Endocrine (1995) 3, 579-584

LH/hCG-receptor is coupled to both adenylate cyclase and protein kinase C signaling pathways in isolated mouse Leydig cells

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The aim of this study was to examine whether or not a protein kinase C-dependent pathway is involved in the desensitization process of the LH/hCG-receptor-linked adenylate cyclase system in isolated mouse Leydig cells. Treatment of these cells with the phorbol ester, 4-β-phorbol 12-myristate 13-acetate (PMA) leads to a translocation (and a putative activation) of protein kinase C from the cytosol to the plasma membrane, as evidenced by the Western blotting procedure using particulate and cytosolic fractions of Percoll-purified mouse Leydig cells. A similar translocation is also observed following the treatment of mouse Leydig cells with hCG. Data obtained show that this effect is time-dependent and is mediated specifically through the LH/hCG-receptor. Furthermore, we show that the treatment of Leydig cells with either PMA or hCG leads to a desensitization of the adenylate cyclase stimulated with hCG, hCG plus GppNHp or AlF_4^-. This desensitization was not accompanied by a change in the [^{125}I]-hCG binding to membrane receptors. Thus we provide here direct evidence that hCG is capable of activating protein kinase C. In addition, we postulate that PMA as well as hCG-treatment leads to a lesion located at a site distal to the receptor/G-protein interaction but proximal to the adenylate cyclase activation and that the translocation (and activation) of protein kinase C may be a common mechanism involved in this desensitizing effect caused by both PMA and hCG on Leydig cells

Keywords: cyclic AMP; crosstalk; desensitization; gonadotropin; mouse Leydig cells; hCG

Introduction

Glycoprotein hormones, e.g. gonadotropines, act through G-protein-coupled seven-transmembrane-domain receptors to activate the adenylate cyclase signaling pathway. The activation of a transmembrane signaling process is initiated primarily by the hormone, following its interaction with the cell-surface receptors. However, this process appears to be modulated by several other pathways which are secondarily initiated either by the hormone itself or by other molecules interacting with the target cell simultaneously or sequentially. The biochemical nature of these secondary events determines how such events can be superimposed upon the primary receptor-coupled signaling reaction. Such superimpositions govern the extent and duration of the intracellular response, e.g. the generation of second messengers. Therefore, to understand completely how the hormone-stimulated second messenger formation is regulated, it is important to understand the type and the nature of such secondary reactions taking place intracellularly.

One of the earliest events that occur following the interaction of LH with its receptors in gonadal steroidogenic cells is the increase in intracellular levels of cyclic AMP (Cooke & Rommerts, 1988; Rommerts & Cooke, 1988; for a recent review see Saez, 1994). However, this stimulation of cyclic AMP formation in response to LH in luteal cells for example, can be positively modulated by prior exposure of the cells to EGF via activation of phospholipase D (Budnik & Mukhopadhyay, 1991, 1993a). Similar positive interactions have been shown to occur between the adenylate cyclase system and the protein kinase C signaling systems in a number of cell types including the bovine luteal cells (Budnik & Mukhopadhyay, 1993b) and adrenocortical cells (Bird *et al.*, 1993). The nature of this interaction appears to depend upon the particular isoform of the adenylate cyclase enzyme as well as the class of protein kinase C involved (Yoshimura & Cooper, 1992; Jacobowitz *et al.*, 1993). One of the ways protein kinase C exerts a stimulatory effect on adenylate cyclase has been shown to involve a phosphorylation of type 2 adenylate cyclase (Jacobowitz & Iyenger, 1994).

On the other hand, an inhibitory effect has also been shown to be exerted by protein kinase C on the gonadotropin-receptor-coupled adenylate cyclase system in certain types of cells, e.g. Leydig cells of mouse and rat (Mukhopadhyay *et al.*, 1984; Nikula *et al.*, 1987), in a murine Leydig tumor cell line (Rebois & Patel, 1985), granulosa cells from porcine (Wheeler & Veldhuis, 1988), rodent (Shinohara *et al.*, 1985; Trzeciak *et al.*, 1987) and human (Abayasekara *et al.*, 1993) ovaries. Moreover, $PGF_{2\alpha}$ induced luteolysis in the primate ovary has been shown to be mediated by an activation of protein kinase C (Houmard *et al.*, 1994).

