Mechanism of action of gonadotropin-releasing hormone upon gonadotropin α-subunit mRNA levels in the αT3-1 cell line: role of Ca^{2+} and protein kinase C

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Addition of [D-Trp^6]gonadotropin-releasing hormone (GnRHa) to αT3-1 cells induced a very rapid response upon gonadotropin α-subunit mRNA which was detected after 30–60 min and was abolished by pretreatment with actinomycin D. A similar response was obtained with the protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA), or the Ca^{2+} ionophore, ionomycin. GnRHa (10 nM) also stimulated a secondary rise in α-subunit mRNA levels between 12 and 24 h of incubation. No additivity was obtained (at 60 min) upon the combined addition of GnRHa and PMA, GnRHa and ionomycin, or PMA and ionomycin. The effect of GnRHa upon α-subunit mRNA was blocked by the PKC inhibitors staurosporine or GF 109203X. Down-regulation of endogenous PKC activity resulted in inhibition of the stimulatory effect of gonadotropin-releasing hormone (GnRH), PMA and ionomycin. Removal of extracellular Ca^{2+} abolished the effect of GnRHa and PMA upon α-subunit mRNA levels. Interestingly PMA and ionomycin had no effect on α-subunit mRNA levels at 24 h of incubation; however, the combined addition of the drugs mimicked the late phase of GnRHa (10 nM) action. The data provide evidence that PKC and Ca^{2+} are involved in mediating the early and the late responses of GnRHa upon α-subunit mRNA elevation and that differential cross-talk exists between the messengers.

INTRODUCTION

The gonadotropin luteinizing hormone (LH), follicle-stimulating hormone (FSH), as well as thyroid-stimulating hormone, are a family of glycoprotein hormones which are heterodimers and are composed of a common α subunit and a specific β subunit [1,2]. The neurohormone gonadotropin-releasing hormone (GnRH) regulates the synthesis and release of the gonadotropins from pituitary gonadotrophs (see [2–7] for reviews). Following the binding to its receptor, GnRH activates phosphoinositide turnover, resulting in elevation of cytosolic Ca^{2+} levels and activation of protein kinase C (PKC) (see [4,5,7] for reviews). Molecular events leading to gonadotropin release are well documented (see [4,5,7] for reviews). However, the mechanism by which GnRH activates the gonadotropin genes is not yet known.

Using primary cell cultures of rat pituitaries some laboratories observed increased levels of common α-, but not of LH β-subunit mRNA levels after GnRH stimulation [8–10]. Others reported an increase in LHβ mRNA levels by GnRH and suggested mediation by PKC since the effect was reduced in PKC-depleted cells [11]. Still others reported that both α and LHβ gene transcription, as well as mRNA levels, were elevated after GnRH challenge [12–14] and suggested that PKC and protein kinase A (PKA) participate in GnRH action in a non-additive manner [12]. Studies on the regulation of α-subunit in the pituitary have been hampered so far because of the heterogeneity of the gland and the fact that α is produced in both the gonadotrophs and thyrotrophs. However, a pituitary cell line of a gonadotroph lineage (αT3-1) has been derived by targeted tumorigenesis in transgenic mice [15] and is capable of producing only the common α subunit. Using αT3-1 cells enabled investigators to study the regulation of α subunit and GnRH action in an homogeneous cell population. Horn et al. [16] have shown that prolonged (16 h) stimulation of αT3-1 cells with GnRH increased common α-subunit mRNA levels. While phorbol 12-myristate 13-acetate (PMA) and forskolin mimicked the neurohormone effect, a role for PKA was ruled out. Transcriptional regulation of the α-subunit gene by prolonged stimulation with GnRH has been reported [17,18]. Common to the studies on gonadotropin gene expression is a long incubation protocol with GnRH. Recently we have reported a rapid early phase (30–60 min) of GnRH elevation of common α-subunit mRNA levels in αT3-1 cells and cultured rat pituitary cells [19,20]. Here we report that convergence of signalling by PKC and Ca^{2+} is most likely involved in the early phase of GnRH action upon gonadotropin α-subunit gene expression. Moreoever a different cross-talk between PKC and Ca^{2+} is involved in mediating the early and late phases of GnRH action.

