Role of PKC in the regulation of gonadotropin subunit mRNA levels: interaction with two native forms of gonadotropin-releasing hormone

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GONADOTROPIN-RELEASING HORMONE (GnRH) is a decapeptide that plays a key role in the development and maintenance of reproductive functions in vertebrates. It is best known for its role in the regulation of the synthesis and secretion of pituitary gonadotropin hormones (GtHs), including follicle-stimulating hormone (FSH) and luteinizing hormone (LH). GnRH effects on GtH secretion and gene expression in the goldfish pituitary are of particular interest (27, 41). In mammalian and nonmammalian systems, GnRH-induced GtH release and gene expression are mediated, in part, by PKC (33, 39, 49). The PKC family is composed of 10 isoforms grouped into three classes. Conventional PKC isoforms, including α, γ, and the alternatively spliced Β1 and ΒII, are activated by diacylglycerol (DAG)/phorbol ester, Ca2+, and phosphatidylinositol 4,5-bisphosphate (PIP2). Novel PKC isoforms, including δ, η, ε, θ, and ζ are Ca2+ insensitive and are activated by DAG/phorbol ester and phosphatidylinositol 4,5-bisphosphate. Atypical PKCs, including µ and θ, are Ca2+ and DAG/phorbol ester insensitive and are activated by phosphatidylinositol 3,4,5-trisphosphate (40). Although not all PKCs bind DAG/phorbol esters, there are at least five other types of high-affinity DAG/phorbol ester receptors: chimaerins (a family of Rac GTPase activating proteins), PKD1/PKCµ, Ras-GRPs (gastrin-releasing peptide; exchange factors for Ras/Rap1), Munc13 isoforms (scaffolding proteins involved in exocytosis) and DAG kinase γ (11, 29). In the goldfish pituitary, the signal transduction of GnRH-stimulated GtH subunit gene expression is unknown although the involvement of PKC in GnRH-induced GtH release is well established (reviewed in Ref. 15). The objective of this study was to investigate whether the PKC pathway is involved in the regulation of GtH subunit mRNA levels and the possible involvement of PKC signaling in sGnRH- and cGnRH-II-induced increases in GtH-α, FSH-β, and LH-β mRNA levels.

MATERIALS AND METHODS

Reagents. [Trp7,Leu8]GnRH (sGnRH) and [His4,Trp7,Tyr9]GnRH (cGnRH-II) from American Peptide (Sunnyvale, CA) were solubilized in 0.1 M acetic acid and stored at −20°C. Calphostin C (Cal C), GF109203X (GF), and tetradecanoylphorbol (TPA) were purchased from Calbiochem (La Jolla, CA), dissolved in DMSO, and stored at −20°C, 4°C and 20°C, respectively. 1,2-Dioctanoyl-sn-glycerol (DiC8), also purchased from Calbiochem, was dissolved in DMSO just before use. 4α-TPA was purchased from Sigma (Oakville, Ontario, Canada), dissolved in DMSO, and stored at −20°C. DMSO species. In particular, the goldfish brain and pituitary contain at least two forms of GnRH: salmon (s)GnRH and cGnRH-II (31, 51, 52). Both sGnRH and cGnRH-II stimulate the synthesis and release of GtHs in the goldfish pituitary (15, 32). In addition to GnRH, the production of FSH and LH is also regulated by other neuromodulatory factors including, steroids, catecholamines, amino acids, and gonadal peptides (41, 45, 49). In particular, dopaminergic neurons directly innervate the goldfish pituitary, and dopamine is known to inhibit basal and GnRH-induced GtH release from gonadotropes (27, 41).
REGULATION OF GTH SUBUNIT mRNA LEVELS BY PKC AND GnRH