Apart from activating the adenylate cyclase pathway, LH appears to be also capable of stimulating a phospholipase C signaling cascade, particularly in the ovary (see Davis, 1994, for a recent review). Although, from such information it may be implied that the activation of phospholipase C by LH may potentially cause a stimulation of protein kinase C activity, no data is available in the literature supporting a direct regulation of protein kinase C activity by the gonadotropins (Davis, 1994). Activation of protein kinase C is known to be associated with a translocation of the enzyme from the cytosolic compartment to the membrane (Nishizuka, 1984; Gopalakrishna *et al.*, 1986). Based on this criterion, we report here that in isolated mouse Leydig cells, a translocation (and therefore a putative activation) of protein kinase C is brought about by hCG treatment. Furthermore, we provide evidence suggesting that this activation of protein kinase C by hCG may be responsible for the homologous desensitization observed when the Leydig cells are repeatedly stimulated by LH/hCG.

Results

Preliminary Western blotting experiments carried out with the homogenate of Percoll-purified Leydig cells, using specific antibodies to various protein kinase C isoenzymes, revealed that the protein kinase C type ε was best visualized in these cells. Therefore, we have carried out all further experiments using the specific antibody to protein kinase type ε.

In the first experiment, results of which have been depicted in Figure 1, we have incubated the cells with PMA (10^{-7} M

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Received 23 February 1995; accepted 19 April 1995

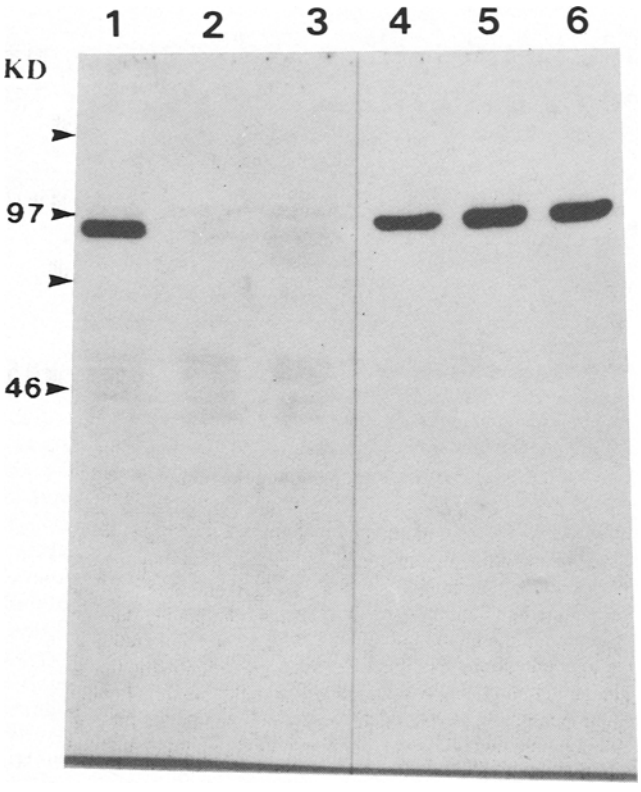

Figure 1 Distribution of cellular protein kinase C between particulate and cytosolic fractions in Leydig cells. Cytosolic (lanes 1–3) and particulate (lanes 4–6) fractions prepared from untreated controls (lanes 1 and 4), from cells pretreated with 10^{-6} M PMA (lanes 2 and 5) and 10^{-7} M PMA (lanes 3 and 6) for 2 h at 36°C, were subjected to SDS PAGE analysis. Western-blotting was performed as described in detail under Material and methods