MATERIALS AND METHODS

GnRH was purchased from Peninsula Laboratories (San Carlos, CA, U.S.A.). The GnRH analogue [D-Trp^6]GnRH (GnRHa) was a gift from Dr. R. Millar (Cape Town, South Africa). The calcium ionophore ionomycin was purchased from Boehringer (Mannheim, Germany). BSA, PMA and actinomycin D were purchased from Sigma (St. Louis, MO, U.S.A.). 1,2-Bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid acetoxymethyl tetraester (BAPTA/AM) was purchased from Molecular Probes (Eugene, OR, U.S.A.). Staurosporine was from Kyowa

Abbreviations used: BAPTA/AM, 1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid acetoxymethyl tetraester; CRE, cyclic AMP response element; DMEM, Dulbecco's modified Eagle's medium; LH, luteinizing hormone; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; GnRHa, [D-Trp^6]GnRH analogue; PMA, phorbol 12-myristate 13-acetate; GADPH, glyceraldehyde-3-phosphate dehydrogenase; PKC, protein kinase C; PKA, protein kinase A.

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Medex Co. (Tokyo, Japan). Bisindolylmaleimide (GF 109203X) was purchased from Calbiochem (Laufelfingen, Switzerland). The rat α-subunit cDNA was kindly provided by Dr. W. W. Chin (Boston, MA, U.S.A.). All media, sera and antibiotics for cell culture were purchased from Biological Industries (Kibbutz Beth Ha'Emek, Israel). [α-^{32}P]dCTP was purchased from Rotem Industries (Beersheba, Israel).

Cell culture

αT3-1 cells were subcultured into 60-mm-diam. tissue-culture dishes (Sterilin, Hounslow, U.K.). Cells were grown in 5 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) fetal calf serum, 5% (v/v) horse serum, penicillin (10 units/ml) and streptomycin (0.1 mg/ml). Cells were grown to 70–80% confluency and, after 3–4 days, the cultures were washed three times with DMEM and stimulants were added in 5 ml of DMEM at the indicated concentration for a given length of time. When the stimulation period was up to 1 h, 10 mM Hepes was added to the medium. For long stimulation periods (more than 6 h), the medium contained 0.1% BSA and antibiotics.

PKC inhibition, depletion and assay

In order to inhibit PKC activity, cells were pretreated with various doses (1–100 nM) of staurosporine for 5 min. Cells were then washed several times in DMEM and further incubated with GnRHa (0.1 nM) for 1 h. Alternatively cells were pretreated for 20 min with various doses (10 nM–2.5 μM) of the PKC inhibitor GF 109203X and subsequently challenged for 1 h with GnRHa (0.1 nM) or with ionomycin (1 μM). In order to down-regulate PKC, PMA (100 ng/ml) was added to cells grown in the culture medium for the last 24 h of the culture period. Control cultures were treated with dimethyl sulphoxide (DMSO, 0.01%). Cells were washed and treated for 1 h with the indicated stimulant. PKC activity in the cells was determined as previously described [21,22].

RNA isolation and analysis

Total RNA was extracted from cells in 5 M guanidinium thiocyanate containing 8% (v/v) 2-mercaptoethanol and isolated by the LiCl method as described by Cathala et al. [23]. RNA samples (4 μg) were blotted on to GeneScreen using a slot blot manifold (Schleicher and Schull, Dassau, Germany) and the samples loaded in each lane were separated into two. One of the two halves of each sample derived from the slot blot analysis was hybridized with α-subunit cDNA and the second corresponding half was hybridized with the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA which was used as internal control. Thereafter filters were washed at high stringency and 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 GAPDH.

