Effect of phorbol ester and phospholipase C on LH-stimulated steroidogenesis in purified rat Leydig cells

Vassilios Papadopoulos, Serge Carreau* and Michel A. Drosdowsky

Laboratoire de Biochimie, GS-CNRS 79, CHU Côte de Nacre, 14033 Caen, France

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When the phorbol ester, 4β-phorbol-12-myristate-13-acetate (PMA) or bacterial phospholipase C (PL-C) is added to a preparation of purified adult rat Leydig cells, containing 2 mM $CaCl_2$, a time- and dose-dependent decrease of LH-stimulated testosterone production is observed. After a 3 h stimulation with oLH (100 ng/ml), PMA (100 ng/ml) and PL-C (1.6 U/ml) do not affect the cell viability or the hCG specific binding, while cAMP accumulation is significantly reduced; cAMP-stimulated steroidogenesis is diminished only in the presence of PL-C. These observations suggest that in vitro: (i) activated Ca^{2+}- and phospholipid-dependent protein kinase is implicated in the regulation of rat Leydig cell steroidogenesis by LH at a step before the adenylate cyclase; (ii) phospholipids play an important role in cAMP-stimulated testosterone synthesis.

Leydig cell *Steroidogenesis* *Phorbol ester* *Phospholipase C*

1. INTRODUCTION

Recent studies (review [1]) have demonstrated that phosphatidylinositol turnover may regulate a novel protein kinase, the protein kinase C (PK-C) which is present in all organs including testes [2,3] but usually inactive. In vivo, this enzyme is activated by diacylglycerol in the presence of both Ca^{2+} and phospholipids [1]. It is also possible to activate PK-C using phospholipase C (PL-C); this enzyme hydrolyses all the phosphatidylinositol content within seconds and produces diacylglycerol which in turn activates PK-C. Direct activation of PK-C is obtained using a tumor-promoting phorbol ester (i.e. PMA) which is able to substitute for diacylglycerol; moreover, PK-C may regulate cell functions via protein phosphorylation [1]. In adrenocortical cells, it has been shown that angiotensin and acetylcholine regulate steroidogenesis via PK-C [4,5] and that PK-C phosphorylates in vitro the cytochrome P-450 involved in cholesterol side chain cleavage [6]. Steroidogenesis of rat Leydig cells is stimulated by LH through activation of a cAMP dependent protein kinase in which phospholipids seem to be implicated [7]. In mouse Leydig cells, phorbol esters inhibit hCG-stimulated testosterone production only if submaximal doses of hCG are used [8]. Here, we have investigated the implication of phosphatidylinositol turnover and PK-C in steroidogenesis of purified rat Leydig cells.

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* To whom correspondence should be addressed

2. MATERIALS AND METHODS

2.1. *Chemicals*

Powdered Ham F12 and DME media were purchased from Seromed. Collagenase-dispase and PL-C from *Bacillus cereus* (spec. act. 800 U/mg protein) were from Boehringer. Soybean trypsin inhibitor (STI), deoxyribonuclease I, 4β-phorbol-12-myristate-13-acetate (PMA), human transferrin and bovine insulin were from Sigma. Iodogen was purchased from Pierce. Ovine luteinizing hormone (NIH-oLH-24) was from NIADDK, highly purified hCG (CR-121,

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13450 IU/mg) was a gift from Dr Canfield (New York). Unlabelled hCG (Pregnyl) was from Organon, testosterone antibody from Biomerieux; cyclic AMP kit used for radioimmunoassay (RIA) and iodine were from Amersham.

2.2. *Purification and incubation of rat Leydig cells*

Adult Sprague-Dawley rats (70–80 days old) were killed by decapitation; the testes were quickly removed and decapsulated. The crude interstitial cells were prepared following collagenase-dispase (0.05%) treatment in the presence of STI (0.005%) and deoxyribonuclease I (0.001%). Purified Leydig cells were obtained after centrifugation on a discontinuous Percoll gradient [9] but without fetal calf serum. The percentage of Leydig cells obtained (≥80%) was determined by histochemical staining for 3β-hydroxysteroid dehydrogenase [10]; the cell viability was around 90% as determined by the NADH-dependent diaphorase activity [11].

All cell preparations and incubations were performed in Ham F12/DME medium (1 : 1, v/v) containing 2 mM $CaCl_2$, buffered with 15 mM Hepes and 13 mM sodium bicarbonate to which were added penicillin (50 U/ml) and streptomycin (50 μg/ml). Leydig cells (10^5) were incubated 3 h at 32°C under O_2/CO_2 (95:5, v/v) in 400 μl of medium supplemented with insulin (10 μg/ml), transferrin (5 μg/ml) and different substrates to be studied. Testosterone and cAMP [12] were measured by RIA in the cell culture media. The inter- and intra-assay coefficients of variation were 5% and the sensitivity 4 pg/tube for testosterone determinations. The inter- and intra-assay coefficients of variation of cAMP measurements were 5 and 9.4%, respectively, and the sensitivity 0.1 pmol/tube.