Effects of PKC inhibitor applied at 24 h after recovery. Initial experiments were carried out to examine the effect of a PKC inhibitor, Cal C, on GnRH-induced GtH subunit gene expression in primary cultures of dispersed goldfish pituitary cells after 24 h of recovery. Cal C targets the DAG/phorbol ester-binding site in the regulatory domain and is a potent and specific inhibitor of PKC (IC_{50}, 5 \times 10^{-8} M) (34). GtH-\(\alpha\), FSH-\(\beta\) and LH-\(\beta\) mRNA levels were determined after 12 h of continuous treatment with varying doses of sGnRH or cGnRH-II (10^{-9} M, 10^{-8} M, or 10^{-7} M) in the absence or presence of Cal C (10^{-7} M) (Fig. 1). Both GnRHs stimulated GtH subunit mRNA levels in a dose-dependent manner with cGnRH-II being the more effective of the two. Cal C significantly stimulated GtH-\(\alpha\), FSH-\(\beta\), and LH-\(\beta\) mRNA levels. These increases were observed with a concentration of Cal C well below the IC_{50} values for its inhibition of PKA or a tyrosine-specific protein kinase (IC_{50},\geq 5 \times 10^{-7} M) (34). Cal C-stimulated GtH-\(\alpha\) mRNA levels were not affected by treatment with low doses of sGnRH, but the response to Cal C tended to be additive to the response to 10^{-7} M sGnRH (Fig. 1). Cotreatments of Cal C with cGnRH-II resulted in GtH-\(\alpha\) mRNA levels that were significantly greater than those observed with either Cal C or cGnRH-II alone. Combination treatments with Cal C and varying doses of sGnRH or cGnRH-II did not result in any further stimulation of FSH-\(\beta\) mRNA levels compared with that observed with Cal C alone. Additive LH-\(\beta\) subunit responses were observed when Cal C was combined with 10^{-8} M and 10^{-7} M sGnRH, as well as with all doses of cGnRH-II.

To further confirm the effects of inhibition of PKC, the effects of another PKC inhibitor, GF, on GnRH-stimulated

at a final concentration of <0.1% had no effect on GtH subunit mRNA levels. Goldfish FSH-\(\beta\) (0.48 kb), LH-\(\beta\) (0.52 kb) and 18s rRNA (0.56 kb) cDNA fragments were cloned in this laboratory (23). The common carp GtH-\(\alpha\) subunit cDNA fragment (0.8–0.9 kb) was a gift from Dr. F. L. Huang (Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan).

Animals and cell preparation. Male and female goldfish, Carassius auratus, ranging from 8 to 12 cm in length were purchased from Aquatic Imports (Calgary, Alberta, Canada) and used throughout the yearly reproductive cycle. The fish were maintained in semicirculating tanks at 17°C on a 16:8-h light-dark photoperiod for acclimation before the experiments and fed a commercial fish diet. Goldfish were anesthetized and killed in accordance with the principles and guidelines of the Canadian Council on Animal Care. Pituitary cells were dispersed using a trypsin-DNase protocol modified from that of (14) and described by Klausen and colleagues (32). Briefly, pituitaries were placed in dispersion medium (medium 199 with Hanks’ balanced salt solution, 25 mM HEPEPS, 2.2 g/l sodium bicarbonate, 0.3% BSA, 100,000 U/l penicillin, 100 mg/l streptomycin, pH 7.2), diced into fragments and treated sequentially with trypsin (25,000 U/ml), trypsin inhibitor (25,000 U/ml), DNase I (0.01 mg/ml), 2 mM EGTA, and 1 mM EGTA. The fragments were dispersed by gentle trituration in calcium-free HBSS with 25 mM HEPEPS, 2.2 g/l sodium bicarbonate, 0.3% BSA, 100,000 U/l penicillin, 100 mg/l streptomycin, pH 7.2). Cell yield and viability were determined using Trypan blue exclusion, and the cells were resuspended in culture medium (medium 199 with Earle’s salts, 25 mM HEPEPS, 2.2 g/l sodium bicarbonate, 100,000 U/l penicillin, 100 mg/l streptomycin, pH 7.2). The cells were plated in culture medium for 2 h at 28°C, 5% CO2, with saturated humidity, after which horse serum was added to a final concentration of 1%. Depending on the experiment, the cells were allowed to recover for 24 or 72 h with a medium change at 48 h.

