illing et al. 1999). Since $InsP_3$ is a classical second messenger known to mobilize intracellular Ca^{2+} from endoplasmic reticular stores, these results would suggest that sGnRH-stimulated GTH-II release has a greater dependence on the integrity of $InsP_3$-sensitive intracellular Ca^{2+} stores. Surprisingly, neither sGnRH- nor cGnRH-II-stimulated GTH-II release is affected by pretreatment with thapsigargin, a Ca^{2+}/ATPase inhibitor known to interfere with refilling of $InsP_3$-sensitive Ca^{2+} stores (Johnson et al. 1998). Whether other thapsigargin-insensitive intracellular Ca^{2+} stores are involved in mediating GnRH stimulation of GTH-II release, and that the two GnRHs are differentially linked to these stores, are currently under investigation. Preliminary evidence suggests that these possibilities are likely. For example, while BHQ (another Ca^{2+}/ATPase inhibitor) appears to be particularly effective in reducing sGnRH-induced GTH-II response, ryanodine treatment appears to preferentially affect cGnRH-II-stimulated GTH-II secretion (J.D. Johnson, and J.P. Chang, unpublished). How the homeostasis of these different Ca^{2+} stores is regulated, and how they are linked to plasma membrane Ca^{2+} entry events in fish gonadotropes, are areas that require further examination.

Analysis of the $[Ca^{2+}]_i$ response kinetics to sGnRH and cGnRH-II in identified goldfish gonadotropes revealed other differences in the acute action of the two GnRHs. In response to sGnRH and cGnRH-II application, three types of $[Ca^{2+}]_i$ response profiles (waveforms) are observed: biphasic, monophasic, and oscillatory (Mollard and Kah 1996; Johnson et al. 1999*b*). With increasing concentrations of both sGnRH and cGnRH-II, the maximal amplitude of the $[Ca^{2+}]_i$ response increases, and the proportion of gonadotropes

responding with biphasic $[Ca^{2+}]_i$ responses increases and predominates. But at these high concentrations, sGnRH elicited a greater percentage of monophasic responses than cGnRH-II. Compared to sGnRH, cGnRH-II-elicited increases in $[Ca^{2+}]_i$ also have a steeper overall slope to peak amplitude; however, the latency, the maximum amplitude of $[Ca^{2+}]_i$ response, and the average increase in $[Ca^{2+}]_i$ over time are not different (Johnson et al. 1999*b*). How these changes in the GnRH-induced $[Ca^{2+}]_i$ responses are related to modulations of Ca^{2+} fluxes from the different intracellular Ca^{2+} stores, extracellular Ca^{2+} entry, and Ca^{2+} buffering systems remain to be investigated. However, interesting inferences in the role of $[Ca^{2+}]_i$ changes in GTH-II cell secretory responses can be made. Given that oscillatory $[Ca^{2+}]_i$ responses are not the most common, and that drug treatments that increase $[Ca^{2+}]_i$ to levels similar to those elicited by GnRH do not always lead to elevations in GTH-II release (Johnson et al. 1998, 1999*a*, 1999*b*; also see section on basal GTH-II release below), spatiotemporal features of $[Ca^{2+}]_i$ responses other than frequency and amplitude may play important roles in signal transduction and information coding in cell signaling by GnRH and other ligands in goldfish GTH-II cells. The importance of spatiotemporal features of $[Ca^{2+}]_i$ signals in the control cell function (including hormone release from goldfish pituitary cells) has been extensively reviewed in a separate article (Johnson and Chang 2000).

GH release

Pharmacological studies indicate that prolonged sGnRH and cGnRH-II stimulation of GH secretion also involve extracellular Ca^{2+} entry through VSCC present on identified goldfish somatotropes (reviewed in Chang et al. 1994, 1996*b*; Fig. 2). However, unlike the situation with GTH-II release (see above), the long-term action of both GnRHs are equally (and entirely) dependent on extracellular Ca^{2+} (Chang et al. 1994, 1996*b*). How GnRH activates VSCC in somatotropes is not known, but probably does not involve apamin-sensitive mechanisms. Treatment with apamin did not affect GnRH-induced GH release in perifusion experiments (P. Kwong and J.P. Chang, unpublished).

Recent results confirm that acute applications of sGnRH and cGnRH-II elevate $[Ca^{2+}]_i$ in identified goldfish somatotropes, and that both peptides can stimulate a $[Ca^{2+}]_i$ response in the same cell (J.D. Johnson, W.K. Yunker, and J.P. Chang, unpublished). Somatotropes also respond to both GnRHs with three types of $[Ca^{2+}]_i$ waveforms: biphasic, monophasic, and oscillatory. But whether sGnRH and cGnRH-II elicit $[Ca^{2+}]_i$ responses with different temporal characteristics, as in gonadotropes, has not been systematically analyzed. Preliminary results indicate that acute GH responses to the two GnRHs are not affected by treatment with different inhibitors of Ca^{2+}/ATPases, including BHQ and thapsigargin (J.D. Johnson and J.P. Chang, unpublished). These findings also suggest that GnRH action on GH release differs from that observed in gonadotropes in terms of their relative dependence on intracellular Ca^{2+} stores (see GTH-II section, above). As in the case of GTH-II release, how the different intracellular Ca^{2+} stores interact with one another and their relationships with plasma membrane Ca^{2+} channels remain to be elucidated.

Calmodulin involvement

GTH-II release

In many systems, effects of increased $[Ca^{2+}]_i$ are mediated by calmodulin (CaM). CaM and CaM kinase II also participate in mediating sGnRH and cGnRH-II effects on GTH-II secretion in goldfish (Fig. 1). However, CaM and CaM kinase appear to participate in long-term, but not acute, GnRH-stimulated GTH-II release, suggesting that this transduction mechanism plays an important role in the recruitment of hormone into the releasable pool or other events required for sustained release. A differential sensitivity of the actions of the two GnRHs to modulation by CaM and CaM kinase inhibitors is also seen, with cGnRH-II effects being more sensitive than sGnRH to these perturbations (reviewed in Jobin et al. 1996; Chang et al. 1996*b*). The possibility that Ca/CaM-sensitive pathways differentially mediate the effects of the two native GnRH on long-term availability of GTH-II for release has not been addressed.

