Differential Activation of Protein Kinase C δ and ε Gene Expression by Gonadotropin-releasing Hormone in αT3-1 Cells

AUTOREGULATION BY PROTEIN KINASE C*

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The effect of gonadotropin-releasing hormone (GnRH) upon protein kinase C (PKC) δ and PKCe gene expression was investigated in the gonadotroph-derived αT3-1 cell line. Stimulation of the cells with a stable analog [d-Trp6]GnRH (GnRH-A) resulted in a rapid elevation of PKCδ mRNA levels (1 h), while PKCe mRNA levels were elevated only after 24 h of incubation. The rapid elevation of PKCe mRNA by GnRH-A was blocked by pretreatment with a GnRH antagonist or actinomycin D. The PKC activator 12-O-tetradecanoylphorbol-13-acetate (TPA), but not the Ca2+-ionophore ionomycin, mimicked the rapid effect of GnRH-A upon PKCe mRNA elevation. Additionally, the rapid stimulatory effect of GnRH-A was blocked by the selective PKC inhibitor GF109203X, by TPA-mediated down-regulation of endogenous PKC, or by Ca2+ removal. Interestingly, serum-starvation (24 h) advanced the stimulation of PKCδ mRNA levels by GnRH-A and the effect could be detected at 1 h of incubation. The rapid effect of GnRH-A upon PKCe mRNA levels in serum-starved cells was mimicked by TPA, but not by ionomycin, and was abolished by down-regulation of PKC or by Ca2+ removal. Precactivation of αT3-1 cells with GnRH-A for 1 h followed by removal of ligand and serum resulted in elevation of PKCe mRNA levels after 24 h of incubation. Western blot analysis revealed that GnRH-A and TPA stimulated (within 5 min) the activation and some degradation of PKCδ and PKCe. We conclude that Ca2+ and PKC are involved in GnRH-A elevation of PKCδ and PKCe mRNA levels, with Ca2+ being necessary but not sufficient, while PKC is both necessary and sufficient to mediate the GnRH-A response. A serum factor masks PKCδ but not PKCe mRNA elevation by GnRH-A, and its removal exposes preactivation of PKCδ mRNA by GnRH-A which can be memorized for 24 h. PKCδ and PKCe gene expression evoked by GnRH-A is autoregulated by PKC, and both isotypes might participate in the neurohormone action.

The protein kinase C (PKC) family is a family of serine/threonine protein kinase isoforms, which play key roles in signal transduction (1–3). Conventional PKCs (α, βI, βII, and γ) are activated by Ca2+, diacylglycerol (DAG), and phospholipid such as phosphatidyserine (PS) and are tightly coupled to phosphoinositide turnover (1–3). Novel PKCs (δ, ε, η, and θ) are Ca2+-independent but DAG- and PS-activated isoforms. Atypical PKCs (ζ and λ) are Ca2+ - and DAG-independent but PS-activated isoforms and are also stimulated by other lipiderived mediators (1–4). PKCμ takes an intermediate position among the novel PKC and atypical PKC isoforms and is a Ca2+- and DAG-independent isoform. Whereas relatively much is known about regulation of PKC at the protein level including cofactor requirements, translocation to the membrane, substrate phosphorylation, and degradation (1–9), very little is known about ligand regulation of PKC gene expression (10–12). Previous work has implicated PKC in gonadotropin-releasing hormone (GnRH) action upon gonadotropin secretion and gonadotropin subunits gene expression in pituitary and αT3-1 cells (5, 6, 12–26). Recently, while examining conventional PKC regulation, we have shown that GnRH-A increases the levels of PKCδ, but not PKCα, mRNA levels in αT3-1 cells, while PKCγ is not expressed in the cells (12). Since PKCδ and PKCe of the novel PKC group are major subspecies in the pituitary (26), we decided to investigate the effect of GnRH-A on the mRNA levels of both isotypes in the αT3-1 cell line. Here we demonstrate that GnRH-A directs differential autoregulation of PKCδ and PKCe gene expression, which is dependent upon growth conditions and Ca2+, and reveals a memory mechanism, which might participate in PKCδ autoregulation.