GtH subunit mRNA experiments. Cells were prepared as described above and plated on 6- or 24-well Primaria plates (VWR, Edmonton, Alberta, Canada) at a density of 0.75–1.25 million cells/ml of medium. Unless otherwise indicated, cells were treated continuously with stimuli for 12 h. For studies involving the inhibition of PKC, inhibitors were added 30 min before the addition of stimuli. Continuous treatment with GnRH or cGnRH-II has previously been shown to be effective in stimulating GtH-\(\alpha\), FSH-\(\beta\), and LH-\(\beta\) mRNA levels in primary cultures of dispersed goldfish pituitary cells (32).

Northern blot analysis of goldfish GtH subunit mRNA levels has been previously validated and described (22, 23, 30, 32). Briefly, total RNA was extracted from the cells using Trizol Reagent (Invitrogen, Burlington, Ontario, Canada), and its purity was determined from ratios of the sample absorbances at 260 and 280 nm (A_{260}:A_{280}). Three to five micrograms of RNA was fractionated on 1.4% agarose/2% formaldehyde gels and blotted in the presence of 20X SSPE (3 M sodium chloride, 0.23 M sodium phosphate, 20 mM EDTA, pH 7.4) onto Hybond-XL membranes (Amersham Biosciences, Baie d’Urfé, Quebec, Canada). RNA was fixed to the membranes by baking at 80°C for 2 h. Purified cDNA fragments (GtH-\(\alpha\), FSH-\(\beta\), LH-\(\beta\), and 18s rRNA) were labeled by the random primer method using the T7 Quickprime kit (Amersham Biosciences) and [\alpha-^{32}P]dCTP (3,000 Ci/mmol; Perkin Elmer Life Sciences, Woodbridge, Ontario, Canada). Membranes were prehybridized for 1 h and hybridized for 2 h at 60°C in rapid hybridization buffer (Amersham Biosciences) with the specific probe of interest. The membranes were then washed in a series of increasing stringency washes up to 0.1X SSC (15 mM sodium chloride, 1.5 mM sodium citrate, pH 7.0) in the presence of 0.1% SDS and exposed to Kodak X-Omat blue XB-1 film (Perkin Elmer Life Sciences). Additional hybridizations on the same membranes were carried out after stripping with repeated washings in boiling 0.1% SDS. 18s RNA was used as an internal standard for normalizing mRNA levels. Autoradiograms were scanned and quantified with a computerized densitometry program (Image 1.62, National Institutes of Health, Bethesda, MD). mRNA levels are expressed with respect to 18s rRNA levels for the same sample and given as a percentage change with respect to a time-matched control (means ± SE, where control shows 0% change with respect to itself). mRNA and rRNA densitometry readings were all within the linear detection range of the system. The results from a minimum of three independent experiments performed on separate cell cultures were pooled, log-transformed, and analyzed by a one-way ANOVA. If the ANOVA analysis showed that a significant difference existed among the groups, the Student-Newman-Keuls test for multiple comparisons of means was performed to identify the treatment groups that were different from one another. Means were considered statistically different if \(P < 0.05\) and are indicated by different letters.

