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Effects of phorbol esters on steroidogenesis in small bovine luteal cells

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The possible influence of an activator of protein kinase C, the tumor-promoting phorbol ester, PMA (phorbol-12-myristate-13-acetate), upon small bovine luteal cell steroidogenesis was investigated in vitro. PMA had no significant effect on basal and dibutyryl cyclic AMP (dbcAMP)-stimulated progesterone production but markedly modulated the LH-stimulated progesterone and cAMP productions. PMA potentiated the LH-stimulated cAMP accumulation whatever the dose of LH used. It also potentiated the LH-induced progesterone production in the presence of low doses of LH. Paradoxically, in the presence of maximal or submaximal effective doses of LH, PMA exerted a time- and dose-dependent inhibition of progesterone synthesis. Diacylglycerol was able to mimic the effects of PMA on LH-induced steroidogenesis. These observations suggest that the Ca^{2+}- and phospholipid-dependent protein kinase C can modulate the regulation by LH of small bovine luteal cell steroidogenesis at a step before the synthesis of cAMP. They also suggest that the interaction between LH and its receptor is able to trigger a negative regulatory signal which would be only expressed for high doses of LH and in the presence of an activator of PKC.

Luteinizing hormone; cAMP; Progesterone; Phorbol ester; (Luteal cell)

1. INTRODUCTION

Phosphatidyl inositol metabolism is involved in several hormone actions. Diacylglycerol produced by phosphatidylinositol hydrolysis activates, in the presence of calcium, a membrane protein kinase (protein kinase C) [1] which is known to play a crucial role in signal transduction for substances involved in cellular differentiation and division. Direct activation of protein kinase C (PKC) can be obtained in vitro using the tumor-promoting phorbol ester (PMA) which is able to substitute for diacylglycerol. Several observations have suggested that PKC could be involved in the regulation of steroidogenesis in ovarian, testicular and adrenocortical cells [2–4]. Indeed, PKC is able to phosphorylate in vitro the mitochondrial cytochrome P-450 involved in cholesterol side chain cleavage [5]; the presence of PKC has been demonstrated in the bovine corpus luteum [6]; steroidogenesis in this tissue can be stimulated by luteinizing hormone (LH) through activation of the cAMP-dependent protein kinase, by a mechanism in which phospholipids may be involved [7]. However little is known about the role of PKC in luteal steroidogenesis. Recently, the enhancement of PMA of basal and LH-stimulated progesterone production has been reported in bovine luteal cells [8]. The present report concerns the in vitro influence of PMA on progesterone and cAMP production by selected small bovine luteal cells which, unlike the large ones, can be stimulated by physiological concentrations of LH [9].

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2. MATERIALS AND METHODS

2.1. *Chemicals*

N^6,O^2-$(Bu)_2$-cAMP, phorbol-12-myristate-13-acetate (PMA) and 1-oleyl-2-acetyl-rac-glycerol (OAG) were purchased from Sigma (St. Louis, MO). Bovine 5-NIH-b-LH was a gift from NIH (Bethesda, MD). Progesterone antibody was a gift from the Fondation de Recherche en Hormonologie (France). cAMP radioimmunoassay kit was obtained from Amersham (France).

2.2. *Preparation of small luteal cell suspensions*

Bovine luteal cells were obtained by enzymatic dissociation of corpora lutea of pregnant cow and the small cells were selected by sedimentation at unit gravity as described [9]. Cell viability was about 95% as determined by the Trypan blue exclusion test [10]. The cells were not contaminated by red blood cells or large luteal cells.

2.3. *Incubations*

Cells were incubated in triplicate in the presence of indicated substances in a Kreb's Ringer phosphate buffer, pH 7.2, with 0.2% glucose, 0.1% bovine serum albumin and 25 mM Hepes.

2.4. *Measurements*

At the indicated time, the incubations were stopped by freezing the content of the incubation tubes. Extra- and intracellular progesterone were measured in duplicate by radioimmunoassay as previously outlined in [9]. The intra- and inter-assay coefficients of variation were 6% and 13%, respectively, and sensitivity was 30 pg/tube. Cyclic AMP production was determined by radioimmunoassay after treatment with cold ethanol as described in [11].