2.3. *^{125}I-hCG binding studies*

Highly purified hCG was iodinated using iodogen and the specific activities obtained ranged between 35 and 45 μCi/μg. The binding of ^{125}I-hCG in the presence of an excess of rat testis membranes ranged between 30 and 40%. Leydig cells (0.25×10^6) were incubated with or without PL-C (1.6 U/ml) or PMA (100 ng/ml) in presence of labelled hCG (2 ng). Non-specific binding was determined in parallel incubations to which an excess of unlabelled hCG was added (100 IU Pregnyl). All incubations were performed at 32°C for 3 h, then the cells were washed twice with 2 ml ice-cold phosphate-buffered saline containing 0.1% BSA and centrifuged (2500 × *g*, 15 min, 4°C). The washed pellet was dissolved in 1 ml of 0.5 N sodium hydroxide and the radioactivity counted.

2.4. *Statistical analysis*

The data obtained were analyzed using the Student's *t*-test.

3. RESULTS

A saturating concentration of LH (100 ng/ml) caused a 10-fold stimulation of purified rat Leydig cell testosterone synthesis after 3 h incubation (10.16 ± 0.4 ng vs 1 ± 0.2 ng, basal production per 10^5 cells). The LH stimulatory effect was inhibited by PL-C and PMA in a dose-dependent manner (fig.1); the ED_{50} values were 0.75 U/ml for PL-C and 44 ng/ml for PMA. Using very low concentrations of PL-C (0.08 U/ml) and PMA (1 ng/ml), a significant decrease of LH-stimulated testosterone

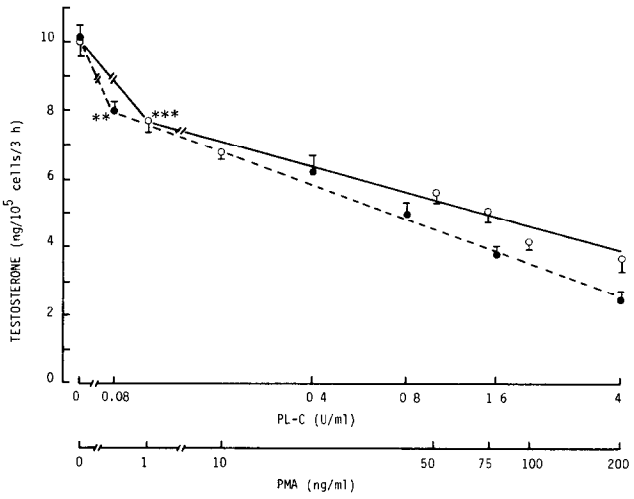

Fig.1. Testosterone production in purified rat Leydig cells incubated in the presence of oLH (100 ng/ml) and increasing concentrations of PL-C (●---●) or PMA (○—○). Results are means ± SE of 3 separate experiments performed in duplicate; ** $p < 0.01$, *** $p < 0.001$. Log scale on abscissa.

synthesis was observed (22 and 25%, respectively, with PL-C and PMA).

For further studies, we chose doses of 1.6 U/ml PL-C and 100 ng/ml PMA which caused 60% inhibition. Basal testosterone production (fig.2) and cAMP extracellular accumulation (table 1) were unaffected by either PL-C or PMA. Cell viability also remained stable (about 90%) as well as the specific binding of ^{125}I-hCG to LH/hCG membrane receptors (table 2). However, under LH stimulation, the cAMP levels were significantly decreased ($p < 0.001$) by PL-C and PMA (table 1).

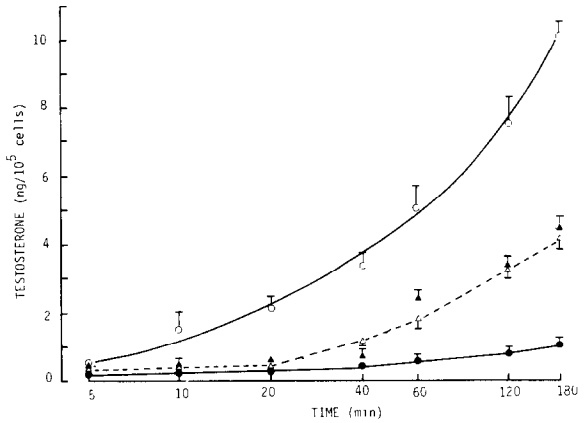

Fig.2. Time-related effects on the secretory pattern of testosterone in Leydig cells alone (●——●) or incubated either with PL-C (1.6 U/ml), PMA (100 ng/ml) (●——●) or with oLH (100 ng/ml) (○——○), oLH + PL-C (▲ ··· ▲), oLH + PMA (△---△). Values are means ± SE (n = 3). Log scale on abscissa.