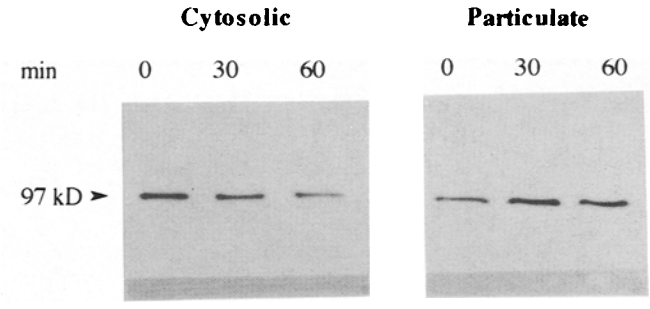

Figure 2 Redistribution of protein kinase C from the cytosol to the particulate fraction following hCG-stimulation. The blot marked as 'Cytosolic' shows the amount of protein kinase C in the cystolic fraction 0 min (lane 1), 30 min (lane 2) and 60 min (lane 3) after stimulation of cells with 0.1 μg/ml hCG. The blot marked 'Particulate' shows the amount of protein kinase C in the particulate-fraction in the corresponding time periods after stimulation

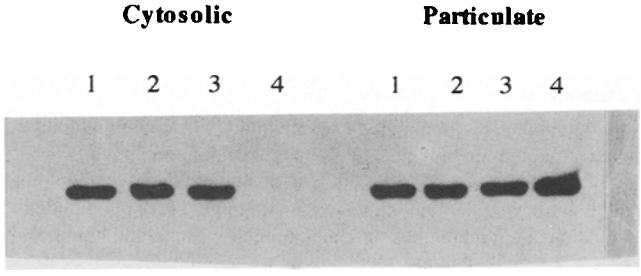

Figure 3 Lack of an effect of hCG on protein kinase C translocation in a pituitary tumor celline (αT3-1). Lane 1 shows protein kinase C-signal of non-pretreated cells, lanes 2 and 3 show protein kinase C-signal of cells pretreated with 0.1 μg/ml hCG for 30 and 60 min, lane 4 shows protein kinase C after pretreatment with 10^{-7} M 4β-PMA

and 10^{-6} M) following which the particulate and cytosolic fractions were prepared. The proteins were resolved first by SDS–PAGE and then transferred to a PVDF membrane. Protein kinase Cε was visualized with the help of a specific antibody and ECL reagents. Figure 1 shows clearly that following incubation with two different concentrations of PMA, protein kinase C disappears from the cytosolic fraction. At the same time the protein kinase C signal gets more intense in the particulate fraction. Thus as expected, PMA treatment results in a clear translocation of protein kinase C from the cytosol to the particulate fraction in mouse Leydig cells. No such effect is observed in untreated control cells.

In order to investigate whether hCG treatment also leads to a translocation of protein kinase C from the cytosolic to the particulate fraction in Leydig cells, a time course experiment was performed, the data of which is presented in Figure 2. The cells were incubated with hCG for different durations, following which the cells were homogenized, and the particulate and cytosolic fractions were separated. Protein kinase C in these two compartments was visualized by Western blotting followed by ECL detection. It is evident that the amount of immunoreactive protein kinase C decreases gradually over a period of 60 min in the cytosolic fraction, whereas it increases in the particulate fraction at the same time.

That the effect of hCG was specifically mediated through its receptors on Leydig cells was demonstrated by carrying out an experiment similar to that described in Figure 2, but instead of Leydig cells, αT3-1 cells were used, which are pituitary gonadotroph-derived cells and do not possess any hCG/LH-receptors. The data in Figure 3 clearly show that protein kinase C is present in these cells and that following treatment with PMA, it is translocated from the cytosolic to the particulate fraction. However, no effect of hCG on protein kinase C in these cells is observed either at 30 min or 60 min of incubation.

In the next series of experiments, we have examined the possibility that this translocation/activation of protein kinase C from the cytosol to the particulate fraction induced by hCG/PMA may constitute to be an underlying mechanism for a homologous (induced by LH/hCG) or heterologous (induced by PMA) desensitization.

The results summarized in Tables 1 and 2 compare the characteristics of the desensitizing effect produced by an incubation of mouse Leydig cells with either 10^{-7} M PMA (Table 1) or 10 ng/ml hCG (Table 2). Adenylate cyclase activity was measured in the particulate fractions prepared from untreated control and from treated cells. From the data obtained it was obvious that a preincubation of the cells with either PMA or hCG did not produce any significant effect on basal adenylate cyclase activity measured in the absence of any addition. In contrast however, both PMA and hCG produced a significant decrease in the adenylate cyclase activity measured in the presence of either hCG alone or hCG plus GppNHp.

