Arachidonic acid and lipoxygenase products stimulate protein kinase C β mRNA levels in pituitary α T3-1 cell line: role in gonadotropin-releasing hormone action

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The cross-talk of arachidonic acid (AA) and its lipoxygenase products with protein kinase C β (PKC β) mRNA levels during the action of gonadotropin-releasing hormone (GnRH) was investigated in the pituitary α T3-1 cell line. The addition of AA or its 5-lipoxygenase products 5-hydroxyeicosatetraenoic acid (5-HETE) or leukotriene C $_4$ (LTC $_4$) for 30 or 60 min stimulated PKC β , but not PKC α mRNA levels (3–5-fold); PKC γ is not expressed by the cells. Other HETEs or leukotrienes tested showed no significant effect. The range of effective concentration for LTC $_4$ and 5-HETE (around 10 $^{-10}$ M) is the range found in GnRH-stimulated pituitary cells. Although PKC β mRNA levels were preferentially elevated by LTC $_4$ and 5-HETE at early time

points, PKC α mRNA levels were elevated at 6–12 h of incubation when PKC β mRNA levels returned to basal levels. The addition of the phospholipase A $_2$ inhibitor 4-bromophenacyl bromide or the selective 5-lipoxygenase inhibitor L-656,224 abolished [D-Trp 6]GnRH (GnRH-A) elevation of PKC β mRNA levels, whereas PKC α mRNA levels were not increased by this neuro-hormone. The cyclo-oxygenase inhibitor indomethacin elevated basal PKC β mRNA levels and potentiated the GnRH-A response. Cross-talk exists between AA and some of its lipoxygenase products and PKC β gene expression during cell signalling. AA, 5-HETE and LTC $_4$ participate in the rapid stimulation of PKC β mRNA levels by GnRH.

INTRODUCTION

Protein kinase C (PKC), a serine/threonine protein kinase family of related subtypes, plays an important role in a variety of cellular functions such as synaptic transmission, memory, learning, growth, differentiation, metabolism, contraction, exocytosis, gene expression, transformation and regulation of ion channels [1,2]. We and others have proposed that PKC is involved in gonadotropin-releasing hormone (GnRH)-induced gonadotropin secretion and synthesis [3–8]. Previous reports have also presented evidence for a potential role for arachidonic acid (AA) and some of its lipoxygenase products in GnRH-induced gonadotropin secretion and synthesis [9–12]. More recently we and others have demonstrated the formation of lipoxygenase products of AA such as leukotriene C $_4$ (LTC $_4$) and 5-hydroxyeicosatetraenoic acid (5-HETE) by GnRH in rat pituitary cells [13,14]. Whereas relatively a lot is known about regulation of PKC at the protein level [1,2], very little is known about PKC gene expression. We have recently demonstrated that a GnRH analogue (GnRH-A) stimulates a rapid increase (30 min) in PKC β , but not PKC α , mRNA levels in the pituitary α T3-1 cell line [15]. Since cross-talk between AA and PKC has been demonstrated [1,2,16–19], we decided to investigate the effect of AA and its lipoxygenase products on the mRNA levels of conventional PKC subtypes in pituitary α T3-1 cells and to examine its relevance to the action of GnRH.

MATERIALS AND METHODS

Materials

α T3-1 cells were kindly provided by Dr. P. Mellon (La Jolla, CA, U.S.A.). The GnRH analogue [D-Trp 6]GnRH (GnRH-A) was a

gift from Dr. R. Millar (Cape Town, South Africa). Indomethacin and 4-bromophenacyl bromide (BPB) were from Sigma (St. Louis, MO, U.S.A.). Hydroxy fatty acids (15-, 12- and 5-HETE), peptidoleukotrienes (LTC $_4$, LTD $_4$, LTE $_4$) and the lipoxygenase inhibitor 7-chloro-2-[(4-methoxyphenyl)methyl]-3-methyl-5-propyl-4-benzofuranol (L-656,224) were gifts from Dr. Ford-Hutchinson of Merck Frosst Canada Inc. (Point Duval, Quebec, Canada). Media and sera for cell culture were from Biological Industries (Kibbutz Beth Ha'Emek, Israel). [α - 32 P]dCTP was purchased from Rotem (Beersheba, Israel). PKC α and β cDNAs were obtained from ATCC/NIH (American Type Culture Collection, Rockville, MD, U.S.A.). β -Tubulin cDNA was kindly provided by Dr. I. Ginzburg (Rehovot, Israel).