Table 1

Effect of PL-C and PMA on cAMP extracellular accumulation

Treatment	cAMP (pmol/10^5 cells per 3 h)
–	6.29 ± 0.75 (8)
PL-C (1.6 U/ml)	6.79 ± 0.87 (8)
PMA (100 ng/ml)	5.79 ± 0.84 (7)
LH (100 ng/ml)	29.28 ± 1.31 (8)
LH + PL-C	15.91 ± 0.44 (7)
LH + PMA	20 ± 0.69 (4)

Values are means ± SE (number of experiments in parentheses)

The inhibitory action of PL-C and PMA on LH-regulated Leydig cell testosterone production is a time-dependent process (fig.2): a first period of complete inhibition of LH-stimulated testosterone synthesis is observed, the length of this period being 20 min when cells are incubated with PMA and 40 min with PL-C; after this time, steroidogenesis started increasing under LH. When dbcAMP (1 mM) was added to Leydig cell medium, a 9-fold increase of testosterone synthesis was observed (8.82 ± 0.5 ng) (fig.3); the addition of PL-C reduces this process (56%) while PMA is ineffective (fig.3).

Table 2

Effects of PL-C and PMA on ^{125}I-hCG specific binding on purified Leydig cells

Treatment	^{125}I-hCG, specific binding
–	100 ± 8.6
PL-C (1.6 U/ml)	103 ± 8.8
PMA (100 ng/ml)	96 ± 8.7

Values are expressed as percentage of control (n = 3)

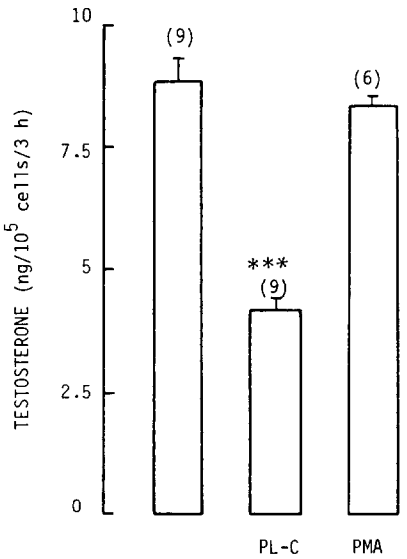

Fig.3. Effects of PL-C (1.6 U/ml) and PMA (100 ng/ml) on dbcAMP (1 mM) stimulated testosterone synthesis in Leydig cells. Values are means ± SE; *** $p < 0.001$.

4. DISCUSSION

Two different approaches have been used to study the role of PK-C in the regulation of steroidogenesis in purified rat Leydig cells: (i) directly with PMA, a potent activator of PK-C, and (ii) indirectly using PL-C which increases the intracellular pool of diacylglycerol and so activates PK-C [1]. Leydig cells are incubated in medium containing 2 mM $CaCl_2$ which promotes a maximal activation of PK-C [1]. PL-C and PMA do not affect Leydig cell viability or ^{125}I-hCG binding as well as basal cAMP and testosterone production. These observations demonstrate that any non-specific effects of PL-C or PMA on Leydig cell membranes can be excluded and that activation of PK-C, in the absence of physiological stimuli, does not alter Leydig cell function. Under a saturating concentration of LH, which stimulates maximally cAMP and testosterone syntheses, PL-C and PMA inhibit this process in a dose- and time-dependent manner. These results are different from those of Mukhopadhyay et al. [8] who found that PMA has no effect on testosterone synthesis in mouse Leydig cell suspensions in the presence of a saturating concentration of LH; but, when submaximal doses of LH are used, PMA inhibits testosterone synthesis. Similar results to ours have been observed with purified bovine luteal cells (P. Leymarie, personal communication). The above results and the absence of any effect of PMA, but not of PL-C, on dbcAMP-stimulated testosterone synthesis show that PMA inhibits LH-regulated steroidogenesis at a step before adenylate cyclase which is in agreement with the results of Mukhopadhyay et al. [8], while PL-C seems to have two sites of action: the first before adenylate cyclase and the second beyond. As we found no effect of PL-C or PMA on ^{125}I-hCG specific binding to LH-hCG receptors, we suppose that activation of PK-C by PL-C or PMA leads to phosphorylation of membrane substrates which in turn alter the transmission of the hormonal signal or the adenylate cyclase activity and so, inhibit the stimulation of Leydig cell steroidogenesis by LH.

This hypothesis is further supported by the time-related pattern of action of PL-C and PMA on LH-stimulated testosterone production where 2 distinct periods can be observed: (i) a first short period in which LH action is probably inhibited by the phosphorylated substrate of PK-C or by alteration of the membrane structures; (ii) a second period during which the Leydig cells become responsive to LH. Our findings do not permit one to suppose an action of PK-C on the cholesterol side chain cleavage as shown by Vilgrain et al. [6] on adrenocortical cells in vitro. The inhibitory action of PL-C on dbcAMP-stimulated testosterone production is in agreement with the findings of Lowitt et al. [7] showing that phospholipids are involved in the cAMP-dependent stimulation of steroidogenesis.

In conclusion, the above data demonstrate that the activated Ca^{2+}- and phospholipid-dependent protein kinase exerts a negative control on LH-stimulated steroidogenesis in purified rat Leydig cells in vitro. These results are in agreement with those of Culty et al. [4] showing that PMA inhibits ACTH-induced steroidogenesis in adrenocortical cells.

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