Thus the results obtained so far confirm and extend previous observations that both PMA and hCG treatment of mouse Leydig cells *in vitro* caused a marked desensitization of the hCG-responsive adenylate cyclase activity (Mukhopadhyay *et al.*, 1984; Schumacher *et al.*, 1984).

It has been previously demonstrated that the homologous desensitization of mouse Leydig cells induced *in vitro* by pretreatment of the cells with hCG does not involve any change in the binding characteristics of LH/hCG receptors (Schumacher *et al.*, 1984). We show now in Figure 4 that also the PMA-induced desensitization does not affect the

Table 1 Effect of treatment of Leydig cells with PMA on adenylate cyclase activity

Additions	Adenylate cyclase activity (pmol cyclic AMP/mg protein/30 min) untreated control	PMA-treated
none	117 ± 4	108 ± 12
hCG	329 ± 32	182 ± 6*
hCG + GppNHp	2138 ± 25	1173 ± 99**

Mouse Leydig cells were pretreated with PMA (5×10^{-7} M for 3 h) and were washed three times. Subsequently, particulate fractions were prepared and incubated in the presence of hCG (0.4 μg/ml), hCG (0.4 μg/ml) plus GppNHp (0.1 mM). Adenylate cyclase activity was measured as described in Materials and methods. Values are means ± SD of triplicate determinations from one of three different experiments. *$P<0.001$, **$P<0.0001$ compared to untreated control

Table 2 Effect of treatment of Leydig cells with hCG on adenylate cyclase activity

Additions	Adenylate cyclase activity (pmol cyclic AMP/mg protein/30 min) untreated control	hCG-treated
none	126 ± 6.7	93 ± 6.2
hCG	230 ± 8.2	102 ± 7.8**
hCG + GppNHp	1040 ± 69.5	375 ± 10.4*

Experimental setup was identical to Table 1, except that the cells were pretreated with hCG (10 ng/ml) instead of PMA prior to preparation of particulate fractions. *$P<0.001$, **$P<0.00001$ compared to untreated control

[^{125}I]-hCG binding characteristics on Leydig cells. The cells (2×10^5 cells per ml) were incubated for 3 h at 36°C in the absence or presence of PMA (5×10^{-7} M), following which the cells were washed once with medium and subsequently incubated with [^{125}I]-hCG and various concentrations of unlabeled hCG as described in Materials and methods. The receptor numbers and dissociation constants (K_d) determined by Scatchard-Plots (data not shown) were 23 957 ± 7396 per cell and 217 ± 74 pM for untreated cells and 20 630 ± 5717 per cell and 202 ± 42 pM for PMA-pretreated cells.

Above results show a striking similarity between the desensitizing effects of hCG and PMA, pointing to possibly an identical lesion caused by these two agents. We have therefore investigated if this lesion could be located proximal to the adenylate cyclase but distal to the receptor/G-protein interaction. For this we have further examined whether both PMA and hCG will affect AlF_4^--stimulated adenylate cyclase activity, since AlF_4^- is able to activate adenylate cyclase activity independent of hormonal stimulation. We have incubated Leydig cells in the presence of 5×10^{-7} M PMA or in its absence for 3 h and then prepared particulate fractions from these cells to carry out a time course kinetics of adenylate cyclase activity in the presence of AlF_4^-, i.e. $AlCl_3$ plus NaF. It is obvious from the data presented in Figure 5a that at all time points examined, adenylate cyclase activity in the particulate fraction prepared from PMA-treated Leydig cells registered a marked decrease (approximately 30%) compared to untreated control cells. A similar decrease in AlF_4^--stimulated activity was noted when the enzyme activity in the preparations of particulate fractions from hCG-treated and untreated cells were compared (Figure 5b).