EXPERIMENTAL PROCEDURES

Materials

αT3-1 cells were kindly provided by Dr. P. Mellon (University of California San Diego, La Jolla, CA). The GnRH analog [d-Trp6]GnRH (GnRH-A) was a gift from Dr. R. Millar (University of Cape Town Medical School, Cape Town, South Africa). A potent GnRH antagonist [D-Glu(P)2,CI(Phe)3,D-Trp6]GnRH was kindly provided by Dr. D. Coy (Tulane University School of Medicine, New Orleans, LA). Ionomycin was purchased from Boehringer (Mannheim, Germany). The PKCe-selective inhibitor bisindolylmaleimide (GF 109203X) (27) was purchased from Calbiochem (Laufelfingen, Switzerland). Bovine serum albumin, TPA, and other chemicals were purchased from Sigma (Rehovot, Israel). Media and sera for cell culture were from Biological Industries (Kibbutz Beit Ha’Emek, Israel). [a-32P]dCTP was purchased from Rotem (Beer-sheba, Israel). PKCδ and PKCe cDNAs were kindly provided by Dr. H. Mischak (Institute of Clinical Molecular Biology, GSF, Munich, Germany) and Dr. F. Mushinsky (NH, Bethesda, MD) (27), and the respective antibodies were obtained from Sigma.

Methods

Cell Culture—αT3-1 cells were subcultured into 60-mm tissue culture dishes (Sterilin, Hounslow, United Kingdom). Cells were grown in 5 ml of Dulbecco’s modified Eagle’s medium (DMEM) containing 5% fetal calf serum, 5% horse serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. After 3–4 days, when cells were 70–80% confluent, the

DAG, diacylglycerol; PS, phosphatidyserine; PKM, catalytic moiety of PKC.

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§ The abbreviations used are: PKC, protein kinase C; PKCs, PKC subtypes; GnRH, gonadotropin-releasing hormone; GnRH-A, GnRH analog; TPA, 12-O-tetradecanoylphorbol-13-acetate; DMEM, Dulbecco’s modified Eagle’s medium; DAG, diacylglycerol; PS, phosphatidyserine; PKM, catalytic moiety of PKC.
Western-blotted (right panels) as described under “Methods” using anti-PKC with 0.25 M sucrose, 1 mM phenylmethylsulfonyl fluoride, 10% (100,000 cytosol and membrane fractions were obtained by ultracentrifugation syringe. Following removal of nuclei (1200 rpm for 5 min at 4 °C), were harvested with ice-cold Tris-buffered saline, pH 7.2, utilizing anti-PKC8 (upper panel) and anti-PKCε (lower panel) antibodies. Right panels show the separation (right arrows) of PKCδ and PKCe and their degradation products (left arrows indicate the size shift of PKCδ). Left panels show the densitometric quantitation of PKCδ and PKCe and their degradation products (insets, p42 and p50, respectively) in the membrane fractions. cultures were washed three times with fresh DMEM, and stimulants were added in 5 ml of DMEM at the indicated concentrations for the given length of time. For short period incubations (up to 1 h) 10 mM Hepes was added to the medium. When the stimulation period was longer than 9 h, the medium was supplemented with 0.1% bovine serum albumin.

**RNA Extraction and Analysis—**At the end of the stimulation period, total RNA was isolated from cells by extraction in guanidium thiocyanate containing 8% β-mercaptoethanol by the LiCl method as described by Cathala et al. (28). For Northern blot analysis, total RNA (15 µg) was fractionated on 1.2% denaturing agarose gel and transferred to GeneScreen membranes (DuPont NEN). Alternatively, RNA samples (8 µg) were slot blotted onto GeneScreen using a slot manifold (Schleicher & Schull). Following baking and prehybridization, the membranes were hybridized overnight with the specific cDNA probes labeled to high specific activity using a random primer labeling kit (Boehringer). Half of each lane was hybridized with a PKC cDNA, and the second half was hybridized with glyceraldehyde-3-phosphate dehydrogenase cDNA as an internal control. Thereafter, filters were washed at high stringency and were autoradiographed at −70 °C. Steady state levels of mRNAs were quantified with densitometric scanning of autoradiograms. The data were corrected for variability in loading by calculation as a ratio to glyceraldehyde-3-phosphate dehydrogenase.

