The inhibitory GTP-binding protein (G_i) occurs in rat Leydig cells and is differentially modified by lutropin and 12-*O*-tetradecanoylphorbol 13-acetate

Elizabeth A. PLATTS,* Dennis SCHULSTER† and Brian A. COOKE*‡

*Department of Biochemistry and Chemistry, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF, and †Department of Endocrinology, National Institute for Biological Standards and Control, South Mimms, Herts. EN6 3QG, U.K.

Luteinizing-hormone (LH)-stimulated cyclic AMP production in rat testis Leydig cells was desensitized by both LH and 12-*O*-tetradecanoylphorbol 13-acetate (TPA). However, TPA, but not LH, enhanced the subsequent response to cholera toxin. Treatment of the cells with pertussis toxin potentiated cyclic AMP production in both control and LH-desensitized cells, but did not potentiate further the responses obtained by TPA pretreatment. The results implicate the presence of an inhibitory GTP-binding protein (G_i), which may be inhibited by TPA. The presence of a G_i -like protein within the plasma membrane of Leydig cells was demonstrated by pertussis-toxin-catalysed [32 P]ADP-ribosylation of a M_r -40000–41000 protein.

INTRODUCTION

The control of adenylate cyclase involves at least two GTP-binding membrane proteins, namely G_s and G_i , which respectively mediate stimulation or inhibition of cyclic AMP production by stimulatory and inhibitory agonists (for reviews see Rodbell, 1980; Northup, 1985). In the Leydig cell, stimulation of adenylate cyclase by LH is mediated by the coupling of the LH receptor to adenylate cyclase via a putative G_s protein. The Leydig-cell G_s is presumed to be similar to G_s in other tissues, because Leydig-cell adenylate cyclase is activated by cholera toxin and forskolin in whole cells and by NaF and non-hydrolysable GTP analogues in membrane preparations (Dix *et al.*, 1982; Levi *et al.*, 1982). Whether the control of adenylate cyclase by LH is also mediated by G_i is not known. However, the presence of G_i in rat Leydig cells has been inferred from other studies (Adashi *et al.*, 1984; Khanum & Dufau, 1986).

Interactions between different transducing systems, i.e. adenylate cyclase and protein kinase C, have been reported in both mouse and rat Leydig cells (Mukhopadhyay & Schumacher, 1985; Themmen *et al.*, 1986; Dix *et al.*, 1987) and in many other cell types (Nishizuka, 1984). Treatment with the phorbol ester TPA can mimic hormone-induced desensitization, and has been demonstrated both in tumour Leydig cells (Dix *et al.*, 1987; Rebois & Patel, 1985) and in other cell types, e.g. avian erythrocytes (Sibley *et al.*, 1984). However, differences between hormone- and TPA-induced desensitization have also been noted (Dix *et al.*, 1987, and further references cited therein).

In the studies reported here, using testis Leydig cells, it was found that, although both LH and TPA induced desensitization of adenylate cyclase, there were marked differences between the effects of these compounds on adenylate cyclase activity subsequently stimulated by

cholera toxin. This effect may be due to either a modification of G_s such that activity is increased, or to an inhibitory effect on the putative G_i protein. These differential effects of LH and TPA were therefore investigated further by using pertussis toxin (which ADP-ribosylates and hence inactivates G_i) to examine whether (a) G_i protein is present and (b) this protein is inhibited by TPA. In addition, the proteins ADP-ribosylated in the presence of pertussis toxin were investigated by SDS/polyacrylamide-gel electrophoresis. A preliminary report of this work has been presented (Platts *et al.*, 1987).

EXPERIMENTAL

Materials

Ovine LH (oLH) was obtained from the Endocrinology Study Section, NIH, Bethesda, MD, U.S.A. Pertussis toxin was purchased from Porton Products Ltd., Porton Down, Salisbury, Wilts., U.K. Cholera toxin, TPA and PDD were from Sigma Chemical Co., Poole, Dorset, U.K. [32 P]NAD $^{+}$ (1000 Ci/mol) was purchased from New England Nuclear, DuPont (U.K.) Ltd., Stevenage, Herts. U.K., and autoradiography film was X-Omat S from Kodak. Acrylamide and bisacrylamide (Electran) were bought from BDH, Poole, Dorset, U.K. Dulbecco's modified Eagle's minimum essential medium (DMEM) was purchased from GIBCO.