RESULTS
REGULATION OF GtH SUBUNIT mRNA LEVELS BY PKC AND GnRH

GtH subunit mRNA levels were also examined after 24 h of recovery. GF targets the ATP-binding site in the catalytic domain and is a potent and selective inhibitor of PKC (IC₅₀ for various isoforms, 10⁻⁸ to 2 × 10⁻⁷ M) (46). mRNA levels were quantified after 12 h of treatment with sGnRH or cGnRH-II (10⁻⁷ M) in the absence or presence of GF (10⁻⁸ M, 10⁻⁷ M or 10⁻⁶ M) (Fig. 2). Dose-dependent stimulation of GtH-α, FSH-β, and LH-β mRNA levels was observed following GF treatment. These elevations were observed with a concentration of GF (10⁻⁷ M) well below the IC₅₀ values for inhibition of receptor tyrosine kinases (6.5 × 10⁻⁵ to 10⁻⁴ M) or PKA (2 × 10⁻⁶ M) (17, 36, 46). Treatment with cGnRH-II significantly elevated GtH subunit mRNA levels and significantly enhanced the effects of GF in at least an additive manner. Treatment with 10⁻⁷ M sGnRH alone produced significant elevations only in GtH-α mRNA levels although slight increases were also observed with FSH-β and LH-β subunits. This lack of effect may be related to seasonal changes in responsiveness to GnRH that are observed in goldfish (30). Nevertheless, general additive effects were often seen when sGnRH was applied with higher doses of GF.

Effects of a PKC activator applied at 24 h following recovery. To study the effect of PKC activation, cells were treated continuously for 12 h with varying doses of TPA (10⁻⁸ M, 10⁻⁷ M, or 10⁻⁶ M) (Fig. 3A). GtH-α mRNA levels were increased by 10⁻⁸ M TPA, whereas higher concentrations had no effect. Significant elevations in FSH-β mRNA levels were observed with all concentrations of TPA, although higher concentrations were less effective. Increases in LH-β mRNA levels were also observed with 10⁻⁸ M and 10⁻⁷ M TPA.

With the use of Western blot analysis, it has been demonstrated that prolonged TPA treatment downregulates conventional PKC levels in goldfish pituitary cells (25). Thus a modified treatment protocol, in which cells were treated for 2 h with TPA followed by 10 h in normal medium (12 h total), was used to control for depletion of PKC by prolonged treatment with TPA (Fig. 3B). GtH-α mRNA levels were significantly increased after treatment with 10⁻⁸ M and 10⁻⁷ M TPA. Elevated FSH-β mRNA levels were observed with all concentrations of TPA, although higher concentrations were again less effective. Although LH-β mRNA levels were increased by 10⁻⁸ M TPA, higher doses were either ineffective or resulted in a significant reduction in LH-β mRNA.

Determination of dopamine levels. The ability of PKC inhibitors alone to elevate GtH subunit mRNA levels suggests the presence of a PKC-mediated suppression of basal GtH subunit mRNA expression. This could result either from the presence of a basal, tonic inhibitory PKC effect or that PKC mediates the action of an inhibitory regulatory presence in the culture system. We hypothesized that dopamine, which has been shown to be a potent inhibitor of LH secretion in goldfish (41), is present in the cell cultures and is inhibiting basal GtH subunit mRNA expression. To evaluate the possible presence of dopamine in the cell cultures, we determined dopamine levels in the medium and cell contents by HPLC analysis (Fig. 4). Dopamine was easily detected in intact goldfish pituitaries (Fig. 4A), consistent with the known presence of dopaminergic terminals among the hypothalamic neuronal innervation of fish pituitaries (27). In contrast, dopamine was not detected in cell culture medium after 24, 48, or 72 h of recovery (data not shown). Although dopamine was detected in the cell contents at 24 h of recovery, the levels observed were 15-fold lower than in pituitary tissue (Fig. 4B). Dopamine levels in the cell contents were further reduced by 48 h, and by 72 h the levels were at or below the detection limit.