Statistical analysis

The hybridization signals for α-subunit mRNA in each group were normalized to the hybridization signals for the housekeeping gene GAPDH. An arbitrary unit of 1 represents the control values. Statistical comparisons between control and treatment groups were performed using Student's t-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

RESULTS

Effect of GnRHa, PMA and ionomycin on α-subunit mRNA levels in αT3-1 cells

To identify potential mediators of GnRH action upon α-subunit gene expression we compared the effect of PMA and a Ca^{2+} ionophore, ionomycin, with the response induced by GnRHa. As shown in Figure 1, low concentrations of GnRHa (0.1 nM) induced a very rapid response upon α-subunit mRNA accumulation, which is detected already after 30–60 min of incubation as previously shown [19]. Higher concentrations of GnRHa (10 nM) stimulated a biphasic response upon α-subunit mRNA levels with a similar early phase and additional late phase of mRNA elevation which was detected between 12 and 24 h of incubation [19].

Addition of a known PKC activator PMA (100 ng/ml) to αT3-1 cells resulted in a rapid (30–60 min) increase of α-subunit mRNA. The increase was followed by a sustained decrease (3–24 h) slightly below basal levels (Figure 1). Similar results were obtained with a lower dose of PMA (10 ng/ml). Addition

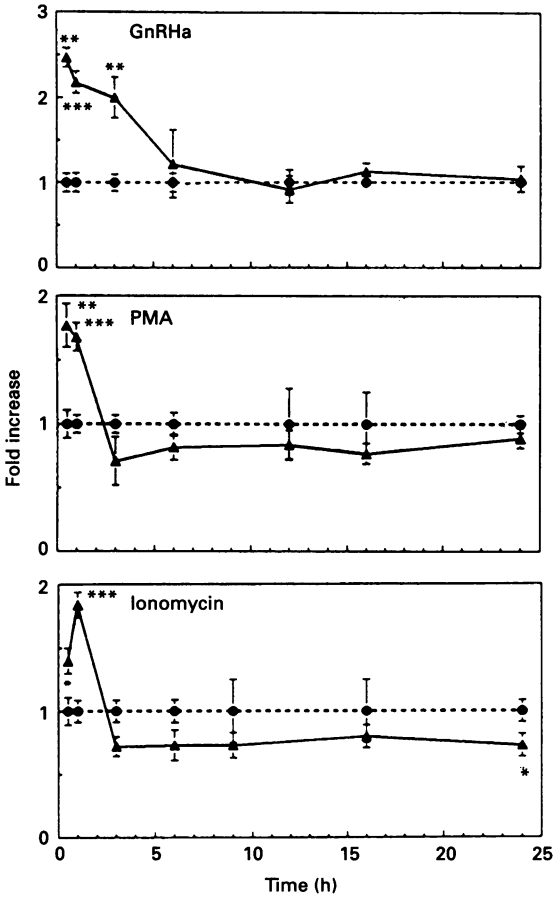

Figure 1 Time response of GnRHa (0.1 nM), PMA (100 ng/ml) and ionomycin (1 μM) effect upon α-subunit mRNA levels in αT3-1 cells

αT3-1 cells were grown in 60-mm-diam. dishes in DMEM containing 5% (v/v) fetal calf serum, 5% (v/v) horse serum and antibiotics. When cells reached 70–80% confluence the cultures were washed and treated with stimulants (triangles) added in 5 ml of DMEM for the given length of time. α-Subunit mRNA levels were determined as described in the Materials and methods section. Higher concentrations of GnRHa (10 nM) produced a secondary late rise as seen in Figure 2. An arbitrary unit of 1 represents the control values (circles). Results for all the Figures are mean ± S.E.M. (n = 5–13). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 1 Late effect of GnRHa, PMA and ionomycin alone or in combination upon α-subunit mRNA levels in αT3-1 cells

The cells were treated for 24 h with GnRHa (10 nM), PMA (100 ng/ml), ionomycin (1 μM) or with PMA and ionomycin together. α-Subunit mRNA levels were determined as described in the Materials and methods section. * $P < 0.05$; ** $P < 0.01$.