GH release

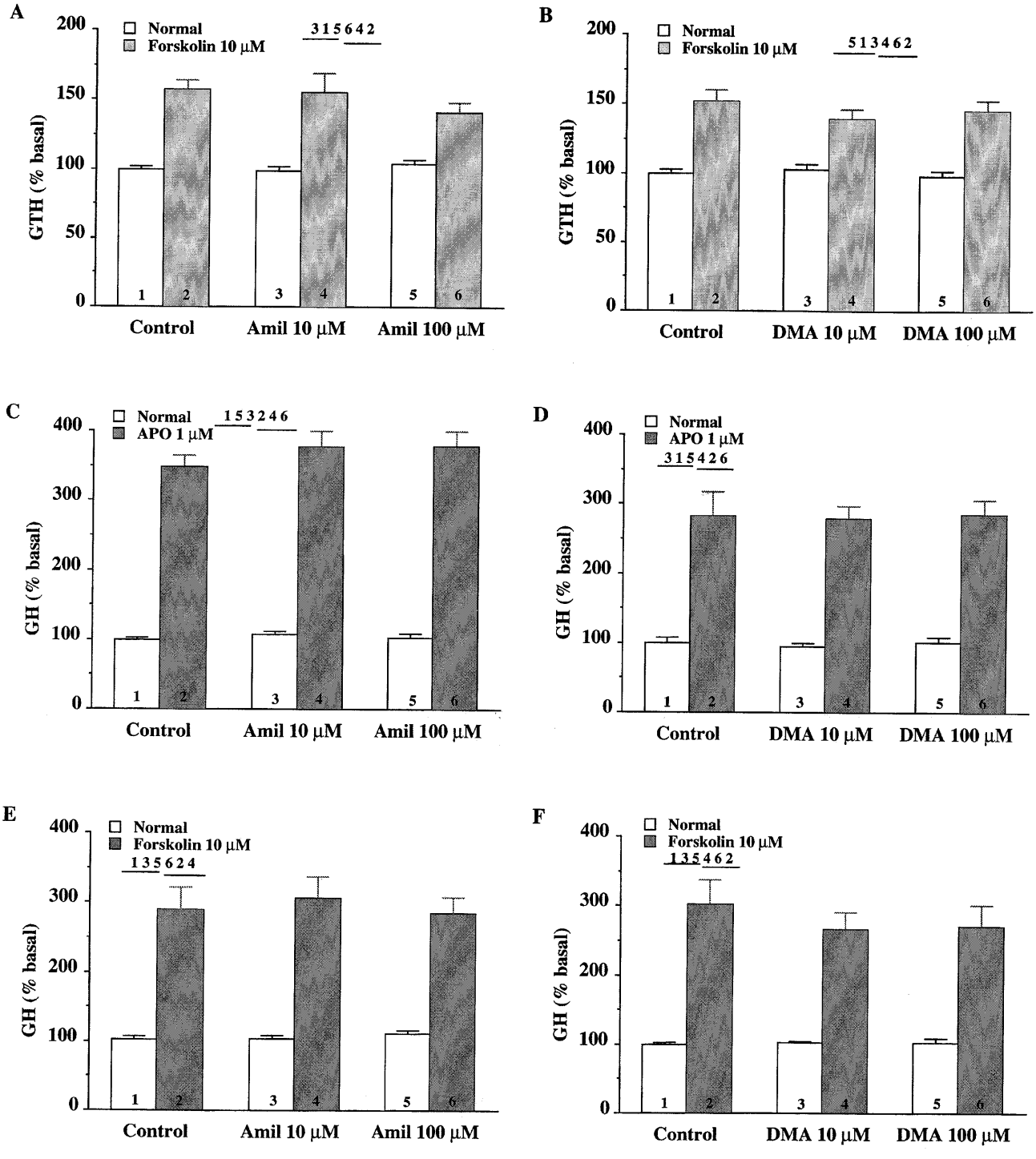
CaM and CaM kinase are also important signaling components mediating sGnRH- and cGnRH-II-induced GH responses (Fig. 2). However, unlike GTH-II release, inhibition of CaM kinase affects both acute and prolonged GnRH action, suggesting a role for CaM kinase in both short- and long-term action of the two GnRHs (Chang et al. 1996*a*).

Protein kinase C

GTH-II release

As indicated earlier, GnRH stimulates PLC activity in goldfish pituitary cells. A product of phospholipase C action is diacylglycerol (DAG), an endogenous activator of protein kinase C (PKC). An involvement of PKC in both the acute and prolonged GTH-II response to both GnRHs have been demonstrated (Fig. 1). Early reports suggest that the response to cGnRH-II is also slightly more sensitive to the action of PKC inhibitors than sGnRH, especially under prolonged exposure conditions (Chang et al. 1991*a*). PKC-stimulated and Ca^{2+}-induced GTH-II release likely share the same GTH-II releasable pool and common distal signaling mechanisms. The GTH-II responses to PKC activators are dependent on extracellular Ca^{2+}, and subject to modulation by VSCC agonists and antagonists (reviewed in Chang et al. 1996*b*). Recently, PKC activation has been shown to increase $[Ca^{2+}]_i$ in identified goldfish gonadotropes, and the effects of K^+ depolarization on GTH-II release are potentiated by subthreshold doses of a PKC activator (Chang et al. 1997; Van Goor 1997). Such close relationships between PKC activation and VSCC/$[Ca^{2+}]_i$-related hormone release events suggest that PKC may be an important element in how GnRH elevates $[Ca^{2+}]_i$ and modulates VSCC activity (Fig. 1). However, direct action of PKC activators on VSCC currents have not been demonstrated. A possible role of PKC in mobilization of Ca^{2+} from intracellular stores is also suggested by the inability of depletion of extracellular Ca^{2+} to totally abolish the $[Ca^{2+}]_i$ increases induced by a synthetic DAG in mixed

Fig. 3. Effects of two Na^+/H^+ antiport inhibitors, amiloride and DMA, on the GTH-II and GH responses to cAMP activators in 2-h static incubation experiments with primary cultures of dispersed goldfish pituitary cells (as in experiments in Van Goor et al. 1996*b*, 1997). (A and B) The GTH-II responses to 10 μM forskolin were unaffected by amiloride and DMA. (C and D) The GH responses to 1 μM apomorphine (APO, a DA agonist) were not inhibited by amiloride and DMA. (E and F) The GH responses to 10 μM forskolin were not altered by amiloride and DMA. Results (expressed as a percentage of basal, control hormone release; mean ± SEM) represent pooled data from at least three separate experiments, each performed in triplicate or quadruplicate. Treatment groups are identified by numbers. Treatments resulting in hormone release responses that are not significantly different (ANOVA followed by Fisher's LSD test; $P < 0.05$) share the same underscore.



populations of goldfish pituitary cells (Jobin and Chang 1992).

Recent results indicate that PKC is also an important target in gonadal steroid positive feedback modulation of GnRH responsiveness on goldfish pituitary cells. Application of testosterone and estradiol to goldfish pituitary cells in vitro enhanced the GTH-II responses to the two GnRHs and PKC activators, but not the responses to Ca^{2+} ionophores (Lo and Chang 1998*a*; Lo et al. 1998). Conversely, application of a PKC inhibitor reduced the potentiating effects of testosterone treatment (Lo and Chang 1998*a*). Taken together, these observations suggest that PKC is an important signal transduction element in the neuroendocrine regulation of GTH-II release by GnRH.