Discussion

In this study carried out on mouse Leydig cells, we have extended and confirmed previous reports (Kimura *et al.*, 1984; Nikula *et al.*, 1987; Pelosin *et al.*, 1991) describing the presence of protein kinase C in Leydig cells isolated from rat testes. Furthermore, we have been able to obtain direct

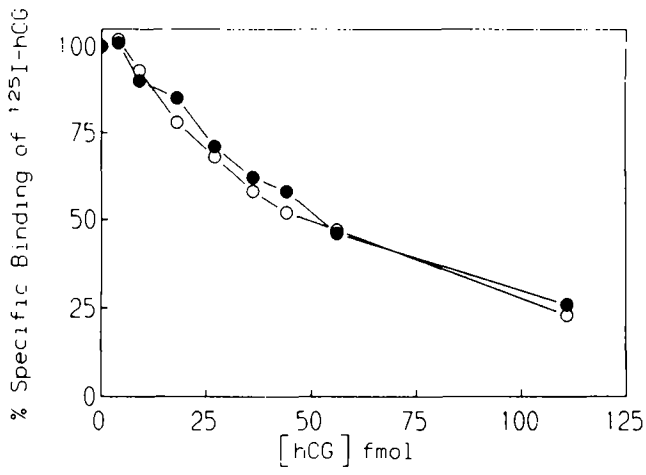

Figure 4 Effect of preincubation of Leydig cells with PMA on [^{125}I]hCG binding to cell-surface receptors. Leydig cells (2×10^5 cells/ml) were incubated for 3 h in the absence (○) or presence (●) of 5×10^{-7} M PMA. Afterwards, cells were washed with medium once and resuspended in medium with ^{125}I-labeled hCG for 21 h at 4°C. Separation of bound from unbound ^{125}I-labeled hCG was achieved by centrifugation of the cells through silicon-oil

evidence documenting the fact that in Leydig cells, a stimulation with hCG leads to a translocation (and putative activation) of protein kinase Cε from the cytosolic compartment to the particulate fraction. A possible stimulation of the protein kinase C signaling pathway following gonadotropin receptor activation in a number of gonadal steroidogenic cells has been postulated (Davis, 1994) but no direct evidence showing that this indeed happens has been so far demonstrated. PMA, an activator of protein kinase C (Niedel *et al.*, 1983) produced a dramatic effect on protein kinase C translocation both in Leydig cells and in a gonadotrop-derived cell line αT3-1. However, hCG used at a concentration that elicited a clear translocation in Leydig cells did not have any effect on αT3-1 cells which are not known to possess any gonadotropin receptors. It could be argued that a contaminating factor present in the hCG preparation used, may have produced the observed translocation of the kinase in a nonspecific manner, and that hCG itself has nothing to do with this phenomenon. This possibility is highly unlikely, as demonstrated by the lack of an effect of hCG on αT3-1 cells.

The effect of hCG on protein kinase Cε was strictly dependent upon the duration of the exposure of Leydig cells to the hormone. A clear effect was seen within 30 min of exposure, and thereafter no further increase of signal-intensity in the particulate fraction was observed.

That a stimulation of protein kinase C by PMA leads to an inhibitory effect on hCG/LH-stimulated cyclic AMP formation and steroidogenesis has been documented in mouse and rat Leydig cells by several groups (Mukhopadhyay *et al.*, 1984; Mukhopadhyay & Schumacher, 1985; Rebois & Patel, 1985; Themmen *et al.*, 1986; Nikula *et al.*, 1987). Rebois and Patel (1985) observed that PMA-treatment did not lead to any change of receptor numbers or their affinity and proposed that the activation of protein kinase C is the mechanism involved in the heterologous desensitization of hCG/LH-receptor linked adenylate cyclase activity in a MTLC-1 tumor Leydig cell line. Thus protein kinase C-dependent pathways have been implicated in the process of desensitization in Leydig cells as evidenced in published literature. Although this is true for PMA-induced desensitization, a role for protein kinase C in the case of hCG-induced desensitization, is yet to be reported.

However, before a role for protein kinase C in hCG-induced desensitization could be discussed, it is imperative to have a clear demonstration that hCG/LH is indeed able to