Cell culture

α T3-1 cells were subcultured into 60 mm tissue culture dishes (Sterilin, Hounslow, Middx., U.K.). Cells were grown in 5 ml of Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum, 5% horse serum, penicillin (100 units/ml) and streptomycin (0.1 mg/ml). After 3–4 days, when cells were 70–80% confluent, the 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.

RNA extraction and analysis

At the end of the stimulation period, total RNA was isolated from cells by extraction in guanidinium isothiocyanate containing 8% 2-mercaptoethanol by the LiCl method as described by Cathala et al. [20]. Total RNA samples (8 μ g) were slot-blotted on to Gene Screen using a slot-blot manifold (Schleicher and

Abbreviations used: PKC, protein kinase C; GnRH, gonadotropin-releasing hormone; LTC $_4$, LTD $_4$, LTE $_4$, leukotriene B $_4$, C $_4$, D $_4$, E $_4$ respectively; HETE, hydroxyeicosatetraenoic acid; AA, arachidonic acid; L-656,224, 7-chloro-2-[(4-methoxyphenyl)methyl]-3-methyl-5-propyl-4-benzofuranol; BPB, 4-bromophenacyl bromide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DMEM, Dulbecco's modified Eagle's medium.

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Schuell, Dassal, Germany). After baking and prehybridization, the membranes were hybridized overnight with the specific cDNA probes labelled to high specific radioactivity using a random primer labelling kit (Boehringer, Mannheim, Germany). Half of each lane was hybridized with a PKC cDNA (ATCC/NIH) and the second half was hybridized with β -tubulin and/or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA as an internal control. Thereafter, filters were washed at high stringency and autoradiographed at -70°C . Steady-state levels of mRNAs were quantified by densitometric scanning of autoradiograms. The data were corrected for variability of loading by calculation as a ratio to β -tubulin and/or GAPDH. An arbitrary unit of 1 represents the control values. Statistical comparisons between control and treatment groups were performed using Students' *t* test; $P < 0.05$ was taken as significant.

RESULTS

We first examined the effect of arachidonate (20 μM), LTC_4 and 5-HETE (each at 10^{-10} M) [14] on PKC β mRNA levels in $\alpha\text{T3-1}$ cells (Table 1). A rapid (30 min) and significant (3–5-fold) increase in PKC β mRNA levels was observed on addition of arachidonic acid and its products. PKC α mRNA levels were not elevated under these conditions, and PKC γ is not expressed in

Table 1 Effect of AA and lipoxygenase metabolites on PKC β mRNA levels in $\alpha\text{T3-1}$ cells

The cells were treated with AA (20 μM), LTC_4 , LTD_4 (0.1 nM each), LTE_4 (1 nM), 5-HETE, 15-HETE (0.1 nM each) or 12-HETE (1 nM) for the time indicated, and PKC β mRNA levels were determined. Results are means $\pm$ S.E.M. ($n = 6$ –12). * $P < 0.05$; ** $P < 0.01$. N.D., not determined.

Treatment	PKC β mRNA (fold increase)	
	30 min	60 min
Control	1.00 $\pm$ 0.18	1.00 $\pm$ 0.09
AA	4.05 $\pm$ 0.72**	3.20 $\pm$ 0.76*
LTC_4	2.85 $\pm$ 0.46**	5.28 $\pm$ 1.21**
LTD_4	N.D.	1.00 $\pm$ 0.12
LTE_4	N.D.	1.44 $\pm$ 0.52
5-HETE	3.16 $\pm$ 0.3**	4.72 $\pm$ 0.95**
15-HETE	N.D.	1.44 $\pm$ 0.52
12-HETE	N.D.	1.44 $\pm$ 0.50

Figure 1 Dose-response of LTC_4 effect on PKC β mRNA levels in $\alpha\text{T3-1}$ cells

The cells were treated with increasing concentrations of LTC_4 for 1 h, and PKC β mRNA levels were determined. Data are presented as means $\pm$ S.E.M. ($n = 4$ –12). ** $P < 0.01$.

Figure 2 Dose-response of 5-HETE effect on PKC β mRNA levels in $\alpha\text{T3-1}$ cells

The cells were treated with various concentrations of 5-HETE for 1 h, and PKC β mRNA levels were determined. Results are means $\pm$ S.E.M. ($n = 4$ –11). * $P < 0.05$; ** $P < 0.01$.

$\alpha\text{T3-1}$ cells at all (not shown). As the effect of the metabolites was more prominent after 1 h of incubation, we also tested other metabolites of AA at this time point. Of the leukotrienes tested, only LTC_4 was effective in elevating PKC β mRNA levels, and of the HETEs tested, only 5-HETE produced a consistent and significant effect (Table 1). No significant elevation of PKC α mRNA levels by AA or its metabolites was noticed after 1 h of incubation (not shown).