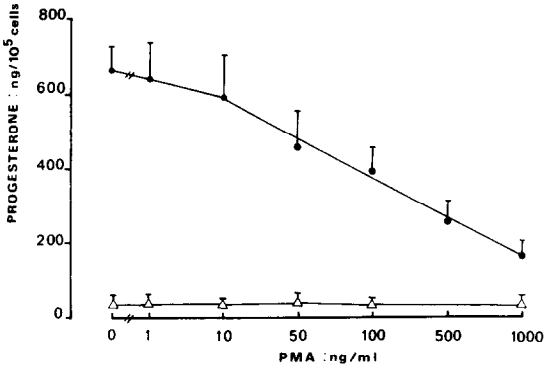

Fig.1. Effects of varying concentrations of PMA on progesterone synthesis in small bovine luteal cells incubated for 1 h in the presence (●—●) or absence (Δ—Δ) of 10^{-7} M bovine LH. Results are given as means ± SE of 4 separate experiments performed in triplicate.

2.5. *Statistical analysis*

Student's *t*-test was used for statistical analysis.

3. RESULTS

Small bovine luteal cells were found to be maximally stimulated by a dose of bovine LH of 10^{-7} M which induces a 30-fold enhancement of progesterone production after incubation for 1 h (21.4 ± 3 ng vs 644 ± 72 ng per 10^5 cells) (fig.1).

This maximal stimulation by LH of progesterone production was inhibited by PMA in a dose-dependent manner (fig.1). In the presence of 1000 ng/ml PMA, a 75% inhibition of LH stimulation was observed whereas, at doses of 1 and 10 ng/ml, there was no significant inhibition. Most studies on the regulation of steroidogenesis by PMA have used a dose of 100 ng/ml. In our model this dose caused an inhibition of about 40% of the LH stimulation and was retained for the rest of this study.

PMA had no effect on basal progesterone production whatever the dose of PMA used (fig.1).

We then investigated the effect of 100 ng/ml PMA on the progesterone synthesis induced by varying doses of bovine LH (from 10^{-13} M to 10^{-7} M) (fig.2). Two distinct effects of PMA were observed: an inhibitory effect for LH concentrations above 10^{-10} M and a stimulatory effect for LH concentrations below 10^{-11} M. The higher the LH concentrations, the more marked was the inhibitory effect. Conversely, the relative stimulatory effect of PMA was highest for doses of LH which did not stimulate steroidogenesis. A similar stimulatory effect was observed with PMA concentrations of 10 or 1 ng/ml (fig.3).

When the cells were incubated in the presence of dibutyryl cyclic AMP (dbcAMP), 100 ng/ml PMA had no effect on steroidogenesis whatever the dose of dbcAMP used. The results of four experiments are shown in table 1.

In order to explore further the site of action of

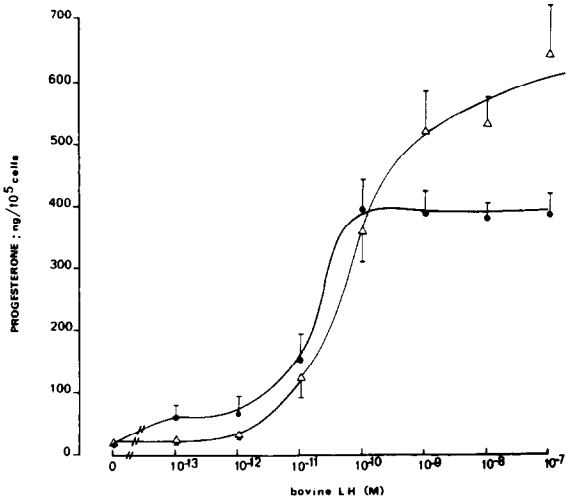

Fig.2. Effects of PMA on LH-stimulated progesterone production in small bovine luteal cells. Cells were incubated for 1 h with increasing concentrations of bovine LH in the absence (Δ—Δ) or presence (●—●) of 100 ng/ml PMA. Results are means ± SE of 6 separate experiments performed in triplicate.

PMA, its effects on LH-stimulated cAMP production were investigated (table 2). PMA had no effect on basal cAMP production whereas in the presence

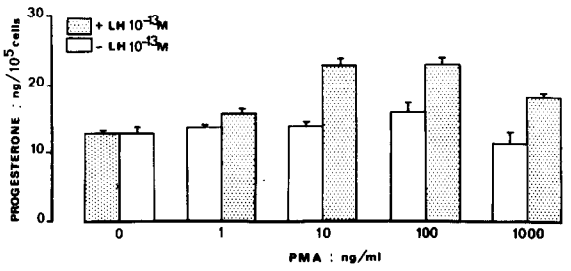

Fig.3. Progesterone production in small bovine luteal cells in the presence (dotted column) or absence (empty column) of 10^{-13} M bovine LH and increasing concentrations of PMA. Results are means ± SE of triplicate determinations.

of LH, a stimulatory effect of PMA on cAMP accumulation was observed whatever the dose of LH used.