Treatment	n	α-Subunit mRNA (fold increase)
Control	21	1.00 ± 0.05
GnRHa (10 nM)	4	1.54 ± 0.01*
PMA (100 ng/ml)	16	0.96 ± 0.04
Ionomycin (1 μM)	24	0.78 ± 0.04*
PMA + ionomycin	17	1.42 ± 0.07**

Table 2 Effect of removal of intracellular and extracellular Ca^{2+} upon GnRHa-induced α-subunit mRNA levels in αT3-1 cells

The cells were grown as in Table 1, washed and transferred to DMEM (control); to DMEM containing BAPTA/AM (20 μM) or to Ca^{2+}-free DMEM containing 250 μM EGTA for 10 min. Cells were then treated with or without GnRHa (1 nM) for 60 min in the respective medium and α-subunit mRNA levels were determined as described in the Materials and methods section. * $P < 0.05$; *** $P < 0.001$.

Treatment	GnRHa (1 nM)	n	α-Subunit mRNA (fold increase)
Control	−	17	1.00 ± 0.05
Control	+	16	2.18 ± 0.16***
BAPTA (20 μM)	−	12	1.25 ± 0.08
BAPTA	+	9	1.65 ± 0.13*
EGTA (250 μM)	−	21	1.13 ± 0.07
EGTA	+	25	1.03 ± 0.07

of the Ca^{2+} ionophore ionomycin (1 μM) to αT3-1 cells resulted in a rapid increase of α-subunit mRNA levels with a peak at 60 min (Figure 1). Thereafter the effect of ionomycin diminished (3–24 h) and reached sub-basal levels. We also examined a lower dose of the ionophore (100 nM) and found similar results (not shown). Pretreatment of αT3-1 cells with actinomycin D (1 μg/ml) blocked the stimulatory effect of GnRHa (0.1 nM) upon α-subunit mRNA levels, suggesting that GnRHa regulates the α-subunit gene at the transcriptional level (not shown).

Incubation of αT3-1 cells with a combination of GnRHa and PMA, or with GnRHa and ionomycin for 60 min, did not produce an additive response upon α-subunit mRNA levels and the effect observed was similar to that obtained with each stimulant alone. Similarly, the combination of PMA and ionomycin also showed no additivity (results not shown).

Although PMA or ionomycin did not stimulate α-subunit mRNA levels at 24 h of incubation (Figure 1), the combined addition of PMA and ionomycin mimicked the secondary stimulatory phase of GnRHa (10 nM) action (Table 1). The comparative studies have revealed that both PKC and Ca^{2+} differentially regulate GnRH effects upon α-subunit mRNA levels in αT3-1 cells in both the early (30–60 min) and the late phase (24 h) of the neurohormone action.

Role of calcium

To investigate the role of Ca^{2+} in GnRH action upon α-subunit mRNA levels, we first added the cell-permeant Ca^{2+} chelator BAPTA/AM to inhibit the mobilization of intracellular Ca^{2+} [24]. As shown in Table 2, addition of BAPTA/AM to αT3-1 cells slightly elevated basal levels of α-subunit mRNA and markedly reduced, but did not abolish, the GnRHa effect on α-subunit mRNA levels after 60 min of incubation. Transfer of the cells to Ca^{2+}-free medium containing EGTA abolished the effect of GnRHa upon α-subunit mRNA levels (Table 2). Hence, Ca^{2+} mobilization and influx are both needed for GnRH action.