Effects of Cal C applied after 72 h of recovery. To minimize the possible compounding effects of inhibitory dopamine in-
fluences, we reexamined the effects of PKC inhibition at a time when dopamine cannot be detected in the cell culture. Cells were treated continuously for 12 h with sGnRH or cGnRH-II (10^{-7} M) in the absence or presence of varying concentrations of Cal C (10^{-8} M, 10^{-7} M, or 10^{-6} M) (Fig. 5). Treatment with Cal C alone significantly increased GtH-α, FSH-β, and LH-β mRNA levels in a dose-dependent manner. Although Cal C at a dose of 10^{-8} M was ineffective in stimulating GtH-α mRNA levels, its presence significantly enhanced the response to sGnRH. Similarly, the effects of higher concentrations of Cal C tended to be additive to those of sGnRH. Additive to sGnRH. Similarly, the effects of higher concentrations of Cal C tended to be additive to those of sGnRH. Additive to sGnRH. Similarly, the effects of higher concentrations of Cal C tended to be additive to those of sGnRH. Additive to sGnRH. Similarly, the effects of higher concentrations of Cal C tended to be additive to those of sGnRH.

Interaction of GnRH with TPA and DiC8. Cotreatment of GnRH with TPA or the cell-permeable and PKC-activating diacylglycerol analog, DiC8, was performed to further investigate the possible involvement of PKC in GnRH-mediated signaling. Cells were allowed to recover for 72 h and treated with varying concentrations of TPA (10^{-8} M, 10^{-7} M, or 10^{-6} M) in the presence or absence of sGnRH or cGnRH-II for 12 h (Fig. 6). Similar to results using cells after 24 h of culture (Fig. 3), all doses of TPA generated significant increases in FSH-β mRNA levels, whereas GtH-α and LH-β mRNA levels were significantly stimulated by only the lower doses. sGnRH- and cGnRH-II-induced increases in GtH subunit mRNA levels were largely additive to those induced by TPA (Fig. 6).

In another experiment, cells were treated with varying concentrations of DiC8 (10^{-6} M, 10^{-5} M, or 10^{-4} M) in the presence or absence of sGnRH or cGnRH-II (Fig. 7). DiC8 structurally resembles the diacylglycerols formed during G_{qσ} coupled G-protein coupled receptor signaling, and represents a more “physiological” signal stimulating PKC activation. In particular, the membrane recruitment and activation of PKC by DiC8 is brief because DiC8, like diacylglycerol, is rapidly metabolized. On the other hand, phorbol esters, which are not readily metabolized, constitutively activate PKC leading to its downregulation (40). All doses of DiC8 induced significant increases in GtH subunit mRNA levels with the higher doses being somewhat more effective. Treatment with sGnRH resulted in significant increases in GtH-α, FSH-β, and LH-β mRNA levels that were additive to those induced by DiC8 (Fig. 7). FSH-β mRNA responses to cGnRH-II were clearly additive to those of 10^{-6} and 10^{-5} M DiC8. Trends toward additivity were also seen with GtH-α and LH-β mRNA responses when cGnRH-II was coincubated with all doses of DiC8.

TPA and DiC8 effects are specific to PKC. It has become apparent that conventional and novel PKCs are not the only targets of phorbol esters and DAG (11, 29). To assess the specificity of phorbol ester-induced responses, we examined the effects of a 4-α-phorbol ester, which is ineffective in activating PKC (10). GtH subunit mRNA levels were not altered after 12 h of continuous treatment with 4-α-TPA (10^{-8} to 10^{-6} M). In contrast in the same experiments, treatment with
resulted in significant increases in GtH-
-of PKC in basal, as well as GnRH-induced, GtH-
inhibitors and activators to examine the possible involvement
dispersed goldfish pituitary cells (32). In this study, we used
stimulate GtH subunit gene expression in primary cultures of
medium (24 h of recovery. Primary cultures of dispersed goldfish pituitary cells were
DISCUSSION
Fig. 3. The effect of TPA on GtH-α, FSH-β, and LH-β mRNA levels after 24 h of recovery. Primary cultures of dispersed goldfish pituitary cells were treated continuously for 12 h (A) or for 2 h followed by 10 h in TPA-free medium (B), with varying concentrations of TPA. An internal standard (18s rRNA) was used to normalize GtH-α, FSH-β, and LH-β mRNA levels. Results (means ± SE, n = 3–5 in replicate experiments) are expressed with respect to control (where control shows 0% change with respect to itself).