GH release

PKC is also an important element in sGnRH- and cGnRH-II elevation of GH release in goldfish under both acute and prolonged stimulation conditions (Fig. 2). VSCC/Ca^{2+}-dependence of PKC-stimulated GH release has also been shown (reviewed in Chang et al. 1994, 1996*b*). In preliminary experiments, the ability of a synthetic DAG to elevate $[Ca^{2+}]_i$ in identified goldfish somatotropes has been demonstrated (W.K. Yunker and J.P. Chang, unpublished). Taken together, these results support the hypothesis that PKC plays a role in mediating GnRH action on Ca^{2+} mobilization and VSCC activity in goldfish somatotropes as in gonadotropes.

Extracellular sodium

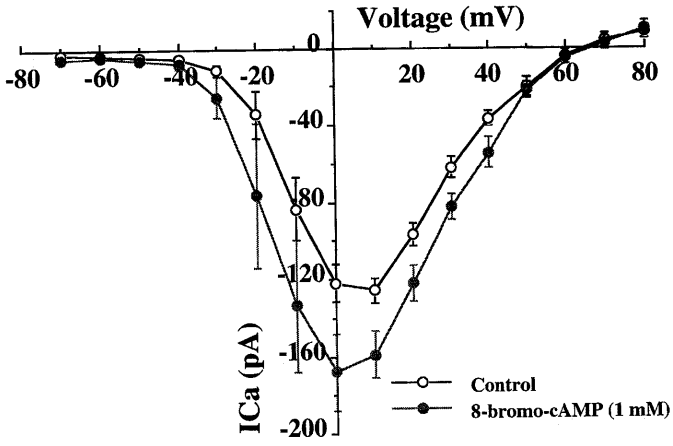
GTH-II release

A dependence on extracellular Na^+ in acute and prolonged sGnRH and cGnRH-II stimulation of GTH-II release has been demonstrated. TTX-sensitive Na^+ channels are present in goldfish gonadotropes. However, evidence from both electrophysiological and hormone release studies suggest that TTX-sensitive Na^+ channels are not involved in mediating GTH-II release responses, but amiloride-sensitive Na^+/H^+ antiports are important in these events (Van Goor et al. 1996*b*; Chang et al. 1997; Fig. 1). Since amiloride and its analog, DMA, block PKC-stimulated GTH-II secretion, but not Ca^{2+} ionophore-, VSCC agonist- (Van Goor et al. 1996*b*), or forskolin (cAMP)-induced GTH-II release (Fig. 3A and B), activation of this antiport system appears to be down-stream of PKC activation. Activation of this antiport may be required to buffer pH changes induced by GnRH activation of PKC (Chang and Jobin 1994; Van Goor et al. 1996*b*). This hypothesis remains to be tested.

GH release

Like gonadotropes, TTX-sensitive Na^+ channels are also present on goldfish somatotropes. The two GnRHs do not alter Na^+ current magnitude and current/voltage properties, but their GH responses are sensitive to the manipulation of extracellular $[Na^+]$. Pharmacological studies suggest that amiloride-sensitive Na^+/H^+ antiports are involved (Fig. 2). As with GTH-II release, PKC-induced GH release, but not ionophore-, VSCC agonist- (Van Goor et al. 1997), or forskolin-elicited GH responses (Fig. 3E and F), are sensitive to manipulations by amiloride and DMA. It is likely that GnRH activation of PKC leads to activation of Na^+/H^+ antiports, which may be important events in the GH release response.

Fig. 4. Effects of 1 mM 8-bromo-cAMP on the current/voltage relationship of peak ionic currents flowing through VSCC in identified goldfish gonadotropes recorded under perforated patch, voltage-clamp conditions. Experimental protocols described in Van Goor et al. (1994, 1996*a*, 1998) were used. Currents were elicited by 40 msec voltage steps from holding potentials of –80 mV. Pooled results (mean ± SEM) from six identified gonadotropes from three different dispersed cell preparations are shown.



Arachidonic acid

GTH-II release

Mobilization of arachidonic acid (AA) via PLA_2, but not DAG lipase, appears to be important in sGnRH stimulation of GTH-II release. Involvement of lipoxygenase, but not cyclo-oxygenase metabolites of AA, is also indicated (Fig. 1). However, the chemical identity of the AA metabolite(s) involved has not been elucidated. Nevertheless, it has been shown that this AA-dependent GTH-II release is independent of the PKC- and extracellular Ca^{2+}-sensitive pathways, and may provide alternate, extracellular Ca^{2+}-independent signaling mechanisms for sGnRH action on GTH-II secretion (reviewed in Chang et al. 1996*b*, 1997). On the other hand, cGnRH-II action on GTH-II release is independent of AA (reviewed in Chang et al. 1996*b*). The differential involvement of AA in the signaling mechanisms of sGnRH and cGnRH-II may contribute to the differences in extracellular Ca^{2+} sensitivity of the GTH-II responses to the two peptides.

GH release

Despite the fact that AA stimulates GH secretion, neither sGnRH nor cGnRH-II action on GH release is affected by inhibition of PLA_2, DAG lipase, cyclo-oxygenase, and lipoxygenase enzymes (Chang et al. 1996*a*). These results strongly suggest that GnRH stimulation of GH secretion does not involve an AA component (Fig. 2). This lack of AA involvement is another major difference in GnRH action between the two cell types.

Cyclic AMP

GTH-II release

Application of cAMP analogs, as well as activation of adenylate cyclase by forskolin, stimulates GTH-II secretion. However, neither of the two GnRHs elevate cAMP levels. Moreover, their actions on GTH-II release are not affected by PKA inhibitors, indicating that the cAMP/PKA cascade does not directly participate in GnRH stimulation of GTH-II release (Fig. 1; reviewed in Chang et al. 1996*b*). The GTH-II responses to forskolin and cAMP analogs are also unaffected by depletion of extracellular Ca^{2+} and application of VSCC antagonists (Chang et al. 1992; J.P. Chang, unpublished). In contrast, activation of cAMP-dependent mechanisms potentiate GnRH- (Chang et al. 1992) and PKC-induced GTH-II release, as well as the effectiveness of VSCC agonist to increase GTH-II secretion (J.P. Chang, unpublished). In perforated-patch, voltage-clamp studies, cAMP also increased the magnitude of Ca^{2+} currents in identified goldfish gonadotropes (maxium current in the presence of 1 mM 8-bromo-cAMP, ICa = 135 ± 8% of control; $n = 6$, $P < 0.05$ paired *t*-test; Fig. 4). cAMP-dependent mechanisms may be important positive modulatory elements for the GnRH-/PKC-/VSCC-dependent GTH-II secretion mechanisms (Fig. 1), and may play an important role in the modulation of GnRH-responsiveness in GTH-II cells by other neuroendocrine regulators (see DA section, below), as well as the control of other cellular functions (see basal GTH-II release section below).

