Inhibition of hCG-stimulated adenylate cyclase in purified mouse Leydig cells by the phorbol ester PMA

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The tumour-promoting phorbol ester, PMA (phorbol 12-myristate 13-acetate), markedly reduced the steroidogenic response of mouse Leydig cells to stimulation by hCG and cholera toxin. However, 8Br-cAMP- and forskolin-stimulated steroidogenesis was not inhibited by PMA. PMA did not inhibit hCG-induced steroidogenesis in the simultaneous presence of 1 μM forskolin. The analysis of intracellular cAM P indicated that the PMA-induced inhibition of steroidogenesis was the result of an impaired cAMP accumulation. Adenylate cyclase in membranes prepared from PMA-treated cells showed a diminished response to hCG, GTP, guanosine 5'-[β,γ-imido]triphosphate [Gpp(NH)p] or to a combination of the stimulants. PMA, however, was unable to inhibit adenylate cyclase when added directly to the membrane preparation from untreated cells. As previous observations have indicated that ^{125}I-hCG binding and phosphodiesterase activity in mouse Leydig cells are not influenced by PMA, it is concluded from the present study that the site of inhibition has to be localised to the regulatory guanine nucleotide binding protein of the adenylate cyclase system.

Mouse Leydig cell *Tumor promoter* *Phorbol ester* *Testosterone production* *Adenylate cyclase*

1. INTRODUCTION

Phorbol esters are a group of structurally related compounds having tumour-promoting activity, originally isolated from seed oil of *Croton tiglium* [1]. Recently, various reports have indicated that the tumour-promoting phorbol ester, phorbol 12-myristate 13-acetate (PMA) can activate a phospholipid-sensitive Ca^{2+}-dependent protein kinase (protein kinase C) [2–4] and that the expression of various cellular functions can be modulated by PMA. In hormone-secreting cells, PMA has been shown to stimulate the release of several anterior pituitary hormones from cultured pituitary cells [5–7], insulin release from pancreatic islets of Langerhans [8] and corticosteroid production by isolated bovine adrenocortical cells [9]. Gonadotropin-induced steroidogenesis by cultured Leydig cells and ovarian cells of the rat has been reported to be inhibited by PMA [10] and this inhibition was shown to result from a modulation of steroidogenic enzymes by PMA at a step beyond hormone-stimulable adenylate cyclase.

Previous reports [11,12] from our laboratory have shown that in mouse Leydig cells, PMA inhibits gonadotropin (hCG/LH)-induced testosterone production and that in contrast to rat gonadal cells, the site of action of PMA in mouse Leydig cells was located beyond hormone-receptor interaction but prior to the activation of steroidogenic cascade by cAMP. To understand the mechanism by which PMA exerts its inhibitory action, we have investigated the effects of this phorbol ester on testosterone production by purified mouse Leydig cells in response to cholera toxin, forskolin, 8Br-cAMP and hCG. Cholera toxin is known to increase intracellular cAMP concentration by causing ADP-ribosylation of the regulatory guanine nucleotide binding protein, which results in activation of adenylate cyclase [13], and forskolin has been proposed to activate the catalytic unit of the enzyme directly [14]. Both cholera toxin

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[15] and forskolin [16] have been shown to stimulate adenylate cyclase activity in mouse Leydig cells. In addition, we have studied adenylate cyclase activity in membranes prepared from PMA-treated and untreated cells.

2. MATERIALS AND METHODS

The sources of various chemicals used were as follows: 8-bromo-cyclic AMP (8Br-cAMP), bovine serum albumin, cholera toxin, dimethyl sulfoxide (DMSO), GTP, guanosine 5'-[β,γ-imido]triphosphate [Gpp(NH)p] and PMA from Sigma, München; minimum essential medium with Earle's salts from Gibco Europe, Karlsruhe; Hepes from Serva, Heidelberg; forskolin from Calbiochem-Behring, Frankfurt; and highly purified human chorionic gonadotropin (hCG, 13 500 IU/mg) from Boehringer, Mannheim.

Preparation and purification of Leydig cells from the testes of adult NMRI mice have been described [15]. In general, purified Leydig cells (10^5 cells) were incubated in 500 μl minimum essential medium containing 25 mM Hepes, pH 7.4 and 0.1% bovine serum albumin (medium) for 3 h at 36°C, with or without various test substances. PMA was dissolved in DMSO at a concentration of 2×10^{-3} M. This stock solution was diluted with medium prior to the addition to the incubation tubes. An appropriate amount of DMSO was added to the control incubation.

Testosterone and intracellular cAMP were measured by radioimmunoassay, as described in [15].

Preparation of plasma membranes of Leydig cells and the adenylate cylase assay was performed as described in [15]. The homogenization medium was modified by including 1 mM EDTA.