TPA for either 12 or 2 h elevated GtH subunit mRNA levels (Table 1). To further verify that TPA and DiC8 effects were mediated via the activation of PKC, we used the PKC inhibitor, GF, which targets the ATP-binding site of PKC to block the effects of TPA and DiC8, which target the DAG/phorbol ester-binding site (Fig. 8). Treatment with GF (10^-6 M) alone resulted in significant increases in GtH-α, FSH-β, and LH-β mRNA levels. Additions of varying concentrations of either TPA or DiC8 to cells pretreated with GF did not produce a further elevation in GtH subunit mRNA levels.

DISCUSSION
We have previously shown that sGnRH and cGnRH-II stimulate GtH subunit gene expression in primary cultures of dispersed goldfish pituitary cells (32). In this study, we used inhibitors and activators to examine the possible involvement of PKC in basal, as well as GnRH-induced, GtH-α, FSH-β, and LH-β gene expression.

Dual effects of PKC on unstimulated GtH subunit gene expression. Cells treated with Cal C or GF after 24 or 72 h of recovery showed dose-dependent increases in GtH-α, FSH-β, and LH-β mRNA levels. These results indicate that PKC is involved in the negative regulation of basal GtH subunit gene expression. Cal C inhibits PKC via the DAG/phorbol ester-binding site in the regulatory domain (34), whereas GF inhibits PKC exclusively via the ATP-binding site in the catalytic domain (46). Thus, despite reports that Cal C also inhibits other DAG/phorbol ester-binding proteins (6, 28, 35, 43), that GF can inhibit MAPKAP kinase-1β and p70 S6 kinase (3), and that the two inhibitors generate the same response is strongly indicative of the involvement of PKC in the tonic suppression of GtH subunit gene expression. Interestingly, PKC-mediated suppression in goldfish pituitary cells is specific to GtH because both PKC inhibitors do not affect basal growth hormone mRNA levels in the same experiments (Klausen, C, Tsuchiya T, Chang JP, and Habibi HR, unpublished observations). In addition, the presence or absence in the cell cultures of measurable dopamine, a known regulator of GtH secretion, does not influence the observed PKC inhibitor-mediated elevation of basal GtH subunit mRNA levels. The exact cause and functional significance of the PKC-mediated suppression of GtH subunit gene expression remain elusive; however, future studies investigating the effects of various paracrine and autocrine factors on GtH subunit mRNA levels will hopefully provide some insight into this question.

The negative regulation of basal GtH subunit gene expression by PKC has not been reported in any other model system. In mammals, treatment with GF did not affect basal GtH-α, FSH-β, and LH-β mRNA levels in cultured rat pituitary cells (8); GtH-α mRNA levels in αT3–1 cells (9); human GtH-α-LUC, rat FSH-β-LUC, and rat LH-β-LUC expression in GGH3-1′ cells (44); equine LH-β-LUC expression in αT3–1 cells (13); and ovine FSH-β-LUC and rat LH-β-LUC expression in LβT2 or LB4 cells (47, 48, 50). In tilapia pituitary

Fig. 4. Determination of dopamine levels in primary cultures of dispersed goldfish pituitary cells by HPLC. Dopamine levels were measured in intact goldfish pituitary tissues as a positive control (A). Cells were allowed to recover for 24, 48, or 72 h, and cellular levels of dopamine were measured at each time point (B). Dopamine concentration was quantified as the integrated area under the curve and compared with that of external standards (a sample curve for each measurement is also shown). Results are expressed as picomol/pituitary equivalent (means ± SE, n = 3 or 4 in replicate experiments), and significant differences are indicated by different letters (P < 0.05).
cells, basal GtH-α mRNA levels were decreased, whereas LH-β mRNA levels were not significantly altered by GF (18, 19, 37). Although we report, for the first time, a negative role for PKC in basal GtH subunit gene expression, PKC-mediated suppression of gene expression has previously been reported in other systems. PKC suppresses human GnRH receptor-LUC activity in αT3–1 cells (16) and GnRH mRNA levels in hypothalamic GT1–7 cells (12).