GH release

Pharmacological treatments that elevate intracellular cAMP levels increase GH release, but cAMP does not participate in mediating sGnRH and cGnRH-II actions on GH secretion (reviewed in Wong et al. 1994*b*; Chang et al. 1994; Peter and Chang 1999). In contrast to GTH-II release, cAMP-stimulated GH release is only additive to GnRH- and PKC-induced responses, indicating that these are parallel transduction systems in the somatotropes, and that the GH releasable pool may be partitioned between cAMP- and PKC-dependent elements (Fig. 2; Wong et al. 1994*b*; Chang et al. 1994).

Nitric oxide

GTH-II release

Recently, the presence of nitric oxide (NO) syntase (NOS)-like immunoreactivity has been demonstrated in goldfish gonadotropes by immunocytochemistry (A.D. Uretsky and J.P. Chang, unpublished). However, the exact isoform of NOS present has yet to be determined. Addition of a NO donor, and treatment with a cGMP analog, increases GTH-II secretion (P. Kwong and J.P. Chang, unpublished). Since NO is known to activate guanylate cyclase in many systems, taken together, these results suggest that NO may be a signaling pathway mediating GTH-II responses (Fig. 1). Whether NO mediates GnRH action on GTH-II release has not been investigated, but remains a possibility. How the NO/cGMP pathway interacts with other second messenger systems in the control of GTH-II release is also not known.

GH release

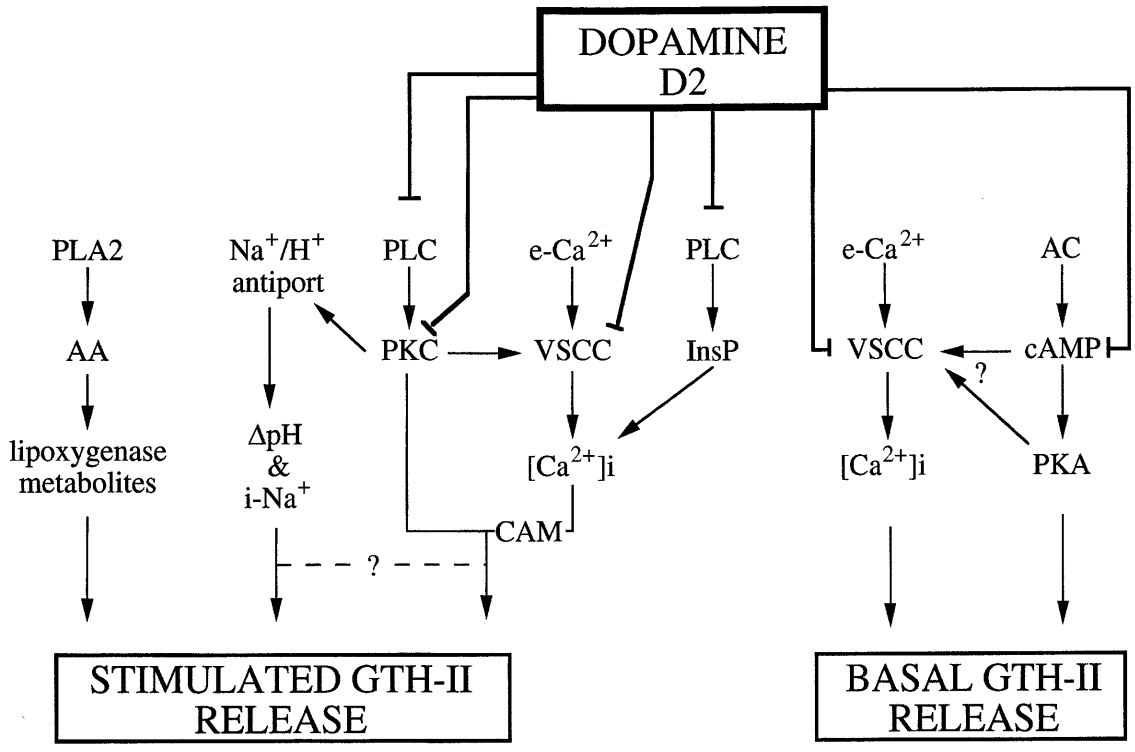
Likewise, NOS immunoreactivity has been detected in goldfish somatotropes (Uretsky and Chang 1999). Addition of NO donors and a cGMP analog elevated GH secretion. Application of a NOS inhibitor reduced the GH responses to sGnRH, suggesting that this signaling pathway participates in GnRH stimulation of GH secretion (Uretsky et al. 1999; Uretsky and Chang 1999; Fig. 2). The exact isoform of NOS present, and how the NO signaling pathway interacts with other second messenger systems in the regulation of GH release, have yet to be explored in detail. Preliminary evidence suggest that NOS activation occurs either independent of or upstream of PKC (Uretsky and Chang 1999). Since NO is a diffusible second messenger that can rapidly exit cells, the potential exists that NO may act as a paracrine/autocrine regulatory factor in addition to an intracrine role to mediate neuroendocrine modulation of GH release. The possibility that NO may play a role in the co-ordination of GH and GTH-II release responses is an interesting avenue for future investigations.

Differences in transduction components in basal GTH-II and GH secretion compared to GnRH-stimulated responses

GTH-II release

Evidence suggests that basal and GnRH-stimulated GTH-II release are not controlled identically. Despite the important roles of VSCC and PKC in GnRH stimulation, addition of organic VSCC blockers or PKC inhibitors does not decrease basal GTH-II secretion (reviewed in Chang et al. 1997). Likewise, removal of extracellular Na^+ also does not decrease basal GTH-II secretion (Van Goor et al. 1996*b*). Conversely, basal GTH-II secretion is reduced by treatment with the PKA inhibitor H89 (P. Kwong, C.J.H. Wong, and J.P. Chang, unpublished) and by inorganic blockers of VSCC in perifusion experiments (Jobin et al. 1996; Chang et al. 1996*b*). These results suggest that unstimulated GTH-II secretion is regulated predominantly by cAMP/PKA, but not PKC- and Na^+-dependent mechanisms; however, some Ca^{2+}/VSCC-dependent components may also be important (Fig. 5). Interestingly, while many treatments that increase $[Ca^{2+}]_i$ in identified gonadotropes leads to GTH-II release, application of the $[Ca^{2+}]_i$ elevating Ca^{2+}/ATPase inhibitor thapsigargin, which does not affect GnRH stimulation of GTH-II release (see GnRH section above), paradoxically suppresses unstimulated GTH-II secretion in perifusion studies (Johnson et al. 1998). This effect of thapsigargin is accompanied by a decrease in cellular GTH-II and GTH-II beta subunit mRNA contents (Johnson et al. 1999*a*). Though these results are suggestive of a link between GTH-II synthesis and basal GTH-II release, this putative connection needs to be systematically studied. In many peptide secretory systems, constitutive release is closely linked to peptide synthesis (Burgess and Kelly 1987; Brion et al. 1992). Results from efforts to investigate whether basal GTH-II secretion occurs via constitutive release pathways, are still inconclusive. Nevertheless, these results with thapsigargin illustrate the complexity of signaling via changes in $[Ca^{2+}]_i$. They also provide insight that specificity in the regulation of cellular functions may be dependent on the sources of Ca^{2+}

Fig. 5. Diagrammatic summary of the signal transduction pathways in DA D2 receptor-mediated inhibition of basal and GnRH-stimulated GTH-II secretion in goldfish. DA inhibition can be exerted at the levels of PLC activation, VSCC currents, and PKC and cAMP actions. DA does not affect AA-stimulated GTH-II release. Inhibitory influences are denoted by blunt-ended lines.



and the way by which changes in $[Ca^{2+}]_i$ are manifested. Whether NOS and AA cascades participate in the regulation of unstimulated GTH-II release is not clear.