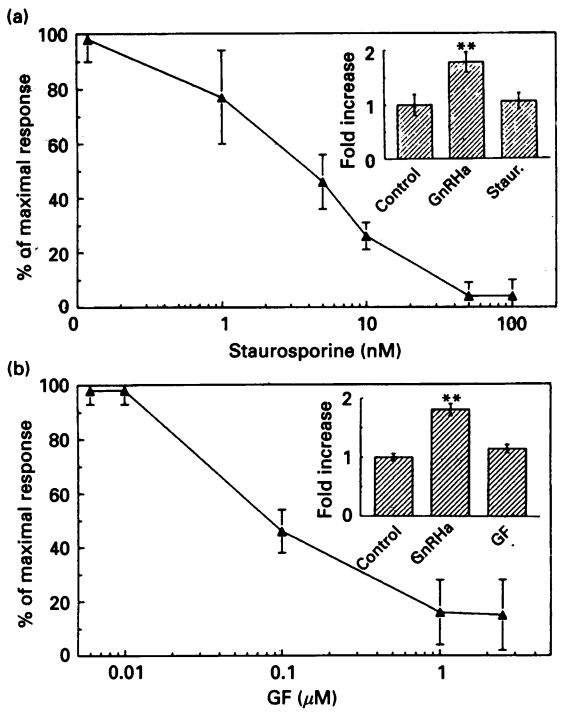

Figure 2 Effect of staurosporine (a) and GF 109203X (b) on GnRHa-induced α-subunit mRNA levels

αT3-1 cells were pretreated with various concentrations of staurosporine for 5 min and the medium was removed and replaced with fresh DMEM. GnRHa (0.1 nM) was then added for an additional 60 min. (**a**) αT3-1 cells were grown as above and pretreated with the PKC inhibitor GF 109203X (GF) at the concentrations indicated for 20 min. The cells were subsequently challenged for 1 h with GnRHa (0.1 nM) in the presence of the drug (**b**). The insets show the effect of the drugs on basal α-subunit mRNA levels in comparison with the GnRHA response. Further details are as described above. Results are means ± S.E.M. ($n = 7$–12). ** $P < 0.01$.

Effect of PKC inhibition

To evaluate further the possibility that PKC mediates GnRH effect upon common α-subunit mRNA levels, we used the PKC inhibitors staurosporine and GF 109203X. Cells were pretreated for 5 min with staurosporine followed by GnRHa challenge (0.1 nM) for 60 min (Figure 2a). Staurosporine (50 nM) did not affect basal α-subunit mRNA levels (Figure 2a, inset). A dose-related inhibition of the stimulatory effect of GnRHa upon α-subunit mRNA levels was achieved by staurosporine. Half-maximal inhibition (IC_{50}) was observed at 4 nM of staurosporine in good agreement with IC_{50} values of PKC inhibition in cellular systems [25]. Pretreatment of the cells with a more selective PKC inhibitor GF 109203X at concentrations known to inhibit PKC in cellular systems [26,27] also resulted in a dose-related inhibition of the stimulatory effect of GnRHa ($IC_{50} = 90$ nM, Figure 2b). GF 109203X had no significant effect upon basal α-subunit mRNA levels (Figure 2b, inset). The results further support a role for PKC as a mediator of GnRH-induced α-subunit mRNA accumulation.

Table 3 Effect of PKC depletion on GnRH- and PMA-induced α-subunit mRNA levels

αT3-1 cells were pretreated with or without PMA (100 ng/ml) for 24 h. Cells were then washed and stimulated with GnRH (10 nM) or PMA (100 ng/ml) for an additional 60 min. Other details are as described in Table 1. Results are means ± S.E.M. ($n = 6$–12). * $P < 0.05$; *** $P < 0.001$.

		α-Subunit mRNA (fold increase)	
Preincubation	Control	GnRH (10 nM)	PMA (100 ng/ml)
None	1.00 ± 0.04	1.93 ± 0.10***	1.75 ± 0.13***
PMA (overnight)	0.89 ± 0.04	1.18 ± 0.08*	0.86 ± 0.10

Table 5 Effect of Ca^{2+} removal on PMA-induced α-subunit mRNA elevation

αT3-1 cells were grown as above, washed several times and transferred to DMEM or to Ca^{2+}-free DMEM containing 250 μM EGTA for 10 min. Cells were then treated with or without PMA (100 ng/ml) for 60 min in the respective medium. α-Subunit mRNA levels were determined as described in the Materials and methods section ($n = 12$–24). ** $P < 0.01$.