Cell and membrane preparations

Rat testis Leydig cells were obtained from 200–250 g Sprague–Dawley rats by collagenase dispersion, as previously described (Aldred & Cooke, 1983). Leydig cells of density 1.070 g/ml were partially purified by using 0–90% linear Percoll gradients, or, where the cells were used for membrane preparations, they were purified by a combination of centrifugal elutriation (Aquilano &

Abbreviations used: G_s and G_i , stimulatory and inhibitory GTP-binding proteins controlling adenylate cyclase; LH, luteinizing hormone (lutropin); oLH, ovine LH; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PDD, phorbol 12,13-didecanoate.

‡ To whom correspondence should be addressed.

Dufau, 1984) and density-gradient centrifugation. The crude Leydig-cell suspension was resuspended in Dulbecco's Modified Eagle's Medium with 0.5% bovine serum albumin, 10 mM-Hepes, 25000 i.u. of heparin/l, 20 mg of DNAase/l and 100 i.u. of kanamycin/l. The cells were loaded into the elutriation chamber of the elutriator (Beckman JE-6B) at a rotor speed of 2000 rev./min and a flow rate of 30 ml/min. A Leydig-cell-enriched fraction was eluted at a flow rate of 35–50 ml/min. These cells were then further purified on a Percoll gradient as described by Aldred & Cooke (1983), and two bands of cells were obtained; the lower band contained cells of density 1.070 g/ml, which were approx. 98% pure Leydig cells.

Plasma membranes were prepared from elutriator- and Percoll-purified cells by the method described by Dix & Cooke (1982). Briefly, cells were homogenized in a Wheaton-Dounce homogenizer, and plasma membranes were purified from the homogenate by using a poly(ethylene glycol)/Dextran two-phase system (Brunette & Till, 1971). Membrane protein was determined by the method of Lowry *et al.* (1951) as modified by Markwell *et al.* (1978), and with bovine serum albumin as a standard.

Cell incubations

Cells were suspended in sterile Dulbecco's modified Eagle's medium with 0.1% albumin, 100 i.u. of kanamycin/ml, 0.5 mM-isobutylmethylxanthine, 10 µg of epidermal growth factor/l, 5 mg of transferrin/l and 100 i.u. of insulin/l, and plated out into 24-well Costar culture plates. The cells were then allowed to attach by incubating the cells at 32 °C for 1 h. In experiments which included pertussis toxin (10 ng/ml) the cells were cultured for an extra 23 h.

Desensitization of the cells was achieved by the addition of oLH, TPA or medium alone for 1 h at 32 °C. The spent medium was then removed, and the cells were washed three times. Fresh medium was added, and the cells were incubated for 1 h further in the presence or absence of oLH or cholera toxin. The incubations were

stopped by addition of HClO₄ (final concn. 0.5 M) and neutralized with K₃PO₄ (final concn. 0.27 M). Cyclic AMP content was measured by radioimmunoassay by the method of Steiner *et al.* (1972), modified by the acetylation procedure of Harper & Brooker (1975).

ADP-ribosylation of plasma-membrane proteins

Pertussis toxin was activated by incubation at 37 °C in 25 mM-dithiothreitol for 30 min, after which 0.075% bovine serum albumin was added to act as an internal standard for protein recovery. Plasma membranes were incubated in 60 µl with activated toxin and [³²P]NAD⁺, in accordance with the method of Ribeiro-Neto *et al.* (1985). The reactions were terminated by addition of 1 ml of ice-cold 20% (v/v) trichloroacetic acid. Precipitated protein was dissolved in Laemmli sample buffer and applied to a SDS/10%-polyacrylamide gel (Laemmli, 1970). After electrophoresis the gel was stained with Coomassie Blue R-250, to determine the reproducibility of albumin recovery, then dried and autoradiographed.