**PKC Translocation—**Following ligand treatment, cells were washed with ice-cold Tris-buffered saline, pH 7.2, harvested with rubber policeman, and pelleted by short spin (1200 rpm for 5 min at 4 °C). Cells were resuspended in 10 mM EGTA, 2 mM EDTA, 20 mM Tris-HCl, pH 7.5, 0.25% sucrose, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 0.5 mM iodoacetic acid, and lysed by 10 strokes of a 25-gauge syringe. Following removal of nuclei (1200 rpm for 5 min at 4 °C), cytosol and membrane fractions were obtained by ultracentrifugation (100,000 × g for 2 h at 4 °C). The proteins were separated on 7.5–18% SDS-polyacrylamide gels (ratio of acrylamide to bisacrylamide, 30:0.5) and electrotransferred to nitrocellulose papers in 50 mM glycine, 50 mM Tris-HCl, pH 8.8 (100 V for 2 h at 4 °C). The papers were blocked for 60 min in 1% bovine serum albumin and 0.5% Tween 20 in Tris-buffered saline and treated overnight with the respective rabbit anti-PKC antibodies (Sigma). The signals were visualized using horseradish peroxidase-conjugated goat anti-rabbit IgG and the ECL method.

**Statistical Analysis—**The hybridization signals for PKC subtypes mRNA in each group were normalized to the hybridization signals for the housekeeping gene for glyceraldehyde-3-phosphate dehydrogenase. An arbitrary unit of 1 represents the control values. Statistical comparisons between control and treatment groups were performed using Student’s t test; in the figures, a single asterisk indicates p < 0.05, a double asterisk indicates p < 0.01, and a triple asterisk indicates p < 0.001.

**RESULTS**

We first studied the cellular redistribution of PKCδ and PKCe following GnRH-A and TPA stimulation, since it is a criterion for PKC activation by extracellular signals (5–8). Both GnRH-A and TPA stimulated an increase in the molecular weight of cytosolic PKCδ within 5 min (Fig. 1), consistent with the size shift reported for PKCδ phosphorylation by Src (29), and with our own finding that GnRH-A and TPA stimulate protein-tyrosine phosphorylation in αT3-1 cells.2 PKCδ in the membrane fraction is already of the high molecular weight form and is further elevated by GnRH-A and even more by TPA. In addition, translocation of PKCδ to the membrane fraction by GnRH-A and TPA is further validated by the appearance of degradation products of 70 and 42 kDa (apparently PKM) in the ligand-treated groups (2-fold; Fig. 1). PKCe activation is manifested by translocation to the membrane fraction and the appearance of 50- and 42-kDa bands (apparently PKM; Refs. 6–8) in the membrane fraction in the ligand-treated groups (3–4-fold stimulation by GnRH-A and TPA; Fig. 1). PKCe activation is manifested by translocation to the membrane fraction and the appearance of 50- and 42-kDa bands (apparently PKM in the ligand-treated groups (2-fold; Fig. 1). Consistent with our previous reports that TPA-mediated down-regulation of endogenous PKC in αT3-1 cells reduced cellular PKC activity by 90% (12, 21, 23), prolonged incubation with TPA (100 ng/ml, 24 h) resulted in loss of most of PKCe and even more by TPA. In addition, translocation of PKCε to the membrane fraction by GnRH-A and TPA is further validated by the appearance of degradation products of 70 and 42 kDa (apparently PKM; Refs. 6–8) in the membrane fraction in the ligand-treated groups (3–4-fold stimulation by GnRH-A and TPA; Fig. 1). PKCe activation is manifested by translocation to the membrane fraction and the appearance of 50- and 42-kDa bands (apparently PKM in the ligand-treated groups (2-fold; Fig. 1). Consistent with our previous reports that TPA-mediated down-regulation of endogenous PKC in αT3-1 cells reduced cellular PKC activity by 90% (12, 21, 23), prolonged incubation with TPA (100 ng/ml, 24 h) resulted in loss of most of PKCe and even more by TPA. In addition, translocation of PKCε to the membrane fraction by GnRH-A and TPA is further validated by the appearance of degradation products of 70 and 42 kDa (apparently PKM; Refs. 6–8) in the membrane fraction in the ligand-treated groups (3–4-fold stimulation by GnRH-A and TPA; Fig. 1).

The regulation of PKCδ and PKCe mRNA levels was determined by treatment of αT3-1 cells with [3H-Trp]GnRH, a stable GnRH analog. Addition of GnRH-A to the cells for 1 h elevated PKCe but not PKCδ mRNA levels in a dose-related fashion.