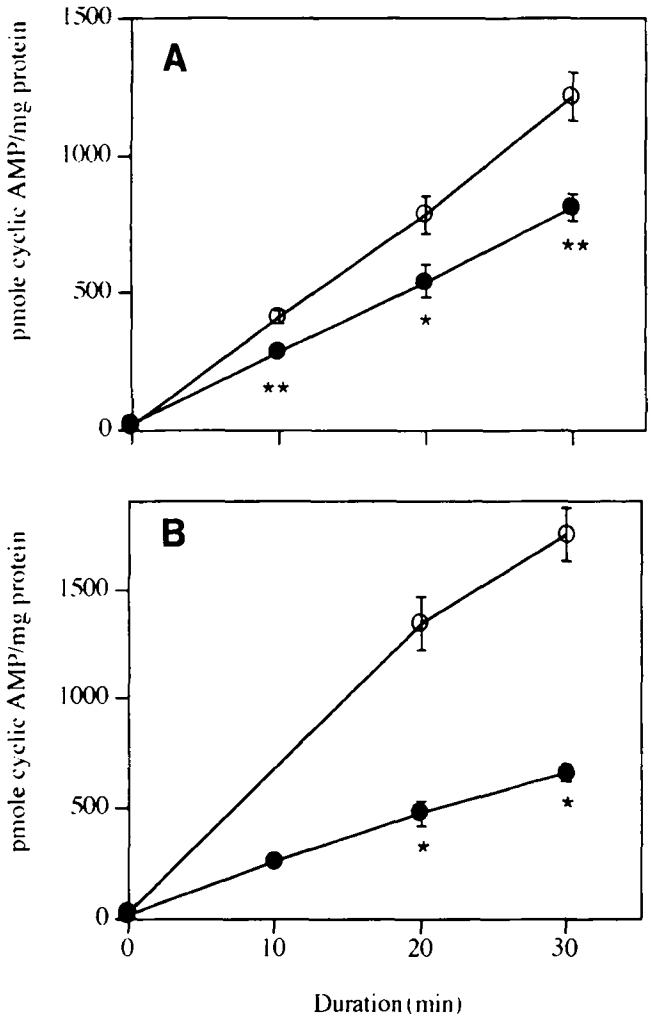

Figure 5 (A) Effect of preincubation of Leydig cells with PMA on the adenylate cyclase activity measured subsequently in the particulate fraction prepared from the cells. Leydig cells (2×10^5 cells/ml) were incubated in the absence (○) or in the presence (●) of 5×10^{-7} M PMA for 3 h at 36°C. After sedimentation (10 min at 100 *g*) and washing with a pH 7.4 buffer, particulate fractions were prepared and adenylate cyclase activity was determined by stimulation of these fractions with 800 mM $AlCl_3$ plus 20 mM NaF. Values represent means from six separate incubations. (**B**) Effect of preincubation of Leydig cells with hCG on the adenylate cyclase activity measured subsequently in the particulate fraction prepared from the cells. Details of experimental setup were as described for (**A**) except that the cells were treated with hCG (10 mg/ml) instead of PMA prior to preparation of particulate fractions. $^*P < 0.0001$, $^{**}P < 0.00001$, treated versus untreated controls

activate this kinase. We have now obtained this evidence in mouse Leydig cells. Moreover, we show that there is a striking parallelism between the effects of PMA and hCG. In both cases, the binding characteristics of the receptor remain unimpaired although the signal transduction processes distal to the event of hormone-receptor interaction is impaired. Based on the data obtained we propose that the lesion is localized at the level of the interaction between the receptor-activated G protein and adenylate cyclase activity, for both PMA and hCG-induced desensitization. The fact that AlF_4^- medicated activation of adenylate cyclase was inhibited by preincubating the cells with eithr PMA or hCG supports this proposal. It has been suggested that fluroaluminates bind to a phosphate of GDP that is bound to the G-protein by forming a hydrogen bond (Bigay *et al.*, 1985). This simulates the presence of a γ-phosphate of GTP, and therefore the G-protein is activated fully bypassing the need for any hormonal stimulation (Sternweis & Gilman, 1982; Bigay *et al.*, 1985).

