Similarities and differences in phorbol ester- and luteinizing-hormone-induced desensitization of rat tumour Leydig-cell adenylate cyclase

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The phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) was shown to mimic luteinizing hormone (LH; lutropin) in causing desensitization of LH-mediated cyclic AMP production in tumour Leydig cells. However, there were differences between LH- and TPA-induced desensitization: (1) TPA induced a more rapid effect than LH; (2) adenosine did not inhibit TPA-induced desensitization, whereas it completely inhibited the LH-induced desensitization; (3) adenylate cyclase activity in plasma membranes from TPA-desensitized cells was not decreased, whereas similar preparations from LH-desensitized cells lost their response to LH and to LH plus guanosine 5'-[β -imido]triphosphate; TPA-, but not LH-, treated cells had a decreased capacity to respond to cholera toxin and forskolin. These results indicate that LH and phorbol esters induce desensitization of adenylate cyclase in rat tumour Leydig cells by different mechanisms.

INTRODUCTION

There have been several reports that phorbol esters decrease hormone-stimulated cyclic AMP production (Heyworth *et al.*, 1984; Kassiss *et al.*, 1985; Kelleher *et al.*, 1984; Rebois & Patel, 1985; Sibley & Lefkowitz, 1985; Sibley *et al.*, 1984). Furthermore, they have been shown to mimic hormone-induced desensitization of adenylate cyclase in the same cell systems, i.e. exposure of the cells to phorbol ester decreases the subsequent response in the presence of the hormone. Sibley *et al.* (1984) and Kelleher *et al.* (1984) have demonstrated that during desensitization phorbol esters increase the phosphorylation of the β -adrenergic receptor in turkey and duck erythrocytes respectively. These studies indicated that the phorbol esters may act in a similar way to the appropriate hormone via stimulation of protein kinase C-mediated phosphorylation of the receptor, thus causing it to uncouple from the adenylate cyclase system, i.e. this may be the mechanism of desensitization.

The effects of phorbol esters on steroidogenesis have been investigated in mouse (Mukhopadhyay *et al.*, 1984) and rat Leydig cells (Themmen *et al.*, 1986a,b; Nikula *et al.*, 1987). It was found that they produced a small stimulation of steroidogenesis when added alone, but an inhibition of submaximally LH-stimulated steroidogenesis (but not dibutyryl cyclic AMP-stimulated steroidogenesis; Themmen *et al.*, 1986b). In the normal testis and tumour Leydig cell, studies have been carried out in our laboratory into the mechanism of desensitization and on the internalization and recycling of the LH receptor (Dix & Cooke, 1981, 1982; Dix *et al.*, 1982, 1984; Habberfield *et al.*, 1986). In the studies reported here, and similarly to the above, we have also found that phorbol esters mimic LH-induced desensitization in the tumour Leydig

cell. While the present studies were in progress, Rebois & Patel (1985) reported similar findings for the mouse tumour Leydig cell. They reported that phorbol esters and LH were similar in all respects in their induction of desensitization. However, we report that, although phorbol esters do mimic LH in some respects, there are important differences which may indicate that different mechanisms are operating. Kassiss *et al.* (1985) have also published evidence that phorbol esters and β -adrenergic agonists mediate desensitization in rat glioma C6 cells by distinct mechanisms. A preliminary account of this work has been presented (Cooke *et al.*, 1986).

MATERIALS AND METHODS

Materials

Sheep LH (NIH S20; 1.9 i.u. NIH SI/mg) and human choriogonadotropin (hCG) (NIH CR115) were gifts from the Endocrinology Study Section, National Institutes of Health, Bethesda, MD, U.S.A. Hepes, cholera toxin, bovine serum albumin (fraction V) and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) were purchased from Sigma Chemical Co. Forskolin (7 β -acetoxy-8,13-epoxy-1 α ,6 β ,9 α -trihydroxy-11,14-dione; M_r 410) was purchased from Calbiochem. Percoll was purchased from Pharmacia Fine Chemicals, and 3-isobutyl-1-methylxanthine (IBMX) was purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A. RO-201724 [4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone] was a gift from Hoffman-La Roche, and Na¹²⁵I (carrier-free) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Dulbecco's modified Eagle's minimum essential medium, without NaHCO₃, was purchased from Gibco. The hCG and 2-*O*-succinyl-

Abbreviations used: LH, luteinizing hormone (lutropin); TPA, 12-*O*-tetradecanoylphorbol 13-acetate; p[NH]ppG, guanosine 5'-[β -imido]triphosphate; hCG, human choriogonadotropin; IBMX, 3-isobutyl-1-methylxanthine.