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Stimulation of PKC\(e\) mRNA levels by GnRH-A was rapid, with a peak at 1 h, declining thereafter to basal levels (Fig. 2B). On the other hand, significant elevation of PKC\(d\) mRNA levels was detected only after 24 h of incubation with GnRH-A (3-fold, \(p<0.001\); Fig. 2B).

The effect of GnRH-A on PKC\(e\) mRNA levels was investigated further, since its rapid nature suggested that it more likely represents a physiological response to the neurohormone. Pretreatment of the cells with a potent GnRH antagonist (Fig. 3A) or with actinomycin D (Fig. 3B) abolished the stimulatory effect of GnRH-A upon PKC\(e\) mRNA levels, indicating a receptor-mediated effect apparently at the transcriptional level (Fig. 3).

The potential role of PKC and \(\text{Ca}^{2+}\) in mediating the GnRH response upon PKC\(e\) mRNA levels was investigated since both messengers were implicated in GnRH action upon gonadotropin release and gonadotropin subunits gene expression (5, 6, 12–26, 30). Addition of the PKC activator TPA to \(\alpha T3-1\) cells for 1 h resulted in elevation of PKC\(e\) but not PKC\(d\) mRNA levels, while the \(\text{Ca}^{2+}\) ionophore, ionomycin, had no effect (Fig. 4, A and C, and data not shown). Elevation of PKC\(e\) mRNA levels by TPA was rapid, with a peak at 1 h and a return to basal levels (Fig. 4B). The similar time responses elicited by GnRH-A and TPA suggest that PKC is involved in GnRH-A stimulation of PKC\(e\) gene expression.

This notion was further supported by inhibition and depletion of PKC. Addition of the selective PKC inhibitor GF 109203X (27, 31) to the cells resulted in a dose-related inhibition of the GnRH-A stimulated PKC\(e\) mRNA levels with half-maximal inhibition (IC\(_{50}\)) observed at 0.8 \(\mu\)M of the drug, in good agreement with IC\(_{50}\) values of PKC inhibition in cellular systems such as Swiss 3T3 fibroblasts (31) (Fig. 5A). The drug alone (1 \(\mu\)M) reduced the basal level by about 50%, suggesting that PKC is also involved in the maintenance of basal PKC\(e\) gene expression. We also used down-regulation of endogenous PKC by prolonged incubation with TPA. Pretreatment of the cells with TPA (100 ng/ml, 24 h) reduced cellular PKC activity by 90% as measured by enzymatic activity assay and Western blot analysis (Fig. 1 and Refs. 12, 21, and 23). The stimulatory effect of GnRH-A and TPA upon PKC\(e\) mRNA levels was abolished in the down-regulated cells (Fig. 5B). In addition, we observed no additivity between GnRH-A and TPA upon PKC\(e\) mRNA levels (Fig. 6), lending further support to the role of PKC in mediating the GnRH-A effect on PKC\(e\) gene expression. The \(\text{Ca}^{2+}\) ionophore, ionomycin, had no effect on basal PKC\(e\) mRNA levels or on the stimulatory response elicited by GnRH-A or TPA (Fig. 6). On the other hand, transfer of \(\alpha T3-1\) cells in triplicate were treated with or without GnRH-A at the indicated concentrations for 60 min (A) or for the time indicated (B).

PKC mRNA levels were determined as described under “Methods.” An arbitrary unit of 1 represents the control values. Results are mean ± S.E. (n = 6). A lane of the dot blot is shown in the inset. *, \(p<0.05\); **, \(p<0.01\); ***, \(p<0.001\).

FIG. 2. Effect of GnRH analog on PKC\(e\) and PKC\(d\) mRNA levels.

\(\alpha T3-1\) cells were treated with or without GnRH-A at the indicated concentrations for 60 min (A) or for the time indicated (B). PKC mRNA levels were determined as described under “Methods.” An arbitrary unit of 1 represents the control values. Results are mean ± S.E. (n = 6). A lane of the dot blot is shown in the inset. *, \(p<0.05\); **, \(p<0.01\); ***, \(p<0.001\).