It is tempting to propose that hCG activates both protein kinase A and C signaling pathways. The stimulation of steroidogenesis is the direct consequence of the activation of the kinase A. Although the activation of protein kinase C is known to cause a stimulation of progesterone production in bovine luteal cells (Brunswig *et al.*, 1986), it can not be considered as an important mechanism in stimulating testosterone production by Leydig cells, as Themmen *et al.*, (1986) have shown that treatment of Leydig cells with PMA caused at best a very modest stimulation of steroid production. Parallel stimulation of these two kinases ensures an effective stimulation of steroidogenesis at one hand and a safety mechanism, on the other, preventing an overstimulation of cells in the presence of continuous or repeated stimuli. A variety of mechanisms based mostly on protein phosphorylation has been considered in the context of agonist-induced/homologous desensitization. Among these, the best studied one is that involves a receptor-specific protein kinase that phosphorylates and desensitizes the β-adrenergic receptors (Hausdorff *et al.*, 1990; Lefkowitz. 1993). In case of hCG/LH-receptors, the nature of the identity of the substrate(s) of protein kinase C remains completely unknown yet. This will require a lot of attention in the future before we can understand the molecular basis of the agonist-induced desensitization of LH/hCG receptors. It may be noted here that in the present study involving mouse Leydig cells, the attention was focussed on only one member of the protein kinase C family, namely the ε type. Further studies will be required to analyse the role of other types of protein kinase C. Also in other types of gonadal steroidogenic cells depending upon the particular class of adenylate cyclase being present in these cells, one can expect completely divergent interactions between protein kinase C and adenylate cyclase ranging from augmentation to desensitization. It will be however interesting to determine whether in ovarian cells as well, hCG is able to translocate and activate protein kinase C.

Materials and methods

Reagents and supplies

Various reagents were obtained as follows: Antibody against rabbit-IgG, aprotinin, ATP, benzamidine, dimethylsulfoxide (DMSO), dithiothreitol (DTT), guanosine 5'-[β,γ-imido]triphosphate (GppNHp), isobutylmethylxanthine (IBMX), creatine-phosphokinase, leupeptine, pepstatin, phenylmethylsulfonylfluoride (PMSF), Triton X-100 were obtained from Sigma (Deisenhofen, Germany); minimum essential medium with Earle's salts and antibody against protein kinase C-ε from Gibco BRL (Eggenstein, Germany); HEPES from Serva (Heidelberg, Germany); GTP and highly purified human chorionic gonadotropin (hCG, 13500 IU/mg) from Boehringer (Mannheim, Germany); chemicals for Enhanced Chemiluminescence (ECL) detection from Amersham (Braunschweig, Germany); 4-β-Phorbol-12-myristate-13-acetate (PMA) from LC Services Corp. (Woburn, MA, USA); and [^{125}I]hCG from NEN (Wilmington, DE, USA).

Preparation and incubation of Leydig cells

The method for the preparation and purification of Leydig cells from the testes of adult NMRI mice has been described previously (Schumacher *et al.*, 1979). Incubation of the purified Leydig cells (2×10^5 cells/ml) was carried out in 25 ml minimum essential medium containing 25 mM HEPES, pH 7.4 and 0.1% bovine serum albumin 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.

Following the incubation, the cells were centrifuged at

100 *g* for 10 min. The pellet was washed with the medium and then used for preparation of the particulate fraction.

Preparation of particulate fraction and adenylate cyclase assay

The preparation of the particulate fraction of Leydig cells, which is the fraction that contains the plasma membranes, and the adenylate cyclase assay was carried out as described by Schumacher *et al.* (1984). In brief, $8-10 \times 10^6$ cells were suspended in 2 ml 50 mM Tris/HCl, pH 7.2 containing 1 mM DTT, 25 μg/ml leupeptin, 25 μg/ml benzamidine, 25 μg/ml aprotinin, 25 μg/ml pepstatin and 1 mM PMSF and homogenized in a Dounce type all-glass homogenizer; this homogenate was centrifuged at 100 *g* and 4°C for 20 min in order to sediment the nuclei and unbroken cells. The pellet was discarded, and the supernatant was used as the fraction that contained the cell membrane components. It was centrifuged at 100 000 *g* at 4°C for 40 min. The pellet was resuspended in 200–400 μl membrane-buffer, and the protein concentration was determined according to Bradford (1976) using reagents provided by Bio-Rad Laboratories (Munich, Germany). Protein concentrations were subsequently adjusted to 0.4 mg/ml.

The incubations were carried out in a final volume of 50 μl at 32°C in conical polystyrene tubes for various times. The incubation mixture consisted of 40 mM Tris-HCl (pH 7.4), 1 mM DTT, 5 mM $MgCl_2$ 1 mM ATP, 1 mM EDTA, 0.5 mM IBMX, 13.2 U/ml creatine phosphokinase, 10 mM creatine phosphate, and 0.1% BSA as well as various stimulators of adenylate cyclase activity as indicated. Incubations were terminated by the addition of 1 ml ice-cold 100% ethanol. The amount of cAMP formed was measured by a specific radioimmunoassay (Mukhopadhyay & Schumacher, 1985), using reagents available from IBL (Hamburg, Germany).