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Fig. 1. Effect of time of pretreatment with LH or TPA on the subsequent LH-stimulated cyclic AMP production

Tumour Leydig cells (100000 cells/incubation) were preincubated at 32 °C for 0, 5, 10, 20 or 40 min with either TPA (10 ng/ml) or LH (10 ng/ml), washed three times, and incubated for 1 h at 32 °C in media containing IBMX (0.5 mM) in the presence of LH (1 µg/ml). The cyclic AMP content was then determined as described in the Materials and methods section. Results, expressed as pmol/h per 10⁶ cells, are means ± S.D. (represented by the bars) for duplicate determinations on triplicate samples.

Fig. 2. Effect of preincubation with LH or TPA on cyclic AMP production by rat tumour Leydig cells

Tumour Leydig cells (100000 cells/incubation) were preincubated with various concentrations of LH or TPA for 30 min at 32 °C, washed three times, and incubated for 1 h at 32 °C in media containing IBMX (0.5 mM) in the absence (basal) or presence of LH (1 µg/ml). The cyclic AMP content was determined as described in the Materials and methods section. Results are means ± S.D. (represented by the bars) for duplicate determinations on triplicate samples.

tyrosine methyl ester derivative of cyclic AMP were radioiodinated as previously described (Dix & Cooke, 1981).

Preparation of tumour Leydig cells

The Leydig tumour used for these studies has been previously characterized (Cooke *et al.*, 1979). It was grown in Fischer rats, and the cells were isolated and purified on Percoll density gradients as previously

described (Dix *et al.*, 1984). Cells with a density of 1.070 g/ml were used in all studies.

Cell incubations

The purified Leydig cells were suspended in Dulbecco's modified Eagle's minimum essential medium, plus 0.1% (w/v) bovine serum albumin and 10 mM-Hepes, pH 7.4. In preincubation experiments, cells were incubated with medium either alone or containing LH or TPA, at the

Fig. 3. Effect of preincubation with TPA on the LH-, cholera-toxin and forskolin-stimulated cyclic AMP production by rat tumour Leydig cells

Tumour Leydig cells (100000 cells/incubation) were incubated with either media alone (□) or TPA (10 ng/ml; ▨) for 30 min at 32 °C, washed three times, and incubated for 2 h at 32 °C in media containing IBMX (0.5 mM) alone or in the presence of LH (1 µg/ml), cholera toxin (CT; 5 µg/ml) or forskolin (F; 100 µM). The cyclic AMP content was determined as described in the Materials and methods section. Results are means ± S.D. (represented by the bars) for duplicate determinations on triplicate samples. The basal productions of cyclic AMP were not detectable.

concentrations indicated in the Figure legends, in a shaking water bath at 32 °C. After preincubation the cells were washed three times by centrifugation (200 g for 10 min) and then resuspended in fresh medium. To inhibit phosphodiesterase activity, the incubation media contained RO-201724 when adenosine was present (Dix *et al.*, 1985), otherwise IBMX was added. The incubations were carried out for various times with various additions as indicated in the Figure legends. At the end of the incubation periods, cyclic AMP was extracted from the cells by the addition of HClO₄ (final concn. 0.5 M) and

then neutralized with K₃PO₄ (final concn. 0.24 M). Cyclic AMP was measured in the total medium and cell extracts by radioimmunoassay by the method of Steiner *et al.* (1972), modified by the acetylation procedure described by Harper & Brooker (1975).

In experiments where ¹²⁵I-hCG binding was determined, a single-concentration saturation assay was used, the details of which have been described previously (Dix & Cooke, 1981).

In experiments where adenylate cyclase activity was determined, plasma membranes were purified from Leydig cells and adenylate cyclase was assayed as previously described (Dix *et al.*, 1982).

Membrane protein was determined by the method of Lowry *et al.* (1951), with the modification described by Markwell *et al.* (1978), and with bovine serum albumin as a standard.