The stimulatory effect of LTC_4 and 5-HETE on PKC β mRNA levels is dose-related, and a maximal response is obtained at metabolite concentrations of about 10^{-10} M (Figures 1 and 2). This range of concentration was noted in GnRH-stimulated rat pituitary cells [14] and thus represents a physiological dose.

The stimulatory effect of LTC_4 (0.1 nM) on PKC β mRNA levels was rapid, and significant elevation was noticed after 30 min of incubation (3-fold; $P < 0.01$; Figure 3). The effect of LTC_4 reached a peak at 1 h of incubation (5-fold; $P < 0.01$), declining thereafter to reach basal levels at 6 h. Significant elevation of PKC α mRNA levels by LTC_4 was noticed at 12 h of incubation. Similarly, the effect of 5-HETE (0.1 nM) on PKC β mRNA levels was rapid (3-fold; $P < 0.01$ at 30 min) with a peak at 1 h of incubation (4.5-fold; $P < 0.01$) and a slower decline thereafter to reach basal levels at 12 h (Figure 4). 5-HETE also stimulated PKC α mRNA levels and the effect was detected after 6–12 h of incubation.

Figure 3 Time-response of LTC_4 effect on PKC α and PKC β mRNA levels in $\alpha\text{T3-1}$ cells

The cells were treated with LTC_4 (0.1 nM) for the time indicated, and PKC α and PKC β mRNA levels were determined. Results are means $\pm$ S.E.M. ($n = 5$ –12). ** $P < 0.01$.

Figure 4 Time-response of 5-HETE effect on PKC α and PKC β mRNA levels

The cells were treated with 5-HETE (0.1 nM) for the time indicated, and PKC α and PKC β mRNA levels were determined. Results are means $\pm$ S.E.M. ($n = 5-12$). * $P < 0.05$; ** $P < 0.01$.

Table 2 Effect of inhibitors of AA metabolism on [D-Trp⁶]GnRH(GnRH-A)-induced PKC β mRNA levels

Cells were pretreated with the phospholipase A₂ inhibitor BPB (10 μ M), the 5-lipoxygenase inhibitor L-656, 224 (3 μ M) or the cyclo-oxygenase inhibitor indomethacin (2 μ M) for 10 min, followed by treatment with the GnRH agonist (GnRH-A; 0.1 nM) for 60 min. PKC β mRNA levels were determined as described in the Materials and methods section. Results are means $\pm$ S.E.M. ($n = 12$). * $P < 0.01$ compared with the control; † $P < 0.01$ compared with control without GnRH-A.

Treatment	GnRH-A	PKC β mRNA (fold increase)
Control	—	1.00 $\pm$ 0.07
Control	+	2.30 $\pm$ 0.18†
BPB	—	1.18 $\pm$ 0.12
BPB	+	1.28 $\pm$ 0.10*
L-656,224	—	1.25 $\pm$ 0.10
L-656,224	+	0.71 $\pm$ 0.07*
Indomethacin	—	2.97 $\pm$ 0.54†
Indomethacin	+	4.00 $\pm$ 0.46*

The possibility that AA and its lipoxygenase metabolites might participate in the GnRH-induced PKC β mRNA increase was examined. Indeed stimulation of PKC β mRNA levels by GnRH-A is rapid, and significant elevation can be detected after 30 min of incubation [15], similarly to the rapid response elicited here by AA, LTC₄ and 5-HETE. Cells were pretreated (10 min) with the phospholipase A₂ inhibitor BPB (10 μ M), the selective 5-lipoxygenase inhibitor L-656,224 (3 μ M) [14] or the cyclo-oxygenase inhibitor indomethacin (2 μ M) [14], followed by GnRH-A (0.1 nM; 60 min) (Table 2). Elevation of PKC β mRNA levels by GnRH-A was abolished by BPB and L-656, 224, which had no effect on basal levels. On the other hand, indomethacin stimulated basal PKC β mRNA levels and augmented the GnRH-A response to that exerted by LTC₄ and 5-HETE. GnRH-A did not elevate PKC α mRNA levels at all the time points examined here (30 min–12 h) as previously described [15].