Radioligand binding assay

Binding assays utilizing ^{125}I-labeled hCG with a specific activity of 80.0 μCi/μg were carried out as follows: For determination of the cell-bound radioactivity, the cells (100 000 per tube) were incubated in a total volume of 250 μl in the presence of 1 ng ^{125}I-labeled hCG and increasing concentrations of cold hCG. The nonspecific binding was determined by adding a 1000-fold excess of unlabeled hCG. The incubation was carried out for 21 h at 4°C and terminated by a 1 min centrifugation of the cells through silicon-oil (specific gravity: $\rho = 1.013$ g/ml) at 10 000 *g*, to separate bound radioactivity from free. The cell-bound radioactivity was measured in a gamma counter. Specific binding was defined as the difference between the total binding and the nonspecific binding.

Western blotting and enhanced chemiluminescence detection

For protein kinase C detection, a Western blotting procedure was done. The particulate fraction was obtained as follows: $8-10 \times 10^6$ cells were suspended in 2 ml 50 mM Tris/HCl, pH 8.0 containing 1 mM DTT and 1 mM EDTA and homogenized in a Dounce type all-glass homogenizer; this homogenate was centrifuged at 100 000 *g* and 4°C for 40 min. The supernatant was used as the cytosolic fraction, whereas the pellet was dissolved in 500 μl 20 mM Tris/HCl, pH 7.5 containing 5 mM EGTA, 2 mM EDTA, 1 mM PMSF, 1 mM DTT, 0.2% Triton X-100 and 10% Glycerol. This was ultrasonicated three times, 10 s each, using a Sonifier (B-12, Branson Sonic Power Company, level 4). The homogenate was centrifuged at 100 000 *g*. The pellet was discarded, and an aliquot of the supernatant was diluted two times with 100 mM Tris/HCl, pH 6.8 supplemented with 24% glycerol, 8% SDS, 0.01% Serva-Blue 6 (Serva, Heidelberg, Germany) and 4% mercaptoethanol prior to resolving on an 8%-SDS–polyacrylamide gel according to Schägger and von Jagow (1987). The proteins were transferred to a PVDF membrane (Immunobilon Millipore, Bedford, MA, USA) by a semi-dry electrophoretic transfer procedure for 1.5 h at 1 mA/cm^2. Rainbow-marker from Amersham (Braunschweig, Germany) was used as molecular weight marker protein mixture. Ponceau staining was done to control whether proteins were transferred to the same extent in all lanes. The membrane was blocked with 1% Blocking Reagent from Boehringer-Mannheim (Mannheim, Germany), prepared in 100 mM maleic acid buffer, pH 7.5, containing 100 mM maleic-acid, 150 mM NaCl and 0.005% Thimerosal for 2 h at room temperature or overnight at 4°C. The membrane was then incubated with a rabbit polyclonal anti-protein kinase Cε antibody, diluted in nine parts TBS-T (0.15 M NaCl, 10 mM Tris/HCl pH 7.4, 0.05% Tween 20) and one part blocking-buffer. Afterwards, the membrane was incubated for 18 h at 4°C and washed three times with TBS-T for 5 min each. The second antibody (anti-rabbit, peroxidase-linked) was diluted 1:500 with the same TBS-T blocking-buffer as above and used for an incubation of the membrane of 1 h at 20°C. The membrane was again washed three times with TBS-T as above. Enhanced chemiluminescence detection of protein kinase C was performed according to the instructions supplied with the ECL reagents, and signals were detected on Fuji-RX film.

Where protein kinase C signals were compared between the cytosolic and particulate fractions, care was taken to adjust protein concentrations of both fractions so that comparable amounts of protein was loaded on the gel in each case.

Statistics

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

Acknowledgements

We acknowledge with thanks the support and the constant encouragement that we have received from Prof F.A. Leidenberger and Prof H.M. Schulte for carrying out this work.

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