Fig.4 shows the 2 h time course of the inhibition by PMA of LH-stimulated progesterone synthesis. An inhibition was observed within 15 min, it reached its maximum value at 60 min and remained fairly constant thereafter.

To rule out a non-specific effect of PMA, we have tested the effects of diacylglycerol (which is known to be an intracellular activator of PKC), on

Table 1

Effect of PMA (100 ng/ml) on dbcAMP-stimulated progesterone production in small luteal cells

dbcAMP concentrations	Progesterone ng/10^5 cells			
	Expt 1	Expt 2	Expt 3	Expt 4
0	21 ± 5	30 ± 6	35 ± 9	17 ± 2
5×10^{-3} M:				
− PMA	549 ± 21	−	1300 ± 30	287 ± 17
+ PMA	507 ± 24	−	1290 ± 90	286 ± 15
2.5×10^{-3} M:				
− PMA	533 ± 38	245 ± 9	890 ± 40	−
+ PMA	481 ± 36	255 ± 17	860 ± 60	−
10^{-3} M:				
− PMA	374 ± 24	193 ± 3	750 ± 45	83 ± 8
+ PMA	340 ± 12	164 ± 10	732 ± 26	81 ± 7
10^{-4} M:				
− PMA	268 ± 6	51 ± 4	144 ± 30	−
+ PMA	261 ± 12	53 ± 2	139 ± 15	−

Results are the means ± SE of triplicate determinations

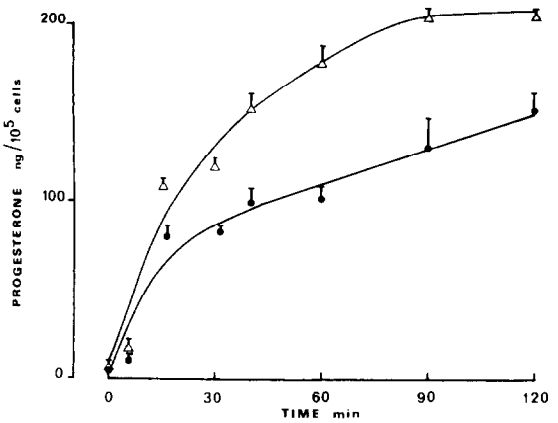

Fig.4. Time course of LH-stimulated progesterone synthesis in the presence (●—●) or absence (Δ—Δ) of 100 ng/ml PMA. Bovine LH was present at 10^{-7} M. Results are the means ± SE of triplicate determinations.

Table 2

Effects of PMA on LH-stimulated cAMP production by small luteal cells

	cAMP production pmol/10^5 cells	
	−PMA	+PMA
Basal	5.3 ± 0.5	5 ± 0.2
LH (10^{-7} M)	16.4 ± 1.8	24.8 ± 2.9[a]
LH (10^{-8} M)	9.8 ± 0.4	14.9 ± 0.9[b]
LH (10^{-10} M)	4 ± 0.5	5.9 ± 0.1[c]
LH (10^{-12} M)	5 ± 0.1	6.6 ± 0.4[a]
LH (10^{-13} M)	5 ± 0.3	5.9 ± 0.4

[a] $p < 0.05$ compared to values obtained in the absence of PMA
[b] $p < 0.01$
[c] $p < 0.001$

Results are the means ± SE of triplicate determinations

LH-stimulated progesterone synthesis (table 3). PMA or OAG had identical effects on LH-stimulated steroidogenesis.

4. DISCUSSION

The present study has demonstrated that the tumor-promoting phorbol ester (PMA) known to specifically activate protein kinase C in vitro has no action on the basal cAMP and progesterone productions by small bovine luteal cells but is able to modulate the stimulatory effect of LH on progesterone and cAMP syntheses.