The results have been presented as mean ± SD of triplicate determinations. The statistical significance of differences between PMA-treated and control groups has been assessed on the basis of Student's *t*-test.

3. RESULTS

The results presented in fig.1 show the effect of PMA (5×10^{-7} M) on testosterone production by mouse Leydig cells stimulated with submaximal steroidogenic doses of hCG, cholera toxin and

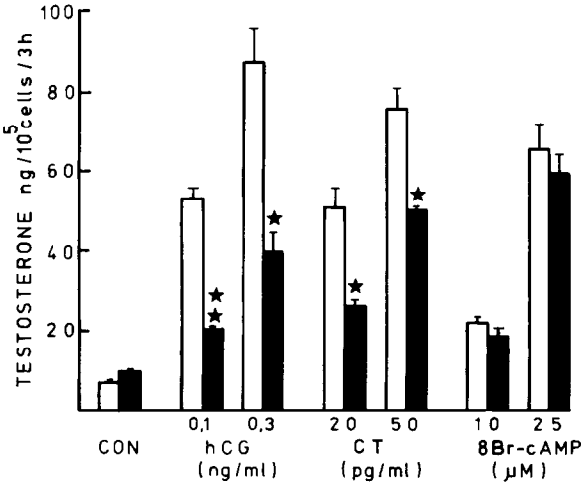

Fig.1. Effect of PMA on basal and on hCG, cholera toxin or 8Br-cAMP-induced testosterone production. Mouse Leydig cells were incubated for 3 h with (solid bars) or without (open bars) 5×10^{-7} M PMA, in the absence or presence of steroidogenic stimulants as indicated. Testosterone accumulated in the medium was assayed by radioimmunoassay. Values are mean ± SD of triplicate determinations from 1 of 3 different experiments. con, control; CT, cholera toxin. $*p<0.01$ and $**p<0.001$ compared to without PMA.

8Br-cAMP. PMA produced a marked inhibition of steroidogenesis stimulated with either hCG or cholera toxin. There was, however, no effect of PMA on 8Br-cAMP-induced steroidogenesis. Basal steroidogenesis was not inhibited by PMA, rather a small but significant stimulation was observed ($p<0.05$, vs untreated cells). The concentration of PMA used was chosen on the basis of published results [11].

Fig.2 shows the effect of forskolin plus/minus PMA on testosterone production by mouse Leydig cells. When the cells were incubated with increasing concentrations of forskolin (fig.2A) the amount of testosterone in the medium increased in a dose-related manner. The maximum stimulation of steroidogenesis was observed at a forskolin concentration of 4 μM. Higher concentrations of forskolin, however, led to a decline of steroidogenesis close to basal level. Addition of PMA (5×10^{-7} M) to forskolin-stimulated cells resulted in an increase in testosterone production rather than an inhibition as seen for hCG or cholera toxin-induced steroidogenesis. The stimulatory effect of PMA on

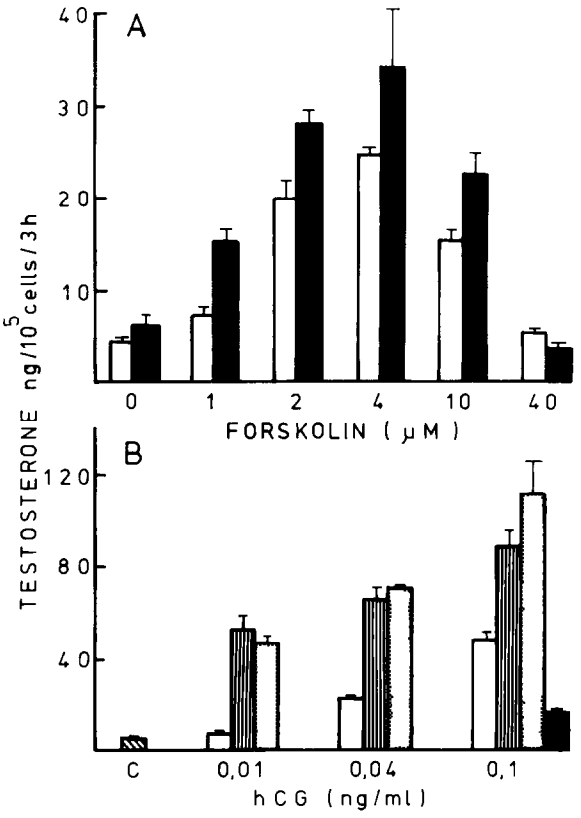

Fig.2. Effect of PMA on forskolin plus hCG-induced steroidogenesis in mouse Leydig cells. (A) The cells were incubated for 3 h with varying concentrations of forskolin in the absence (open bars) or presence (solid bars) of 5×10^{-7} M PMA and testosterone was measured in the medium. (B) The cells were incubated without any addition (C) or with varying concentrations of hCG as indicated (open bars), hCG plus 1 μM forskolin (striped bars) or with hCG, forskolin and 5×10^{-7} M PMA (stippled bars). Testosterone in the medium was measured. The black bar represents the amount of testosterone produced in the presence of 0.1 ng/ml hCG plus 5×10^{-7} M PMA. The values are mean ± SD of triplicate determinations from 1 of 3 different experiments.

forskolin-induced steroidogenesis was observed up to 10 μM forskolin but not with higher concentrations.