GH release

Basal GH secretion appears to be highly sensitive to suppression by the reduction of extracellular $[Ca^{2+}]$, and addition of inorganic blockers of VSCC (Wong et al. 1994*a*; Van Goor et al. 1997), but not by PKC inhibitors (Chang et al. 1991*a*), amiloride, and its analog DMA, and the reduction of extracellular $[Na^+]$ (Van Goor et al. 1997). In addition, perifusion with H89 also reduces unstimulated GH release (P. Kwong, C.J.H. Wong, and J.P. Chang, unpublished). These data suggest that extracellular Ca^{2+} entry and cAMP/PKA, but not PKC and Na^+/H^+ antiport, play an important role in the regulation of basal GH secretion. However, the Ca^{2+}/ATPase inhibitor thapsigargin does not alter either unstimulated GH secretion, or GH protein and mRNA contents (Johnson et al. 1999*a*). In contrast, BHQ elevated GH secretion (J.D. Johnson and J.P. Chang, unpublished). These results with thapsigargin and BHQ illustrate the complexity in the use of alterations $[Ca^{2+}]_i$ as a mechanism in the control of basal GH release. When compared to the results on GTH-II secretion (see above), these observations further illustrate the presence of differences in Ca^{2+} signaling in these two cell types.

Signal transduction in DA inhibition of GTH-II release and stimulation of GH secretion

GTH-II release

DA inhibition of basal and GnRH-stimulated GTH-II release is mediated by D2 receptors, and part of the long-term action of DA is to reduce the number of GnRH receptors in goldfish pituitary (reviewed in Van Der Kraak et al. 1997). In addition, DA inhibition can also be exerted at the level of the intracellular signaling mechanisms (Fig. 5). In recent studies, DA and a D2 agonist decreased the magnitude and slowed the activation kinetics of ionic current through VSCC in identified goldfish gonadotropes (Van Goor et al. 1998). DA D2 agonists have also been shown to attenuate the GnRH-induced elevation of InsP levels and $[Ca^{2+}]_i$ in mixed populations of goldfish pituitary cells, as well as PKC-stimulated GTH-II release, but not Ca^{2+} ionophore- and AA-stimulated GTH-II response (reviewed in Chang et al. 1993, 1997). Reduction of VSCC and PKC activity, as well as mobilization of Ca^{2+}, appear to be important for DA inhibitory influences on GnRH action in gonadotropes. Whether DA affects Na^+ entry into cells is not known, but DA and (or) its agonists inhibit the GTH-II responses to forskolin and cAMP analogues (Chang et al. 1992). The ability of DA to block at sites distal to cAMP formation provides a means for its inhibitory effect on basal GTH-II secretion, and may be an additional mechanism through which modulation of GTH-II release-responsiveness to GnRH can be exerted at the signal transduction level. Whether DA D2 mechanisms involve modulation of the NOS/NO/cGMP pathway has not been studied.

GH release

DA, via D1 receptor-mediated mechanisms, has been shown to be a major stimulatory factor for GH secretion (reviewed in Peng and Peter 1997; Peter and Chang 1999). D1 receptors have been localized to regions of the goldfish pitu-

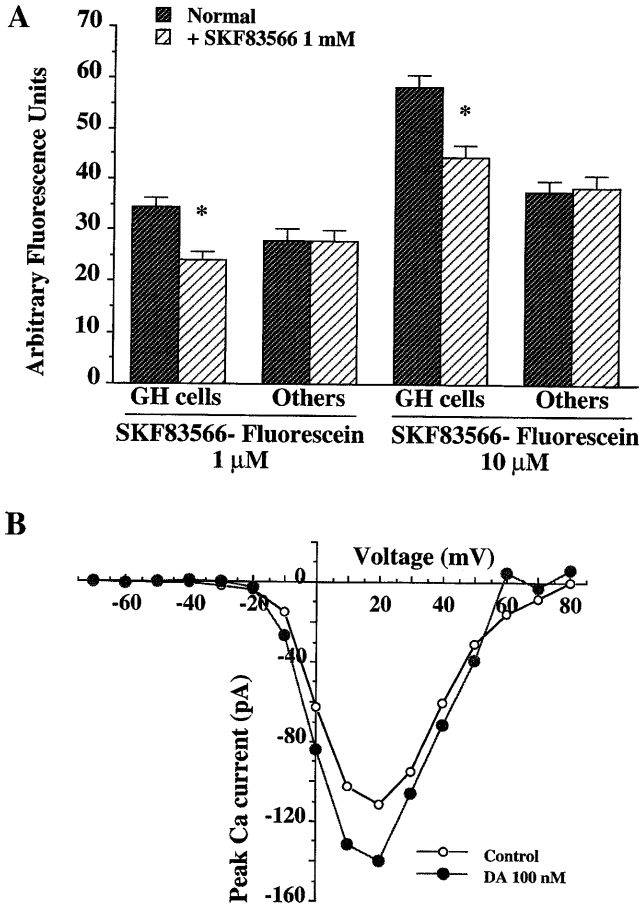


Fig. 6. Presence of D1 receptors on identified goldfish somatotropes and effects of DA on VSCC currents. Dispersed goldfish pituitary cells cultured on poly-L-lysine-coated glass-bottomed petri dishes were used (Van Goor et al. 1994). (A) Displaceable fluorescein-labeled D1 antagonist binding is demonstrated in identified goldfish somatotropes. Dispersed goldfish pituitary cells were incubated with a D1 antagonist conjugated to fluorescein (SKF83566-Fluorescein, 1 and 10 μM), either in the presence or absence of 1 mM unlabeled SKF83566. To ensure the receptor selectivity, serotonin (10 μM) and pimozide (10 μM) were used to block serotonin and D2 binding, respectively. Following washing, fixation, and histochemical processing, somatotropes were identified according to their unique morphology under differential contrast microscopy (Van Goor et al. 1994), their relative positions in the image field recorded, and the fluorescent images captured, digitized, and stored in a computer. Fluorescence intensity (expressed as arbitrary fluorescence units) on identified somatotropes and other pituitary cells (others) were quantified (background fluorescence corrected) using NIH image. Pooled results from 5 different cell preparations are presented (mean ± SEM, n = 27 to 55 for each treatment group). Statistical analysis with Krustal Wallis test indicated the presence of significant differences between treatment groups ($P < 0.05$). *Denotes a significant difference from the corresponding group not treated with excess unlabeled D1 antagonist. (B) The current/voltage relationship of peak VSCC currents in an identified goldfish somatotrope recorded under perforated-patch, voltage-clamp conditions in the absence (control) and in the presence of 100 nM DA. Experimental protocols described in Van Goor et al. (1994, 1996*a*, 1998) were used. Results from one of five experiments using cells from two different dispersed cell preparations are shown. Average peak Ca^{2+} current amplitude in the absence and presence of DA = 78 ± 15 and 102 ± 18 pA, respectively (n = 5, $P < 0.05$, paired t-test).