RESULTS

As reported previously (Dix *et al.*, 1982, 1984), incubation with LH causes a desensitization of tumour Leydig cells, which results in a decreased cyclic AMP production in response to a second challenge with LH. This desensitization is dependent on time (Fig. 1) and the amounts (Fig. 2) of LH added in the first incubation. Exposure of the cells to TPA in place of LH also caused a time- and dose-dependent desensitization of LH-stimulated adenylate cyclase (Figs. 1 and 2). The TPA effect was more rapid than that of LH (Fig. 1); maximum effects were found within 5 min, whereas LH took 40 min to reach the same values. With LH as well as TPA, desensitization was not complete; approx. 30% of the total activity could still be activated by LH in the second incubation. Similar timed experiments in which parallel incubations were carried out with an inactive phorbol ester (phorbol 12,13-didecanoate) in addition to LH and TPA showed that, in contrast with the latter, the cells were not desensitized when challenged with LH. The effects of TPA were not via increases in phosphodiesterase activity, because IBMX (an inhibitor of this enzyme) was present during all the incubations.

In order to determine the site of desensitization, the effect of TPA on cholera-toxin- and forskolin-stimulated cyclic AMP production was studied. In the presence of TPA (10 ng/ml) the LH-stimulated cyclic AMP production was decreased by 53% (Fig. 3), compared with 26% and 24% for cholera-toxin- and forskolin-stimulated cyclic AMP production respectively.

Previous work showed that adenosine potentiates LH-stimulated cyclic AMP production and prevents LH-induced desensitization (Dix *et al.*, 1985). The effect of adenosine on TPA-induced desensitization was therefore investigated. The effects of various concentrations of TPA in the presence of three different concentrations of adenosine on LH-stimulated cyclic AMP production were determined (Fig. 4). In the absence of adenosine, the typical effect of TPA on LH-stimulated cyclic AMP production was found. In the absence of TPA there was a potentiation by adenosine of LH-stimulated cyclic AMP production. However, adenosine did not prevent the desensitizing effects of TPA at any of the doses.

Plasma membranes prepared from LH-desensitized Leydig cells do not respond to LH, nor does LH potentiate the effect of the non-hydrolysable GTP

Fig. 4. Effect of TPA in the presence and absence of adenosine on LH-stimulated cyclic AMP production in rat tumour Leydig cells

Tumour Leydig cells (100000/incubation) were preincubated with TPA at various concentrations for 30 min at 32 °C in media containing RO-201724, and then for 1 h in the presence of adenosine (50 μM; Δ), LH (1 μg/ml; ○) or LH (1 μg/ml) plus adenosine (50 μM, ■; 10 μM, □; 5 μM, ●). The cyclic AMP content was determined as described in the Materials and methods section. Results represent means ± S.D. (represented by the bars) for duplicate determinations on triplicate samples.

analogue p[NH]ppG on adenylate cyclase activity (Dix *et al.*, 1982) (Fig. 5). However, in plasma membranes prepared from TPA-treated cells, the normal response to LH was found, and LH did potentiate the effects of p[NH]ppG, i.e. the desensitization induced by TPA in intact cells is reversed by homogenization and preparation of plasma membranes.

Finally, the effect of TPA on the number of binding sites for hCG was determined. The cells were incubated with medium alone or medium plus LH or TPA for 2 h at 32 °C. The binding of ¹²⁵I-hCG was then determined after a further 2 h incubation in the presence of this labelled ligand. In the cells incubated with medium alone, 8.4 ± 0.72 fmol of ¹²⁵I-hCG/10⁶ cells was bound. TPA

Fig. 5. Effect of preincubation of rat tumour Leydig cells with LH and TPA on the adenylate cyclase activity of purified plasma membranes

Tumour Leydig cells were incubated for 2 h at 32 °C in the presence of media alone, or with LH (1 μg/ml) or TPA (100 ng/ml). Cells were then washed and plasma membranes prepared, and the basal (B; ■), LH- (1 μg/ml; ▨) p[NH]ppG- (0.1 mM; G; □), LH-(1 μg/ml) + p[NH]ppG- (0.1 mM, ▤) and NaF-(10 mM, ▩) stimulated cyclic AMP production was determined as previously described (Dix *et al.*, 1982). Results are means ± S.D. (represented by the bars) for triplicate determinations.

had no effect on the binding (7.38 ± 1.29 fmol of ¹²⁵I-hCG bound/10⁶ cells), whereas a lower binding was found in the LH-treated cells (2.64 ± 0.09 fmol/10⁶ cells); this was due either to occupancy of the receptor sites with LH or to LH-induced down-regulation of the LH receptors.