Table 3

Effects of DAG and PMA on LH-stimulated progesterone production by small luteal cells

	Progesterone ng/10^5 cells		
	LH (10^{-7} M)	LH (10^{-10} M)	LH (10^{-13} M)
No addition	537 ± 17	263 ± 5	37 ± 0.6
PMA (100 ng/ml)	382 ± 19[a]	238 ± 12	54 ± 3[a]
OAG (500 μM)	393 ± 28[a]	253 ± 12	81 ± 6[a]

[a] $p < 0.001$ compared to values obtained in the absence of PMA and OAG

Results are the means ± SE of triplicate determinations

In the presence of LH, PMA at 100 ng/ml increased cAMP production and stimulated or inhibited progesterone synthesis according to the dose of LH used. Identical results were observed in the presence of OAG, known to be an intracellular activator of PKC.

In the presence of 10^{-13}, 10^{-12} and 10^{-10} M LH, a stimulatory effect of PMA or OAG on progesterone production was observed; this effect increased as the dose of LH decreased. At 10^{-11} and 10^{-12} M LH, PMA also increased cAMP synthesis. However, at a concentration of LH of 10^{-13} M, PMA induced a stimulation of progesterone production but no enhancement in cAMP synthesis was detectable. It is likely that a small increase in cAMP production by PMA existed although not detectable.

Considering that PMA had no effect on dbcAMP-stimulated steroidogenesis, the stimulatory effect of PMA on progesterone synthesis seems to occur at a pre-cAMP step only. The stimulatory effect of PMA was only observed in the presence of LH, PMA having no effect on basal cAMP production. This last result is in accordance with the report of Brunswig et al. [8]. These authors observed the stimulatory effect of PMA on steroidogenesis, in the presence of low doses of LH but they also noted stimulation by PMA of basal progesterone synthesis. This slight discrepancy with our results could be explained by

the fact that they used a mixture of both large and small luteal cells while we used small cells only. As to the mechanism of this stimulatory effect of PMA, it could involve a sensitization to LH of small bovine luteal cells, following activation of the membrane kinase C. Kikkawa and Nishizuka [12] have suggested the presence, in certain cell types, of a monodirectional control system, in which PKC is able to potentiate cyclic AMP production. In several studies on the effect of phorbol ester, evidence has been obtained that adenylate cyclase is implicated in the observed phenomenon. In lymphoma cells stimulated by isoproterenol, it has been found that 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), another PKC-activating phorbol ester, stimulates the synthesis of cAMP by acting on the adenylate cyclase and more specifically by facilitating the productive interaction of the stimulatory GTP-binding component (G_s) with the catalytic subunit of the enzyme via phosphorylation of a protein (or proteins) by PKC [13]. Rozengurt et al. [14] have also shown that PKC activation enhances forskolin-stimulated cAMP accumulation in 3T3 cells. As this effect was inhibited by pertussis toxin, these authors presumed that PKC could phosphorylate the subunit of the inhibitory-binding component G_i and change its function from inhibitory to stimulatory.

In the presence of an LH concentration inducing a high stimulation of progesterone synthesis (10^{-7} or 10^{-8} M of LH), PMA or OAG exerted an inhibitory effect on progesterone production. The PMA-induced inhibitory effect on steroidogenesis has already been reported for other steroidogenic systems such as Leydig cells [15] or adrenocortical cells [4]. In their study on bovine luteal cells stimulated by hCG, Brunswig et al. [8] did not observe this inhibitory effect of PMA; however, the highest concentration of hCG that they used was not high enough to induce a maximal stimulation of progesterone synthesis.

Paradoxically the observed inhibitory effect of PMA on LH-stimulated progesterone synthesis was accompanied by a concomitant stimulation of cAMP synthesis. Furthermore, there was no effect of PMA on dbcAMP-stimulated steroidogenesis. In ovine luteal cells, Hoyer and Niswender [16] have already described a situation (luteal cells incubated in the presence of high doses of forskolin) in which an inhibition of progesterone production was observed with a concomitant increase in cAMP accumulation.

It then appears from our results, that the presence of high doses of LH would be requisite to observe an inhibitory effect of PMA on progesterone synthesis. In biological systems, positive signals are often followed by immediate feed-back to prevent overshooting. In view of this, it seems that in our model, one can hypothesize that high doses of LH, in addition to the stimulation of adenylate cyclase, could have released a negative regulatory signal the expression of which would be controlled by PKC activation. The recent suggestion by Davis et al. [17], that at least two second messenger systems exist to mediate the action of LH in the corpus luteum could be pertinent to this hypothesis.

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