itary in which somatotropes are located (Wong et al. 1993). Recently, using a fluorescent-labeled D1 receptor antagonist, the presence of DA D1 receptors on identified goldfish somatotropes has been visualized (Fig. 6A). DA action on GH release is predominantly mediated through a cAMP/PKA mechanism (reviewed in Chang et al. 1994; Peter and Chang 1997, 1999). DA D1 stimulation of GH secretion is accompanied by increases in cAMP formation, and is blockable by an inhibitor of PKA. In contrast, GH responses to D1 stimulation are unaffected by treatment with PKC inhibitors, and the reduction of cellular PKC content. GH responses to D1 stimulation are also additive to GnRH- and PKC activator-induced GH release. These data suggest that the DA D1 cAMP-dependent signaling system forms a separate transduction pathway from the GnRH/PKC pathway, leading to an increase in GH secretion (reviewed in Wong et al. 1994*b*; Chang et al. 1994). In contrast to GnRH action, DA D1- and cAMP-induced GH release is dependent on the mobilization of AA and its metabolism by lipoxygenase enzyme (Chang et al. 1996*a*). In addition, neither DA agonist- nor forskolin-stimulated GH responses are reduced by amiloride and DMA (Fig. 3C–F), suggesting that Na^+/H^+ antiports are not involved in the D1/cAMP pathway (Fig. 2). Although D1 and GnRH stimulation of GH release appears to be mediated by separate pathways, DA D1 action also has a Ca^{2+}- and CaM-dependent component, distal to cAMP formation. Both D1- and cAMP-stimulated GH secretion are reduced by the removal of extracellular Ca^{2+}, the addition of VSCC antagonists, and treatment with a CaM kinase inhibitor (Chang et al. 1994, 1996*a*; Wong et al. 1994*a*, 1994*b*). In preliminary studies, a D1 agonist (Yunker and Chang 1999*b*) and forskolin were found to elevate $[Ca^{2+}]_i$ in goldfish somatotropes (W.K. Yunker and J.P. Chang, unpublished). DA also increased the magnitude of ionic currents through VSCC in somatotropes (Fig. 6B; maximum current in the presence of DA, ICa = 144% ± 13% of control; n = 5, $P < 0.05$, paired t-test). These observations, when viewed together, strongly suggest that activation of the D1/cAMP pathway also leads to increases in $[Ca^{2+}]_i$ via activation of VSCC. Whether DA and GnRH mechanisms activate similar VSCC in somatotropes is not known, but the ability of nifedipine (VSCC antagonist) to attenuate D1 agonist- and PKC activator-induced GH release is additive (J.P. Chang, unpublished), suggestive of the activation of separate channel populations. Like GnRH, D1 agonist-induced GH release is also inhibited by a NOS inhibitor (Uretsky and Chang 1999), indicating that NO also plays a role in mediating D1 action on GH release. In view of the fact that D1 agonist and GnRH can elevate $[Ca^{2+}]_i$ in the same somatotrope (Yunker and Chang 1999*b*), the above data strongly implies that the releasable pool is at least partially partitioned into D1/cAMP- and GnRH/PKC-sensitive components; however, overlapping mechanisms might occur (Fig. 2).

Signal transduction in PACAP action on GTH-II and GH release

GTH-II release

A possible role of PACAP as a hypophysiotropic regulator in goldfish was first suggested by the presence of PACAP immunoreactive neuronal terminals in the pars distalis of goldfish pituitary, where gonadotropes and somatotropes reside (Wong et al. 1998). Recently, a goldfish $PACAP_{38}$ has been cloned, and its mRNA was found to be expressed in the goldfish pituitary. The deduced primary amino acid sequence of goldfish $PACAP_{38}$ is highly homologous to other known vertebrate $PACAP_{38}$ peptides, including mammalian (m)$PACAP_{38}$ (homology ~90%; Leung et al. 1999). In addition, a goldfish PACAP type 1 (PVR1) receptor has been cloned, and its mRNA detected in areas of the goldfish pars distalis where gonadotropes and somatotropes reside. In preliminary studies, $mPACAP_{38}$ increased GTH-II release from dispersed goldfish pituitary cells in perifusion (A.O.L. Wong and J.P. Chang, unpublished). However, the response to $mPACAP_{38}$ was very modest relative to that induced by control sGnRH treatment. Nonetheless, this effect of $mPACAP_{38}$ was reduced by treatment with H89, suggestive of a cAMP-dependent signal transduction pathway (J.P. Chang, unpublished). Consistent with the hypothesized ability of cAMP to enhance GnRH/PKC-dependent GTH-II secretion (see GnRH section above), $mPACAP_{38}$ enhances the GTH-II-releasing ability of sGnRH (J.P. Chang, unpublished). Details of the PACAP signal transduction pathway and how this interacts with signaling mechanisms utilized by other neuroendocrine regulators, are topics that require future examination.

GH release

Applications of $mPACAP_{38}$, zebra fish $PACAP_{38}$, as well as goldfish $PACAP_{38}$, also exert stimulatory influences on GH secretion from dispersed goldfish pituitary cells in vitro (Wong et al. 1998; Leung et al. 1999). Similarly, intraperitoneal injection of goldfish $PACAP_{38}$ elevated serum GH levels in goldfish (Leung et al. 1999). In vitro GH releasing actions of $mPACAP_{38}$ and (or) zebra fish $PACAP_{38}$ are attenuated by inhibitors of the adenylate cyclase and PKA enzymes, and are not additive to maximal stimulation by cAMP analog and forskolin (Leung et al. 1997; N. Wirachowsky and J.P. Chang, unpublished). These results suggest that PACAP action is mediated by the adenylate cyclase/cAMP/PKA system (Fig. 2) and is consistent with the known coupling of the goldfish PVR1 receptors to adenylate cyclase in mammalian cell expression systems (Wong et al. 1998). $mPACAP_{38}$ elevates $[Ca^{2+}]_i$ in identified goldfish somatotropes (W.K. Yunker, J.D. Johnson, and J.P. Chang, unpublished), and the GH responses to $mPACAP_{38}$ and (or) zebra fish $PACAP_{38}$ are reduced by VSCC inhibitors and the reduction of extracellular $[Ca^{2+}]$ (Leung et al. 1997; N. Wirachowsky and J.P. Chang, unpublished). Whether this Ca^{2+}/VSCC-dependent component is distal to, or independent of, PACAP activation of adenylate cyclase is not known at present. Evidence to date has not indicated the presence of a major PKC component in PACAP action on GH release in 2-h hormone secretion experiments (N. Wirachowsky and J.P. Chang, unpublished). However, preliminary results with a NOS inhibitor suggest that this pathway may also be important for PACAP action on somatotropes (Fig. 2; A.D. Uretsky and J.P. Chang, unpublished).