FIG. 3. Inhibition of GnRH-A-induced PKC\(e\) mRNA elevation by a GnRH antagonist (A) and by actinomycin D (B). \(\alpha T3-1\) cells were treated with or without GnRH agonist [\(\text{o-Trp}^6\)GnRH (GnRH-A, 10 nM), GnRH antagonist [\(\text{D-Glu(P)^1,ClPhe(P)^2,D-Trp^3,6}\)GnRH (100 nM], or both for 1 h (A). \(\alpha T3-1\) cells were pretreated with actinomycin D (1 \(\mu\)g/ml) for 60 min followed by GnRH-A (10 nM) for an additional 60 min (B). PKC\(e\) mRNA levels were determined as described under “Methods.” Northern blot analysis for the treatment groups of B is shown in the inset. Results are mean ± S.E. (n = 6). *, \(p<0.05\); **, \(p<0.01\).
cells to Ca\(^{2+}\) free medium, in the presence or absence of EGTA, abolished stimulation of PKC\(\varepsilon\) mRNA levels by GnRH-A (Fig. 7). It therefore seems that Ca\(^{2+}\) is necessary but not sufficient for mediation of the GnRH-A response.

Since GnRH stimulated PKC\(\delta\) mRNA levels only after 24 h of incubation in medium without serum (Fig. 2B), it was possible that growth conditions are involved in PKC\(\delta\) gene expression.

We therefore examined the role of serum in PKC\(\varepsilon\) and PKC\(\delta\) gene expression. Transfer of the cells to medium with low serum (0.5%) for 24 h had no effect on GnRH-A-stimulation of PKC\(\varepsilon\) mRNA levels (Fig. 8). On the other hand, serum starvation advanced the stimulation of PKC\(\delta\) mRNA levels by GnRH-A to 1 h of incubation that could not be observed in serum-grown cells (Fig. 8). Time course of PKC\(\delta\) mRNA levels in serum-starved cells revealed a rapid effect of GnRH-A at 1 h of incubation with no effect at 24 h, as seen in non-starved cells (Fig. 9A). Similarly, serum starvation exposed a rapid response (peak at 30 min) of TPA on PKC\(\delta\) mRNA levels (Fig. 9B), suggesting a role for PKC in mediating PKC\(\delta\) gene expression. Indeed, down-regulation of endogenous PKC by prolonged incubation with TPA abolished GnRH-A and TPA stimulation of PKC\(\delta\) mRNA levels in serum-starved cells (Fig. 10). Transfer of the cells to Ca\(^{2+}\) free medium, in the presence or absence of EGTA, abolished the rapid stimulation of PKC\(\delta\) in serum-starved cells (Fig. 11).

As shown above, when cells are transferred to serum-free medium and exposed to GnRH-A, elevation of PKC\(\delta\) mRNA levels is observed at 24 h, but not at 1 h of incubation (Fig. 12, columns 1–3). On the other hand, when cells are first serum-starved (24 h) and later exposed to GnRH-A, elevation of PKC\(\delta\) mRNA levels is observed after 1 but not 24 h of incubation (Fig. 12, columns 4–6).
12, columns 4–6). We therefore exposed normal cells to GnRH-A for 1 h, washed the cells several times to remove serum and GnRH-A, and further incubated the cells for 24 h. As seen in Fig. 12 (columns 7 and 8), PKC<sub>d</sub>mRNA levels were elevated at 24 h by pretreatment (1 h) with GnRH-A. Thus, the late effect of GnRH-A on PKC<sub>d</sub>mRNA levels (Fig. 12, column 3) is due to generation of a rapid signal (1 h, column 5), which is "memorized" during the long starvation period (t½ > 12 h) required for manifestation of the early signal by means of removal of the inhibitory effect of the serum.

**DISCUSSION**

Whereas much has been learned concerning the regulation of PKC and its subspecies at the protein level (1–9), very little is known about ligand regulation of PKC subtypes gene expression (10–12). Differentiation regulators of the human promyelocytic leukemia cell line (HL-60) such as 1α,25-dihydroxyvitamin D<sub>3</sub>, retinoic acid, and dimethyl sulfoxide, were shown to increase the expression of PKC<sub>a</sub> and PKC<sub>b</sub> mRNA levels (10, 11). Furthermore, transcriptional activation of PKC<sub>a</sub> and PKC<sub>b</sub> expression was reported to result in increased PKC enzymatic activity (10, 11, 32). Here we demonstrate that GnRH-A, which does not promote growth or differentiation, is capable of activating differential nPKC isoforms gene expression. To the best of our knowledge, this is the first demonstration of a natural ligand stimulation of PKC<sub>d</sub> and PKC<sub>e</sub>mRNA levels.