Using varying doses of a phorbol ester (TPA) and a PKC-activating diglyceride (DiC8), we also established a stimulatory role for PKC in the regulation of goldfish GtH subunit mRNA levels. Interestingly, treatment with TPA or DiC8 did not affect growth hormone mRNA levels in the same experiments (Klausen C, Tsuchiya T, Chang JP, and Habibi HR, unpublished observations), indicating that these stimulatory effects on hormone mRNA levels were cell-type specific. Stimulation of GtH subunit gene expression by TPA has been reported in other mammalian systems (8, 9, 13, 44, 47, 48). In fish, GtH-α and LH-β mRNA levels were significantly elevated by TPA in cultured tilapia pituitary cells (18, 37). Similarly, salmon LH-β-CAT expression in transfected αT3–1 cells was also stimulated by TPA (4). We used two approaches to ensure that our responses to TPA and DiC8 were specific to the activation of PKC and not other DAG/phorbol ester-binding proteins. The inactive phorbol ester (4-TPA) had no effect on GtH subunit mRNA levels and, increases induced by TPA or DiC8 were completely blocked by inhibition of PKC via the ATP-binding site with GF. Furthermore, the results from experiments using DiC8 or a 2-h TPA treatment protocol indicate that PKC downregulation is not a significant factor, at least in this system.

It is clear that, in goldfish pituitary cells, PKC isoforms sensitive to Cal C and GF tonically regulate basal GtH subunit mRNA levels in a negative manner. It would appear that only conventional and novel PKCs could be responsible for this type of regulation. Cal C does not target atypical PKCs and GF, at the concentrations used in this study, only marginally inhibits atypical PKCζ (36). Similarly, only the DAG/phorbol ester-responsive conventional and novel PKCs could be responsible for the observed stimulatory role of PKC. To date, isoforms from the conventional (α), novel (δ, and possibly θ) and atypical (ξ) classes of PKC have been detected in the goldfish pituitary (25; Klausen C, Tsuchiya T, Chang JP, and Habibi HR, unpublished observations). It is likely that isoforms within the conventional and novel classes of PKC have radically different effects on GtH subunit gene expression. Isoform specificity has been demonstrated in digitonin-permeabilized rat pituitary cells where the addition of PKCα or β, but not γ, was able to recover TPA-induced GtH secretion (38). More recently, multiple isoforms of PKC (α, β, δ, ε, λ, and θ) were detected in LB2 cells and various inhibitors were used to demonstrate that GnRH- and TPA-mediated signaling may involve distinct sets of PKC isoforms (48). In this context, it is both possible and plausible that the dual, inhibitory, and stimulatory role of PKC in the regulation of GtH subunit mRNA levels in the goldfish pituitary can be achieved via multiplicity of PKC isoforms with differing structures, co-factor requirements, spatial distributions and macromolecular interactions. Further investigation is required to identify the full complement of PKC isoforms expressed in goldfish.
gonadotropes and to determine their individual roles in the regulation of GtH subunit gene expression.