NE mechanisms on GTH-II and GH release

GTH-II release

NE stimulation of GTH-II release is mediated through alpha-1-like adrenergic receptors (Chang et al. 1991*b*). Although the GTH-II response to NE has been reported to be additive to responses elicited by GnRH (Chang et al. 1991*b*), little is known regarding the intracellular signaling mechanisms of NE.

GH release

NE is an effective inhibitor of basal, GnRH-, D1-, and PACAP-induced GH release via alpha-2-like receptor-mediated mechanisms (Leung et al. 1997; Lee et al. 2000; Yunker and Chang 1999*a*). NE and its alpha-2 agonist clonidine reduced the GH responses to forskolin, PKC activators, Ca^{2+} ionophores, and AA. Clonidine also reduced cellular resting cAMP production; however, NE and clonidine did not affect resting $[Ca^{2+}]_i$ levels in identified goldfish somatotropes and mixed populations of dispersed goldfish pituitary cells, respectively (Yunker et al. 2000; Yunker and Chang 1999*a*). Taken together, these data suggest that NE inhibitory action can be exerted at the level of cAMP production, as well as at sites distal to cAMP formation, PKC activation, AA mobilization, and elevations in $[Ca^{2+}]_i$ (Fig. 2). Whether NE also affects AA and Ca^{2+} mobilization and NOS activity, has not been tested. Nevertheless, the known NE sites of action already provide a firm basis for understanding how inhibitory modulation of basal GH release and stimulated GH responses by NE can occur.

Mechanisms mediating SRIF inhibition of GH secretion

SRIF is one of the most potent and effective inhibitors of basal and stimulated goldfish GH release known. Multiple SRIF forms and cDNAs encoding these isoforms are known to exist in goldfish, including $SRIF_{14}$ (reviewed in Lin et al. 1998; Peter and Chang 1999). To date, SRIF signal transduction mechanisms in goldfish somatotropes have only been examined using $SRIF_{14}$. $SRIF_{14}$ reduces the GH responses to forskolin, a cAMP analog, PKC activators, Ca^{2+} ionophores, a VSCC agonist, and an NO donor (Kwong and Chang 1997; Uretsky et al. 1999; Uretsky and Chang 1999). These results indicate that SRIF inhibits GH release by actions on signal transduction sites distal to cAMP formation, PKC activation, increases in of $[Ca^{2+}]_i$, and production of NO. Whether SRIF exerts an inhibitory influence on Ca^{2+} influx through VSCC or other mechanisms causing $[Ca^{2+}]_i$ increases to attenuate stimulated GH release responses, are possibilities that need to be evaluated. However, $SRIF_{14}$ does not alter resting $[Ca^{2+}]_i$ levels in identified goldfish somatotropes in preliminary experiments (Yunker and Chang 1999*b*). Although SRIF and NE target mostly similar sites along the intracellular pathways leading to GH release, SRIF was able to further reduce basal GH release during maximal inhibition by NE (W.K. Yunker and J.P. Chang, unpublished), indicating some non-overlapping actions may exist.

Future studies on the interactions of SRIF and NE at the level of their signal transduction cascades are warranted. Whether other SRIF isoforms act via mechanisms identical to those used by $SRIF_{14}$ is a question that remains to be investigated. With the detection of multiple SRIF forms and SRIF receptor subtypes in the goldfish (reviewed in Lin et al. 1998, 1999; and X.W. Lin and R.E. Peter, personal communications) such investigations are much needed.

Summary perspectives and physiological implications

A significant amount of information has been obtained concerning the signal transduction mechanisms leading to the regulation of GTH-II and GH release in the goldfish. Some of the intracellular pathways by which stimulatory and inhibitory neuroendocrine factors interact to modulate hormone release responses have been identified, and are summarized in Figs. 1, 2, and 5. However, many aspects of the complexity of such interactions, the kinetics of all the various signalling events, their relative functions, as well as the presence and function of many other signaling mechanisms, remain to be investigated and deciphered.

The two native GnRHs clearly utilize partially dissimilar signal transduction mechanisms in stimulating GTH-II and GH release. Furthermore, major differences in the mechanisms of sGnRH and cGnRH-II action also exist within the gonadotropes. Differences between GnRH signaling in gonadotropes and somatotropes may be a consequence of the proposed presence of different GnRH receptors in the two cell types. As a result, the coupling to intracellular pathways are different in somatotropes and gonadotropes. However, how differences in the transduction mechanisms elicited by the two GnRHs are manifested within the gonadotropes, cannot be readily explained by the properties of the two known goldfish GnRH receptor forms, even when assuming that both receptor forms are present on gonadotropes. Available evidence suggests that the GnRH receptors do not differentiate between sGnRH and cGnRH-II in terms of recognition and binding. Certainly, the possibility exists that other GnRH receptors that selectively recognize the two native GnRHs have yet to be discovered. However, it has also been shown that one receptor, including the rat GnRH receptor, can be coupled to more than one type of G-protein in the same cell (Stanislaus et al. 1998). sGnRH and cGnRH-II binding to GnRH receptors on gonadotropes may lead to the establishment of agonist-specific receptor conformations, which allow for differential association and activation of different G-proteins and unique intracellular signaling mechanisms.

Regardless of the exact mechanisms by which the two closely related GnRH peptides generate different intracellular Ca^{2+} signals and other second messengers (e.g., AA) within the gonadotropes, these differences may be of physiological importance. For example, seasonal differences in the direct gonadal feedback influences on sGnRH, and cGnRH-II stimulation of hormone release from gonadotropes, may be manifested through differential actions at the level of these intracellular signaling systems (Lo et al. 1998*b*). In addition, sGnRH has been shown to be more consistently effective in elevating GTH-II beta-subunit mRNA levels than cGnRH-II in the goldfish pituitary (Khakoo et al. 1994). Properties of the intracellular $[Ca^{2+}]_i$ increases (such as duration) are reported to be important in the regulation of gene expression (Dolmetsch et al. 1997, 1998) and the activation of PLA_2 (Hirabayashi et al. 1999). Interestingly, only sGnRH utilizes PLA_2 and AA signaling pathways in GTH-II release, and sGnRH-generated $[Ca^{2+}]_i$ responses contain a greater proportion of monophasic $[Ca^{2+}]_i$ increases, which are also more prolonged. Furthermore, prior exposure to sGnRH affects subsequent thapsigargin influences on basal GTH-II secretion, suggesting that sGnRH interacts with thapsigargin actions (J.D. Johnson and J.P. Chang, unpublished), and thapsigargin-sensitive mechanisms appear to affect GTH-II synthesis (see section on basal release, above). Whether such functional correlates are causal in nature remains to be determined. Nonetheless, they already provide the basis for investigation into the cause-effect relationships in the differential regulation of GTH-II and GH cellular function by different neuroendocrine regulators and second messenger systems.