**FIG. 6.** Lack of additivity between GnRH-A and TPA upon PKC<sub>e</sub>mRNA elevation. αT3-1 cells were treated with or without GnRH-A (10 nM), TPA (100 ng/ml), or ionomycin (1 μM) or in combinations, for 60 min. Results are mean ± S.E. (n = 6). *, p < 0.05; **, p < 0.01.

**FIG. 7.** Effect of Ca<sup>2+</sup> removal upon GnRH-A-induced PKC<sub>e</sub>mRNA levels. αT3-1 cells were transferred to DMEM (control), to Ca<sup>2+</sup>-free DMEM (Ca<sup>2+</sup>-free) or to Ca<sup>2+</sup>-free DMEM + 250 μM EGTA (EGTA) for 10 min. Cells were then incubated with (striped bars) or without (empty bars) GnRH-A (10 nM) for 60 min in the respective medium as indicated. PKC<sub>e</sub>mRNA levels were analyzed as described under "Methods" (n = 6). **, p < 0.01.

**FIG. 8.** Effect of serum starvation upon GnRH-A stimulation of PKC<sub>e</sub> and PKC<sub>d</sub>mRNA levels. αT3-1 cells were preincubated with 10 or 0.5% serum for 24 h. Cells were then washed and incubated in serum-free medium with GnRH-A (10 nM) for 60 min. Results are mean ± S.E. (n = 6). ***, p < 0.01; ****, p < 0.001.

**FIG. 9.** Effect of GnRH-A and TPA upon PKC<sub>d</sub>mRNA levels in serum-starved cells. αT3-1 cells were serum-starved (0.5% serum) for 24 h, washed, and incubated with GnRH-A (10 nM, A) or TPA (100 ng/ml, B) for the indicated time periods. Results are mean ± S.E. (n = 6). ***, p < 0.01; ****, p < 0.001.
expression, possibly via a serum response element.

The differential activation of PKCδ and PKCe mRNA levels by GnRH-A suggests that the isoforms might specialize in different functions. PKCδ is the major subspecies in the 6-day-old rat pituitary and is markedly reduced in the 3-month-old pituitary (26). The opposite is observed for pituitary PKCe, which increases with age (26). Therefore, PKCδ and PKCe might play different roles during pituitary development. It was also shown that while PKCδ is involved in exocytosis, PKCe is autoregulated by PKC.

While overexpression of PKCδ resulted in inhibition of growth rate in NIH 3T3 cells, overexpression of PKCe increased growth rate, and the transformed cells (NIH 3T3 or Rat 6 cells) formed tumors in nude mice (27, 34). Since GnRH affect different responses, it is possible that PKCδ and PKCe are involved in separate functions such as gonadotropin release and gonadotropin subunit gene expression during the hormone action. Elevation of mRNA of a given PKC isoform by ligands in general and by GnRH-A in particular might be a step in the life cycle of PKCs during hormone action to replenish the enzymes after translocation and degradation as shown in Fig. 1.

The rapid effect (peak at 60 min) of GnRH-A upon PKCe and PKCδ (in serum-starved cells) mRNA levels might be physiologically relevant, since GnRH is released from the hypothalamus in a pulsatile manner at intervals of 1–2 h according to the species and its half-life is about 2–4 min (35–37). Thus, prolonged responses such as those observed in Fig. 2 (24 h) are more difficult to interpret, since it is not clear whether multiple pulses of GnRH are capable of eliciting a similar response. Hence, we investigated in more detail the rapid effects of GnRH, which prompted us to identify the second messengers involved in the neurohormone action. Indeed, the PKC activation by GnRH suggests that the isoforms might specialize in separate functions such as gonadotropin release.

While we show here positive regulation of PKCδ and PKCe mRNA levels by GnRH-A and TPA, others have recently reported that PKCe mRNA is down-regulated by TPA (4 h) in the mouse B lymphoma cell line A20 (38). The difference between the results might be due to the presence of a soluble destabilizing factor, which specifically accelerates degradation of PKCe mRNA in A20 cells (38).