RESULTS

Cyclic AMP production from cultured rat testis Leydig cells was stimulated by both oLH and cholera toxin (Fig 1a). Maximum stimulating amounts of LH and cholera toxin were used, but, because cholera toxin has a longer lag time than LH (see Cooke *et al.*, 1977), lower concentrations of cyclic AMP were attained during 1 h incubation. The latter was used to allow comparison of the present results with previous studies; however, the results were essentially similar after 4 h incubations. Pretreatment with oLH for 1 h inhibited, i.e. desensitized, the ability of the cells to respond subsequently to further oLH. In agreement with previous results (Haberfield *et al.*, 1987), it was found that in oLH-treated cells the 'basal' production of cyclic AMP was increased and the addition of further oLH had little effect. When 'basal' values were subtracted, it was found that the response to cholera toxin by oLH-desensitized cells (66.9 ± 8.2 pmol of cyclic AMP/h per 10^6 cells) was not significantly

Fig. 1. Effect of oLH and TPA treatment on rat testis Leydig cells

Cells were pretreated by incubation for 1 h with (a) medium only, (b) oLH (100 ng/ml), (c) TPA (100 nM) or (d) PDD (100 nM). After three washes they were incubated for a further 1 h with medium alone (□), oLH (100 ng/ml; ▨) or cholera toxin (10 µg/ml; ▤). Cyclic AMP content was determined as described in the Experimental section. Results are means $\pm$ S.E.M. for duplicate assays of duplicate incubations from two different experiments.

Fig. 2. Effect of pertussis toxin on control, LH- and TPA-desensitized cells

Leydig cells were cultured with (b, d, f) or without (a, c, e) pertussis toxin (10 ng/ml) for 24 h and desensitized by incubation with medium (a, b), oLH (100 ng/ml; c, d) or TPA (100 nM; e, f) for the last 1 h. After washing, the cells were stimulated with medium (□), oLH (100 ng/ml; ▨) or cholera toxin (10 μ g/ml; ▩). Cyclic AMP was determined as described in the Experimental section. Results are means $\pm$ S.E.M. for duplicate assays of duplicate incubations from three different experiments.

different from that found in the control (76.5 ± 10.0), i.e. oLH did not desensitize the response to cholera toxin.

Pretreatment of the cells with TPA also caused an inhibition of subsequent oLH-stimulated cyclic AMP production, from 188.3 ± 4.8 to 120.6 ± 4.1 pmol/h per 10^6 cells. However, TPA-desensitized cells were found to have an enhanced response to cholera toxin (189.5 ± 8.8 pmol of cyclic AMP/h per 10^6 cells) compared with control cells (76.5 ± 10.0 pmol/h per 10^6 cells) (Fig. 1c). This effect was not due to a decrease in the lag time for cholera toxin to activate maximally adenylate cyclase, because TPA also enhanced cholera-toxin-stimulated cyclic AMP production during a 4 h incubation. These effects were not observed in cells treated with PDD (Fig. 1d), a phorbol ester which does not activate protein kinase C.

To examine whether this effect was due to an interaction of TPA with the putative G_i protein, cells were incubated in the presence of pertussis toxin before being desensitized. In preliminary experiments (results not shown) it was found that pertussis toxin had little or no effect during incubations of the cells for 2–4 h. Longer incubations, of up to 24 h, were therefore carried out. The cells cultured for 24 h were found to respond to oLH and cholera toxin to a similar degree as freshly isolated cells, and the effects of both oLH and TPA were the same after 24 h culture as were observed after 2 h culture (Figs. 2a, 2c and 2e).

Pertussis toxin potentiated cyclic AMP production in both basal and stimulated cells (Fig. 2b). Pertussis toxin also potentiated oLH- and cholera-toxin-stimulated cyclic AMP production in oLH-desensitized cells; however, the effect of oLH on pertussis-toxin-treated oLH-desensitized cells was still found to be less than the effect of oLH on pertussis-toxin-treated non-desensitized cells (Figs. 2b and 2d). Thus inactivation of G_i did not fully reverse oLH-induced desensitization.