The largely parallel cAMP/PKA- and PKC-dependent transduction system leading to GH secretion provides a system by which GH release can be fine-tuned by the many neuroendocrine factors depending on the physiological requirements. At present, GnRH and DA, two of the major stimulatory factors, are known to utilize PKC and cAMP, respectively. PACAP action also appears to be highly dependent on the cAMP-dependent pathway. Whether other known regulators of goldfish GH release use one or both of these major transduction systems, or any other mechanisms, remain to be investigated.

The participation and interactions of PKC- and cAMP-dependent mechanisms in the regulation of GTH-II and GH release may form the basis for the neuroendocrine interactions of SRIF, DA, GnRH, and PACAP in the control of these two hormones during gonadal recrudescence and spawning. It has been shown that serum GTH-II and GH levels increase slowly during gonadal recrudescence, coinciding with both somatic and gonadal growth in late winter and early spring. Rapid increases in serum GTH-II levels occur during final gonadal maturation and in the peri-spawning period. Following spawning, GTH-II levels decrease, while GH levels remain relatively high during the late spring and early summer, reflecting the continuation of somatic growth (Habibi and Peter 1991; Peter and Marchant 1995; Peng and Peter 1997). Co-ordination of GH and GTH-II secretion is also important, since GH is known to potentiate the gonadal steroid response to GTH-II (Van Der Kraak et al. 1997). The effectiveness of DA D1 stimulation of GH release is maximal at early gonadal recrudescence, and that of PACAP is maximal at late gonadal recrudescence (reviewed in Peter and Chang 1997, 1999). The GH and GTH-II responsiveness to GnRH increases throughout gonadal recrudescence, and decreases immediately post-spawning (Habibi and Peter 1991; Peter and Chang 1997). It can be hypothesized that during early gonadal recrudescence, the inhibitory influence of SRIF declines (reviewed in Peng and Peter 1997), reducing its inhibitory actions on GH release at multiple signal transduction sites. At this time, gonadal and other inhibitory influences on GTH-II secretion may be transiently relaxed (reviewed in Van Der Kraak et al. 1997; Lo and Chang 1998*a*), and the ability of GnRH (via PKC-dependent mechanisms) to stimulate GTH-II as well as GH release would be

important. Although it is known that the total GnRH receptor number in the goldfish pituitary increases during early gonadal recrudescence, coinciding with the beginning of the gradual rise in serum GTH-II and GH levels, the exact complement of GnRH receptor types, as well as their differential expression in gonadotropes and somatotropes have not been studied. DA through D2 mechanisms can modulate the responsiveness to GnRH on GTH-II release, keeping the rise in GTH-II release low. At the same time, DA provides an additional excitatory input to the somatotropes via the D1/cAMP-dependent signaling system. A co-ordinated increase in serum GTH-II and GH is important, since GH potentiates the gonadal steroid response to GTH-II. In this regard, it is also interesting to note that the potential exists that the pituitary NOS system in the gonadotropes and somatotropes may play paracrine and autocrine roles in such neuroendocrine regulation. At late gonadal recrudescence, and shortly prior to spawning, DA tone is reduced, allowing increases in serum GTH-II levels to be accelerated in preparation for spawning under the influence of GnRH. At this time, PACAP may not only provide a continual stimulatory input on GH release via cAMP-dependent mechanisms in somatotropes, but through the cAMP signaling pathway, also increases the responsiveness to GnRH in gonadotropes. Whether this increased cAMP activity stimulates GTH-II synthesis in the goldfish, as in mammalian study models (reviewed in Chang and Jobin 1994), is not known, but is a distinct possibility that would lead to a parallel increase in GTH-II release capacity. In addition, the high circulating gonadal steroids levels encountered at this stage of the reproductive cycle would positively influence the effectiveness of PKC-induced GTH-II release in the pituitary cells and further magnify the GTH-II release responsiveness to GnRH. As a result of these and other central mechanisms (reviewed in Trudeau and Peter 1995), the peri-ovulatory increases in serum GTH-II and GH levels are manifested. It is also possible the NE, through alpha-1 action, contributes to the serum GTH-II increase during early gonadal recrudescence (Chang and Peter 1984; Chang et al. 1991*b*), while exerting a negative modulatory influence on the GH release response through alpha-2 mediated actions on somatotropes.

It is also clear from the studies on goldfish gonadotrope and somatotrope signal transduction, that cell specificity in signaling mechanisms exists. For example, in addition to the differential involvement of AA-dependent signaling pathways in sGnRH action on GTH-II and GH release, treatment with the Ca^{2+}/ATPase inhibitor BHQ attenuates sGnRH-induced GTH-II, but not GH release. Other results also reveal a high degree of complexity in the use of changes in $[Ca^{2+}]_i$ in cell signaling. The multiple GnRH-induced $[Ca^{2+}]_i$ waveforms (discussed in the GnRH section, above) are also accompanied by the existence of responses that are distributed either globally, or localized regionally across the cell-image plane in gonadotropes (Johnson et al. 1999*b*), as well as in somatotropes (J.D. Johnson and J.P. Chang, unpublished). These observations indicate that temporal, as well as spatial $[Ca^{2+}]_i$ response characteristics may be of importance in these, as in other cell types (Clapham 1995; Petersen et al. 1994; Johnson and Chang 2000). Furthermore, results from experiments with Ca^{2+}/ATPase inhibitors (see sections on GnRH action and basal hormone release, above) suggest that multiple, agonist- and function-specific intracellular Ca^{2+} stores may exist within both somatotropes and gonadotropes. Recent analysis of spatial properties of some regionalized GnRH-induced $[Ca^{2+}]_i$ suggests that the perinuclear area is also a potential intracellular source for Ca^{2+} mobilization (Johnson et al. 1999*b*). Whether mitochondrial Ca^{2+} buffering and fluxes play a role in mediating GTH-II and GH release is also under ongoing consideration.

Knowledge of the signal transduction pathways mediating GTH-II and GH release in goldfish by multiple neuroendocrine factors is far from complete. Nonetheless, the complexity of transduction pathways already identified within the goldfish gonadotropes and somatotropes provides the intracellular substrates through which regulation of cell functions can be affected by neuroendocrine inputs. Functional- and agonist-specificity of some of the transduction mechanisms already revealed indicate that results from continual investigations may yield additional insight into the neuroendocrine regulation of goldfish GTH-II and GH release, as well as contribute to the basic understanding of control of specific cell functions.

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