Removal of Ca2+ abolished the effect of GnRH-A upon PKCδ and PKCe mRNA levels, but Ca2+ ionophore had no stimulatory effect. We therefore conclude that Ca2+ is necessary but not sufficient, while PKC is both necessary and sufficient to mediate the GnRH response. Furthermore, since removal of extracellular Ca2+ per se is not sufficient to block Ca2+ mobi-

![FIG. 10. Effect of PKC depletion upon GnRH-A and TPA-induced PKCδ mRNA levels in serum-starved cells. αT3-1 cells were serum-starved (0.5% serum) in the presence or absence of TPA (100 ng/ml) for 24 h to deplete endogenous PKC activity. Cells were then washed and incubated with or without GnRH-A (10 nM) or TPA (100 ng/ml) for 60 min. Results are mean ± S.E. (n = 6). ***, p < 0.001.

![FIG. 11. Effect of Ca2+ removal upon GnRH-A-induced PKCδ mRNA levels in serum-starved cells. αT3-1 cells were preincubated with 0.5% serum for 24 h. Cells were then transferred to DMEM (control), to Ca2+-free DMEM (Ca2+ free) or to Ca2+-free DMEM + 250 μM EGTA (EGTA) for 10 min. Cells were then incubated without (empty bars) or with (striped bars) GnRH-A (10 nM) for 60 min in the respective medium as indicated. PKCδ mRNA levels were analyzed as described under "Methods" (n = 6). ** p < 0.01.

![FIG. 12. Effect of pretreatment with GnRH-A on PKCδ mRNA levels in serum-starved cells. αT3-1 cells grown in serum (10%) were washed and stimulated with GnRH-A (10 nM) for 1 or 24 h in serum-free medium (columns 1–3). The second group was serum-starved (0.5% serum) for 24 h, washed, and incubated with GnRH-A (10 nM) for 1 or 24 h (treat=strev, columns 4–6). The third group was grown in 10% serum, washed and stimulated with GnRH-A (10 nM) for 1 h, and then serum-starved for 24 h (treat=strev, columns 7 and 8). Results are mean ± S.E. (n = 6). **, p < 0.01; ***, p < 0.001.](http://www.jbc.org/content/13539/5/13539.f012)

![FIG. 13. Effect of pretreatment with GnRH-A on PKCδ mRNA levels in serum-starved cells. αT3-1 cells grown in serum (10%) were washed and stimulated with GnRH-A (10 nM) for 1 or 24 h in serum-free medium (columns 1–3). The second group was serum-starved (0.5% serum) for 24 h, washed, and incubated with GnRH-A (10 nM) for 1 or 24 h (treat=strev, columns 4–6). The third group was grown in 10% serum, washed and stimulated with GnRH-A (10 nM) for 1 h, and then serum-starved for 24 h (treat=strev, columns 7 and 8). Results are mean ± S.E. (n = 6). **, p < 0.01; ***, p < 0.001.](http://www.jbc.org/content/13539/5/13539.f013)
stimulated PKC and the hormone of the reproductive cycle. Since both Ca\(^{2+}\) are required to identify the PKC isoforms and the site of Ca\(^{2+}\) action in the GnRH-A response.

In addition to mediation by Ca\(^{2+}\) and PKC, elevation of PKC\(^{d}\) but not PKC\(^{e}\) mRNA levels by GnRH-A and TPA required removal of a serum factor. Therefore, although PKC\(^{d}\) and PKC\(^{e}\) gene expression share some common mechanisms, which are mediated by Ca\(^{2+}\) and PKC, they differ in sensitivity to the serum factor. Changes in the concentrations of the serum factor under physiological conditions might therefore enable preferential activation by GnRH of the two isoforms. Moreover, it was possible to first stimulate the cells with GnRH-A, and remove the hormone and serum for 24 h, at the end of which elevation of PKC\(^{d}\) mRNA levels by GnRH-A was detected. The observation is in contrast to previous observations, in which GnRH-stimulated LH release in perfused pituitary cells was terminated immediately after removal of the neurohormone (Ref. 40 and data not shown). Since PKC is the main mediator of GnRH actions (5, 6, 12–26, 30), it is likely that the half-life of the phosphoproteins involved in exocytosis is very short (Ref. 40 and data not shown). Since PKC is the main mediator of GnRH-A response.

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