	α-Subunit mRNA (fold increase)	
Treatment	Ca^{2+}	Ca^{2+}-free
None	1.00 ± 0.05	1.08 ± 0.07
PMA (100 ng/ml)	1.69 ± 0.15**	1.26 ± 0.11

Effect of PKC depletion

We also used PKC-depleted or 'down-regulated' cells as another approach to evaluate the role of PKC in the rapid effect of GnRH. Incubation of αT3-1 cells with PMA (100 ng/ml) for 24 h down-regulated endogenous PKC activity as measured by histone IIIS phosphorylation [control: 0.01 % DMSO-pretreated cells (53 pmol of ^{32}P/min per mg) versus PMA-treated cells (7 pmol of ^{32}P/min per mg)]. The PMA pretreatment down-regulated phorbol ester-sensitive PKC subspecies activity so that a subsequent addition of PMA (100 ng/ml) for 60 min was ineffective in stimulating α-subunit mRNA levels (Table 3). PMA pretreatment slightly decreased α-subunit mRNA levels, consistent with the data presented in Figure 1. GnRH (10 nM)-induced α-subunit mRNA accumulation was inhibited by 70 % (Table 3).

Cross-talk between PKC and Ca^{2+}

To evaluate the cross-talk between the two messengers of GnRH we examined the effect of PKC inhibition and depletion upon ionomycin-induced α-subunit mRNA elevation in αT3-1 cells. As shown in Table 4, down-regulation of endogenous PKC by PMA pretreatment reduced the Ca^{2+} ionophore response by 70 % while GF 109203X abolished the stimulatory effect of ionomycin upon α-subunit mRNA elevation.

Similarly removal of the cells to Ca^{2+}-free medium containing EGTA abolished the stimulatory effect of PMA upon α-subunit mRNA elevation in αT3-1 cells (Table 5).

DISCUSSION

The effect of GnRHa is very rapid and can be detected as soon as 30–60 min after incubation, while higher concentrations of GnRHa (10 nM) also stimulated a secondary rise in α-subunit mRNA levels which is detectable after 12–24 h of incubation. Our results with actinomycin D inhibition of GnRHa action suggest regulation of the α-subunit gene at the transcriptional level. This is supported by a recent report demonstrating that GnRH induces a transient burst of α-subunit gene transcription which was followed by induction of mRNA stability [28].

Since GnRH is released from the hypothalamus in a pulsatile manner [29] at intervals of 1–2 h according to species and its half-life is 2–4 min, the first rapid response of GnRH action (30–60 min) seems to be physiologically more relevant and was therefore investigated in more detail in the present study. Time-course studies with the PKC activator PMA and the Ca^{2+} ionophore ionomycin revealed a rapid response similar to that induced by GnRHa. No additivity was obtained when GnRHa was added together with PMA or ionomycin, indicating that both Ca^{2+} and PKC are involved in the early phase of GnRH action upon α-subunit mRNA elevation. Also no additivity was observed upon the combined addition of PMA and ionomycin, suggesting that Ca^{2+} and PKC act sequentially and/or converge to a common effector unit. Interestingly, recent studies in cultured rat pituitary cells revealed additivity in terms of LH release by the combined addition of PMA and ionomycin, while no additivity was obtained in terms of gonadotropin subunit gene expression [20]. It is therefore possible that different mechanisms are involved in exocytosis versus gene expression in pituitary gonadotrophs. Indeed using the αT3-1 cells, we demonstrated dissociation between GnRH-induced α-subunit release and mRNA elevation [19].

Different mechanisms are also observed concerning the role of Ca^{2+} and PKC in the first phase (60 min) versus the secondary phase (24 h) of GnRHa action. During the first phase PMA or ionomycin could each mimic the GnRHa response and the combined addition showed no further elevation of α-subunit

Table 4 Effect of PKC inhibition and depletion on ionomycin-induced α-subunit mRNA level

Cells were pretreated with or without the PKC inhibitor GF 109203X (GF; 1 μM, 20 min) or PMA (100 ng/ml for 24 h) as described above and in the Materials and methods section. Cells were then treated with or without ionomycin (1 μM) for 60 min. α-Subunit mRNA levels were measured as described above. Results are means ± S.E.M. ($n = 10$–17). * $P < 0.05$; *** $P < 0.001$.