The data in fig.2B shows the effect of forskolin and/or PMA (5×10^{-7} M) on hCG-induced steroidogenesis. In accordance with an earlier report [16], addition of forskolin (1 μM, which causes only a poor stimulation on its own) markedly enhanced the steroidogenic response of the cells to various concentrations of hCG. Furthermore, PMA was unable to inhibit steroidogenesis induced by a combination of hCG and forskolin, in contrast to the observed inhibition of PMA on steroidogenesis stimulated by hCG alone.

Since the PMA-induced inhibition of steroidogenesis appears to reflect an impaired cAMP accumulation, we have studied the effect of the phorbol ester on intracellular cAMP formation in response to varying concentrations of hCG. As summarised in table 1, PMA had no effect on the level of intracellular cAMP in unstimulated cells, whereas the formation of intracellular cAMP induced by 0.2 and 0.8 ng/ml hCG was effectively inhibited by PMA. These 2 doses of hCG were submaximal in terms of the steroidogenic response. The effect of PMA on the cAMP response in cells treated with a higher concentration of hCG ($\geqq$2.0 ng/ml) was found to be insignificant. This is in accordance with an earlier observation that inhibition of steroidogenesis by PMA is observed only when submaximal and not maximal steroidogenic doses of gonadotrophin were used [11].

Adenylate cyclase activity was also measured in membranes prepared from cells incubated with or without PMA. The results presented in fig.3 show that the basal enzyme activity is similar in membranes from PMA-treated or untreated cells. However, adenylate cyclase activity measured in

Table 1

Effect of PMA on accumulation of intracellular cyclic AMP in Leydig cells stimulated with varying concentrations of hCG

hCG added (ng/ml)	Intracellular cyclic AMP (pmol/10^5 cells)	
	Without PMA	With PMA
0	0.081 ± 0.003	0.083 ± 0.01
0.2	0.38 ± 0.02	0.26 ± 0.01*
0.8	2.79 ± 0.18	1.97 ± 0.14*
2.0	6.98 ± 0.71	5.52 ± 0.59

*$p < 0.001$ compared to without PMA

Mouse Leydig cells were incubated with varying concentrations of hCG with or without PMA (5×10^{-7} M for 3 h. Intracellular cAMP was measured as described in section 2. Values are means ± SD of triplicate determinations from 1 of 2 different experiments

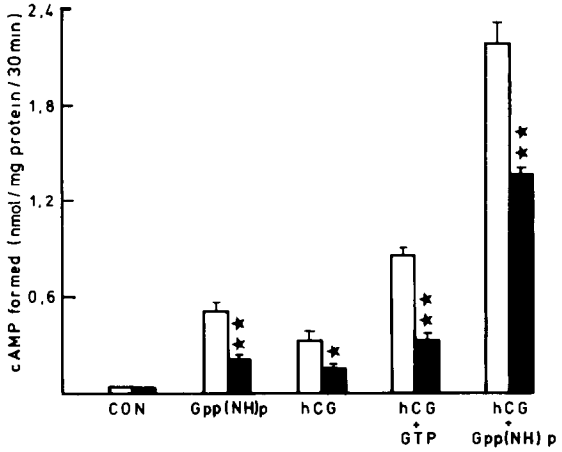

Fig.3. Adenylate cyclase activity in membranes from mouse Leydig cells pretreated with and without PMA. Leydig cells (10^6 ml) were pretreated in triplicate with (solid bars) or without (open bars) 5×10^{-7} M PMA for 2 h (36°C). After sedimentation ($150 \times g$, 10 min) and washing with 5 ml medium the cells were resuspended in 1 ml homogenization mixture containing 10 mM Tris/HCl (pH 7.5), 1 mM dithiothreitol and 1 mM EDTA. The replicates were pooled and homogenized in a Dounce homogenizer. The membrane preparation and the adenylate cyclase assay (30 min) were conducted exactly as described in [15]. Adenylate cyclase activity was determined in the absence of any addition (CON) and in the presence of Gpp(NH)p (0.1 mM), hCG (5 mg/ml), hCG + GTP (20 μM) or hCG + Gpp(NH)p. $^*p < 0.01$ and $^{**}p < 0.001$ compared to untreated cells.

the presence of hCG, hCG plus GTP and hCG plus Gpp(NH)p was markedly reduced in untreated cells. In contrast, PMA had no significant effect on the stimulation of adenylate cyclase when it was directly added to the membrane preparation (not shown).