DISCUSSION

In agreement with studies with other hormones, and also with those of Rebois & Patel (1985) on mouse tumour Leydig cells and LH, it has been found that TPA, like LH, will cause a decrease in LH-stimulated cyclic AMP production in rat tumour Leydig cells and also desensitize the cells after LH stimulation. However, there are differences between TPA- and LH-induced desensitization. (1) The time taken to induce desensitization is much shorter for TPA (5 min, compared with 30–40 min for LH). (2) The TPA-induced desensitization, unlike LH-induced desensitization, is not prevented by addition of adenosine. (3) Adenylate cyclase activity in plasma membranes from TPA-desensitized cells is not decreased, whereas similar preparations from LH-desensitized cells lose their ability to respond to LH and LH plus p[NH]ppG. (4) TPA-induced cells also have a decreased capacity to respond to cholera toxin and forskolin. No such changes were observed with LH-treated cells (Dix *et al.*, 1982, 1985). These results point to different mechanisms for LH- and phorbol-ester-induced desensitization. It is established that phorbol esters activate

protein kinase C in many cell types, including Leydig cells (Mukhopadhyay *et al.*, 1984; Themmen *et al.*, 1986a,b; Nikula *et al.*, 1987); however, activation of other pathways by TPA cannot be excluded.

With respect to the mechanism of LH-induced desensitization, it was found in previous studies that the lesion probably occurred at the level of the receptor interaction with the GTP regulator protein (N_s) (Dix *et al.*, 1982, 1984), i.e. the receptor and/or part of N_s that interacts with the LH receptor is modified so that activation of N_s is decreased. The ability of N_s to activate the adenylate cyclase remained intact, because effects of cholera toxin, forskolin and p[NH]ppG were unaltered.

The results of this study on the effects of treatment of intact Leydig cells with TPA or LH on subsequent LH-stimulated cyclic AMP production are essentially in agreement with those of Rebois & Patel (1985) on cells from a mouse tumour Leydig cell line, i.e. they both caused a time- and dose-dependent desensitization, and to the same degree. Also TPA did not cause a decrease in the numbers of hCG receptors, i.e. this is not the mechanism of its action. However, Rebois & Patel (1985) reported that TPA or hCG treatment of cells had similar inhibitory effects on the ability of membrane adenylate cyclase to respond to p[NH]ppG and NaF. Furthermore, these changes were not always consistent, but they did occur in parallel for TPA and hCG. This is clearly different from the present study. Even with LH we have not observed a decrease in p[NH]ppG or NaF responses in plasma-membrane adenylate cyclase (Dix *et al.*, 1982); this would seem to indicate a different type of desensitization in the mouse tumour cells, i.e. it has the characteristics of heterologous desensitization. However, it would be expected that a decrease in response to forskolin would have been obtained (Dix *et al.*, 1984), which was not the case (Rebois & Patel, 1985).

In the studies of Kassis *et al.* (1985), exposure of rat glioma C6 cells to either isoprenaline or TPA caused a desensitization of isoprenaline-stimulated adenylate cyclase activity and a decrease in the affinity of β -receptors for isoprenaline. N_s was found to be unchanged. Receptors from isoprenaline-treated cells were less active in 'coupling' to foreign adenylate cyclase than were receptors from untreated cells. However, receptors from TPA-treated cells were fully active. These and other results from these workers indicated that β -agonists and phorbol esters desensitize adenylate cyclase by distinct mechanisms. In the present study it was found that plasma membranes isolated from LH-treated cells were unable to respond to LH, whereas TPA-treated cells retained their full response to LH and p[NH]ppG; these results are similar to those of Kassis *et al.* (1985).

It is apparent from available data that, although hormones and phorbol esters are able to induce desensitization, they may have different mechanisms of action, depending on the cell type and hormone used. This is hardly surprising, in view of the wide variation in the degree and mechanism of hormone-induced desensitization.

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