DISCUSSION

We have recently demonstrated that GnRH-A stimulates a rapid increase (30 min) in PKC β but not PKC α mRNA levels in α T3-1 cells [15]. We and others have also reported that GnRH

stimulates AA release and production of its 5-lipoxygenase metabolites, 5- and 15-HETE, LTC₄, LTD₄ and LTE₄, in cultured pituitary cells [9,13,14]. Indeed AA and some of its lipoxygenase metabolites such as LTC₄ [9–14] as well as PKC [3–8] were implicated in GnRH-induced gonadotropin secretion and synthesis [3–14]. Although AA is considered to be a potential second messenger (see ref. [21] for a review), very little is known about the role of AA and its metabolites in gene expression. It was therefore of great interest to examine the cross-talk between AA (and its metabolites) and PKC β gene expression. Here we demonstrate for the first time that AA, LTC₄ and 5-HETE, but not the other lipoxygenase metabolites tested, elevate PKC β mRNA levels in the gonadotroph-derived α T3-1 cell line. Interestingly, the levels of the metabolites achieved by GnRH challenge (about 100–150 pM for pM for LTC₄ and 5-HETE) in cultured rat pituitary cells [14] are sufficient to induce elevation of PKC β mRNA levels as observed here, supporting the notion that LTC₄ and 5-HETE cross-talk with PKC β might represent a physiological response during GnRH action. However, whereas the effect of GnRH-A lasts for about 4 h before declining to basal levels at 12 h [15], the stimulatory effect of LTC₄ and 5-HETE is more transient and declines after 1 h of incubation. The data suggest that LTC₄ and 5-HETE might be involved in GnRH-A action at the early phase of the neurohormone action. This is supported by the observation that GnRH-A does not elevate PKC α mRNA levels as do LTC₄ and 5-HETE at 6–12 h of incubation. Enhanced metabolic clearance of LTC₄ and 5-HETE in GnRH-A-treated cells might terminate their additional biological activities. Alternatively, other mediators of GnRH-A action such as PKC [3,4] might be inhibitory to PKC α gene expression [15]. Indeed we have proposed that GnRH-A elevation of PKC β mRNA levels is autoregulated by PKC [15].

AA and its active metabolites LTC₄ and 5-HETE might elevate PKC β mRNA levels by activation of a novel transcription factor similar to other second messengers. Alternatively AA and its metabolites might directly activate PKC subspecies which are involved in regulation of PKC β gene expression. Indeed AA or oleate were shown to activate PKC β II, γ , ϵ , ζ and δ [2,17–19,22,23] and PKC has been implicated in PKC β gene expression [15,24,25]. It is not yet known whether LTC₄ and 5-HETE also activate various PKC subspecies [18] and what is the nature of the PKCs involved in PKC β gene expression. Similar mechanisms might be involved in the delayed effect of LTC₄ and 5-HETE on PKC α mRNA increases. Still another possibility is that AA does not act intracellularly but rather is first converted to an active metabolite such as LTC₄. The leukotriene exits the cell and binds to neighbouring or the same cells to specific receptors which are coupled to phospholipase C [26]. Activation of phosphatidylinositol turnover might then mobilize and activate the relevant PKCs needed for PKC β gene expression as discussed above. If this is the case, an autocrine/paracrine loop of amplification cycle might be operating during GnRH action converting LTC₄ into a first messenger [14].

The stimulatory effect of GnRH-A on PKC β mRNA levels was blocked by the phospholipase A₂ inhibitor (BPB) and the selective 5-lipoxygenase inhibitor (L-656, 224) [27]. The results suggest that LTC₄ and 5-HETE participate in GnRH-A elevation of PKC β mRNA levels. The cyclo-oxygenase inhibitor indomethacin elevated basal PKC β mRNA levels and potentiated the GnRH-A response. It is therefore possible that prostaglandins or other cyclo-oxygenase products exert an inhibitory effect on PKC β mRNA levels and their removal by indomethacin elevates basal and GnRH-A-stimulated PKC β mRNA levels. This might explain the observation that the effect of GnRH-A on PKC β mRNA levels was smaller than the effect elicited by LTC₄ and 5-

HETE, whereas the effect was similar in the presence of indomethacin. Alternatively, indomethacin might increase formation of the active lipoxygenase products by increasing the availability of AA to the 5-lipoxygenase pathway.

PKC and Ca^{2+} have been implicated in the action of GnRH-A on PKC β gene expression [15]. Release of AA by GnRH-A reveals a lag of at least 1 min [12] whereas stimulation of phosphoinositide turnover and Ca^{2+} mobilization were demonstrated after 30 s [8]. Thus AA release and formation of active metabolites are most likely downstream of Ca^{2+} mobilization and PKC activation.

The demonstration here of cross-talk between AA, LTC $_4$, 5-HETE and PKC β mRNA levels appears to be physiologically relevant as it plays a role during the action of GnRH, which is the first key hormone of the reproductive cycle.

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