		α-Subunit mRNA (fold increase)		
Treatment	Pretreatment ...	None	GF (1 μM)	PMA (100 ng/ml)
Control		1.00 ± 0.05	1.11 ± 0.12	0.89 ± 0.04
Ionomycin (1 μM)		1.93 ± 0.13***	1.26 ± 0.10	1.18 ± 0.07*

mRNA levels. On the other hand, during the second phase (24 h), PMA or ionomycin showed no stimulatory effect on their own but together they mimicked the effect of the high dose of GnRHa (10 nM) upon α-subunit mRNA elevation. Therefore the cross-talk between Ca^{2+} and PKC differs between the two phases of GnRH action.

The role of Ca^{2+} in the rapid effect of GnRH was investigated by the use of the intracellular Ca^{2+} chelator BAPTA, which reduced the GnRHa response, and by incubating the cells in Ca^{2+}-free medium in the presence of EGTA, which abolished the GnRHa response. Therefore both Ca^{2+} mobilization and influx are required for GnRHa elevation of α-subunit mRNA levels, similar to the requirements of exocytosis of LH by GnRH in cultured rat pituitary cells [7,30].

The role of PKC in GnRH action was investigated by two different approaches. The PKC inhibitors staurosporine and GF 109203X exerted a dose-related inhibition upon GnRHa elevation of α-subunit mRNA levels with IC_{50} values of 4 and 90 nM respectively, in good agreement with the concentrations known to inhibit PKC-mediated responses in other cellular systems [26,27]. The second approach utilized the phenomenon of down-regulation of endogenous PKC by prolonged exposure to PMA [31,32]. Indeed down-regulation of PKC in αT3-1 cells resulted in about 90% loss of PKC activity accompanied by inhibition of GnRH and PMA elevation of α-subunit mRNA levels. Surprisingly the stimulatory effect of ionomycin was also inhibited (70%) by down-regulation of PKC or by the PKC inhibitor GF 109203X, suggesting that Ca^{2+} and PKC act sequentially. Furthermore, the stimulatory effect of PMA on α-subunit mRNA levels was abolished in Ca^{2+}-free medium. We suggest that Ca^{2+} and PKC act sequentially and converge to a gene proximal or common effector unit. Furthermore it seems that Ca^{2+} acts both up- and down-stream to PKC.

Hormonal regulation of gene transcription can be mediated by multiple signal transduction pathways [33,34]. The α-subunit gene is known to have a cyclic AMP response element (CRE) [35]. Nevertheless, we did not investigate the role of cyclic AMP in GnRH action since the second messenger was ruled out as a mediator in GnRH-induced gonadotropin release [4,36,37] and α-subunit gene expression [16,18]. On the other hand the CRE is known to mediate also Ca^{2+}-induced responses [38] and cross-talk exists between CRE and the PMA response element TRE [38]. Deletion analysis of the α-subunit gene indicated that sequences between −507 and −205 were sufficient to mediate the transcriptional response to GnRH, PMA and cyclic AMP [17,18]. Since cyclic AMP was ruled out as a mediator of GnRH action [4,16,36,37], it is possible that Ca^{2+} interacts with the CRE as mentioned above, or with the GnRH response element. Convergence of signalling for Ca^{2+} and PKC might be mediated by a single transcription factor or via functional cooperation of different transcription factors acting on separate or composite response elements [33,39–41].

GnRH is the first key hormone of the reproductive system and its mechanism of action is not yet fully understood. Elucidation of the signal transduction cascade, involved in α-subunit synthesis, will contribute to our understanding of GnRH regulation of LH and FSH release and synthesis during the reproductive cycle.

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