PKC and GnRH-stimulated increases in GtH subunit gene expression. The main objective of this study was to determine whether PKC is involved in GnRH-induced increases in GtH-α, FSH-β, and LH-β mRNA levels in goldfish pituitary cells. Although there are some exceptions (5, 7, 20, 50), it is well established that PKC mediates GnRH effects on GtH secretion, as well as gene expression in mammalian model systems (33, 39). In tilapia, PKC is involved in GnRH-stimulated LH release, as well as LH-β and GtH-α, but not FSH-β, transcription (19, 49). Numerous studies in goldfish have provided sound evidence for the involvement of PKC in sGnRH- and cGnRH-II-stimulated LH release (15). Results from the present study investigating the involvement of PKC in GnRH-induced increases in GtH subunit mRNA levels are more variable; nevertheless, GtH subunit mRNA responses to GnRH are generally additive to those induced by PKC inhibi-

Fig. 6. Additivity of TPA- and GnRH-induced effects. Primary cultures of dispersed goldfish pituitary cells were allowed to recover for 72 h and then treated continuously for 12 h with varying concentrations of TPA in the absence or presence of sGnRH or cGnRH-II (10^{-7} M). An internal standard (18s rRNA) was used to normalize GtH-α (A), FSH-β (B), and LH-β (C) mRNA levels. Results (means ± SE, n = 3 or 4 in replicate experiments) are expressed with respect to control (where control shows 0% change with respect to itself), and significant differences are indicated by different letters (P < 0.05).

Fig. 7. The effects of 1,2-dioctanoyl-sn-glycerol (DiC8) and GnRH are additive. Primary cultures of dispersed goldfish pituitary cells were allowed to recover for 72 h and then treated continuously for 12 h with varying concentrations of DiC8 in the absence or presence of sGnRH or cGnRH-II (10^{-7} M). An internal standard (18s rRNA) was used to normalize GtH-α (A), FSH-β (B), and LH-β (C) mRNA levels. Results (means ± SE, n = 3 or 4 in replicate experiments) are expressed with respect to control (where control shows 0% change with respect to itself), and significant differences are indicated by different letters (P < 0.05).
Table 1. Dose-dependent effects of TPA and 4-α-TPA on GtH subunit mRNA levels in goldfish pituitary cells following 72 h of recovery

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Values are expressed as means ± SE; n = 3 or 4 in replicate experiments. Primary cultures of dispersed goldfish pituitary cells were allowed to recover for 72 h and then treated continuously for 12 h with TPA or 4-α-TPA at the concentrations indicated. Alternatively, cells were treated with TPA for 2 h followed by 10 h in TPA-free medium (total of 12 h). An internal standard (18s rRNA) was used to normalize gonadotropin-releasing hormone-α (GtH-α), follicle-stimulating hormone-β (FSH-β) and luteinizing hormone-β (LH-β) mRNA levels. Results are expressed with respect to control (where control shows 0% change with respect to itself, and significant differences between doses for a given GtH subunit are indicated by different letters (P < 0.05).
of GtH-α and LH-β. In tilapia, GnRH-stimulated FSH-β gene expression differs from that of GtH-α and LH-β in that it does not involve PKC (19). The differential regulation of the individual GtH subunits may result from differences in the elements contained within the promoter regions of each gene or it may be the product of their expression in distinct cells. Thus the intracellular signaling mechanisms involved in the regulation of the three GtH subunits likely shares common, as well as independent, elements and will be a fascinating area for future research.

In summary, we used primary cultures of dispersed goldfish pituitary cells to investigate the possible involvement of PKC in GnRH-induced increases in GtH-α, FSH-β, and LH-β subunit mRNA levels. We report for the first time a novel role for PKC in the negative regulation of basal GtH subunit gene expression. In addition, a stimulatory role for PKC is also established in studies with phorbol ester and a PKC-activating diglyceride. PKC inhibitor- and PKC activator-induced effects are generally additive to those of GnRH, indicating that conventional and novel PKCs are unlikely to be the primary mediators of GnRH-induced increases in GtH subunit mRNA levels in the goldfish pituitary. These data, along with previous work on LH release in goldfish pituitary cells, suggest that the role of PKC in GtH secretion and basal vs. stimulated GtH gene expression is exceptionally complex.

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