4. DISCUSSION

Our data confirm recent reports [10–12] that PMA inhibits gonadotrophin-stimulated steroidogenesis and provide further insight into the mechanism by which the phorbol ester exerts its negative action. Recent results from this laboratory [11,12] have shown that specific binding of ^{125}I-hCG and 8Br-cAMP-induced steroidogenesis of mouse Leydig cells are not affected by PMA. These observations indicate that the inhibitory action of PMA has to be localised prior to the accumulation of cAMP, which has now been verified by the measurement of cellular cAMP levels. The impaired cAMP response appears not to be due to an increase in phosphodiesterase activity, since the PMA-induced inhibition was also obvious in the presence of a phosphodiesterase inhibitor (cf. [12]). Our data are in contrast to a recent report [10] demonstrating that the inhibitory effect of PMA in cultured rat gonadal cells is located beyond the generation of cAMP. These authors reported that the inhibitory influence of PMA was exerted by modulating the activity of steroidogenic enzymes. Whether this discrepancy is due to differences in species or in methodology is not clear at present.

For a more precise localisation of the PMA-induced block within the adenylate cyclase system we have analysed the effect of PMA on cholera toxin- and forskolin-stimulated steroidogenesis. Both stimulants activate the enzyme system at different sites (see section 1). Using intact cells, we usually measured the cAMP-mediated stimulation in terms of testosterone production; stimulation of steroidogenesis in the submaximal range is a more sensitive indicator of changes in the cAMP concentration than direct cAMP measurement. The observation that PMA inhibited cholera toxin, but not forskolin-induced steroidogenesis indicates that the site of inhibition is localised at the regulatory guanine nucleotide-dependent protein (N protein) or at the level of coupling between N protein and the catalytic unit and not at the catalytic unit of adenylate cyclase itself. The PMA-induced inhibition was also observed when adenylate cyclase activity was analysed in membranes from PMA-treated cells in the presence of GTP, the non-hydrolysable GTP analogue Gpp(NH)p and/or hCG, which all stimulate catalytic activity through activation of N protein. PMA appears not to inhibit adenylate cyclase by an unspecific mechanism since the enzyme activity was not reduced when the phorbol ester was directly added to the membrane prepared in the presence of EDTA. Furthermore, basal cAMP levels and basal testosterone production were generally not inhibited by PMA at the concentrations used.

Tumour-promoting phorbol esters like PMA may have a variety of pharmacological actions in the cell. One, which has been well studied is the ac-

tivation of protein kinase C [2–4], an enzyme which appears to be present almost universally in eukaryotes [17]. This enzyme is activated by a diacylglycerol [2,18] formed transiently during the turnover of membrane phospholipids. PMA is apparently capable of substituting for diacylglycerol [2] and the kinase has been shown to be a receptor for these compounds [3]. In mouse Leydig cells a synthetic diacylglycerol, 1-oleoyl-2-acetyl glycerol (OAG), has been shown to mimic the inhibitory effect of PMA on gonadotrophin-induced steroidogenesis [12]. Thus, the inhibition of the hormone-responsive adenylate cyclase in mouse Leydig cells by PMA might be mediated by the activation of protein kinase C. A similar mechanism has recently been proposed for the PMA-induced inhibition of glucagon-responsive adenylate cyclase in rat liver cells [20], in which protein kinase C activity has been demonstrated. The available data suggest that protein kinase C plays a role in the expression of the gonadotrophin-induced stimulus by modulating adenylate cyclase activity at the N protein site. The involvement of protein kinase C in the action of PMA is supported by the observation that the phorbol ester is only active in intact cells but not in EDTA-washed membranes which are devoid of the enzyme [19]. In one experiment, in which membranes were prepared in the absence of EDTA, a significant direct inhibition by PMA was observed, probably due to association of protein kinase C with the membrane preparation (not shown). That protein kinase C is partially associated with the particulate fraction of rat testis homogenate has been recently reported [21].

In conclusion, we have shown that in mouse Leydig cells the inhibition of gonadotrophin-induced steroidogenesis by PMA results from a suppression of hormone-responsive adenylate cyclase and that the PMA-induced block is localised at the level of the N-protein. The action of PMA in these cells appears to be mediated via activation of protein kinase C. The demonstration of this enzyme in the mouse Leydig cell and its precise role in the expression of hormone-induced steroidogenesis, however, remain subjects for further studies.

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