The effects of TPA were not modified by culture with

pertussis toxin (Figs. 2e and 2f). The fact that pertussis toxin was not able to potentiate further the enhanced response to cholera toxin implied that TPA and pertussis toxin have a common site of action.

In order to confirm that the pertussis-toxin substrate was a membrane protein with the characteristics of G_i , purified plasma membranes were treated with pertussis toxin and [32 P]NAD $^+$, and ribosylated proteins were then examined by SDS/polyacrylamide-gel electrophoresis and autoradiography. A single radioactive band was observed, of apparent M_r 40 000–41 000 (Fig. 3).

DISCUSSION

The rat testis Leydig cell, like the tumour Leydig cell (Dix *et al.*, 1984), is desensitized by LH, giving rise to a refractoriness to LH but not to cholera toxin, suggesting that the lesion giving rise to desensitization lies at or proximal to the LH-receptor- G_s coupling. This locus has also been cited for the corticotropin-induced desensitization observed in rat adrenal cells (Schulster *et al.*, 1984). The testis Leydig cell, however differs from the tumour Leydig cell (Dix *et al.*, 1987) in that TPA enhances the cell's response to cholera toxin. That this effect is not potentiated further by pertussis toxin demonstrates that the difference between LH- and TPA-induced desensitization is probably due to a modification of G_i by TPA, but not by LH. Phosphorylation of G_i and attenuation of G_i activity in platelets and S49 lymphoma cells by TPA has been demonstrated (Katada *et al.*, 1985; Jakobs *et al.*, 1985; Bell & Brunton, 1986). The difference between LH- and TPA-induced desensitization may be that LH brings about homologous desensitization, whereas TPA desensitized in a heterologous manner, i.e. it inhibits G-protein function generally (Sibley & Lefkowitz, 1985).

The stimulation of Leydig-cell adenylate cyclase activity by agonists which couple receptor and adenylate

Fig. 3. Autoradiograph of pertussis-toxin-catalysed ADP-ribosylated rat testis Leydig-cell plasma-membrane proteins

Purified plasma membranes were treated with pertussis toxin (10 $\mu\text{g}/\text{ml}$) and [^{32}P]NAD $^{+}$ as described by Ribeiro-Neto *et al.* (1985). Then 100 μg of membrane protein was subjected to electrophoresis, and the gel was exposed to X-ray film. Lane A, membranes treated with [^{32}P]NAD $^{+}$ and pertussis toxin; lane B, membranes treated with [^{32}P]NAD $^{+}$. M_r values of standard proteins are indicated on the right.

cyclase via G_s is well documented. However, only two previous reports have indicated that G_i may also have a role in the control of Leydig-cell cyclic AMP production. Adashi *et al.* (1984) found that pertussis toxin potentiated human-choriogonadotropin-stimulated steroidogenesis, but they did not assess the effect of the toxin on adenylate cyclase activity. Khanum & Dufau (1986) have suggested that a G_i protein is activated in preference to G_s by low doses of forskolin, thus inhibiting cyclic AMP production. Although this effect could be prevented by pertussis toxin, the effect of pertussis toxin alone was not assessed. The results presented above indicate that testis Leydig-cell plasma membranes do contain a pertussis-toxin substrate which has inhibitory control of adenylate cyclase.

A number of pertussis-toxin substrates have been identified. The M_r values of the α subunits of these G proteins are within the range 39000–42000 (Gilman, 1984; Northup, 1985). The apparent M_r of the protein detected in testis Leydig cells by SDS/polyacrylamide-gel electrophoresis is also within this range. Furthermore, it is shown that, when it is inactivated by pertussis toxin, adenylate cyclase is released from a negative control,

implying that G_i can modify cyclic AMP production (although it is not yet known whether the LH receptor is coupled to G_i).

An alteration of G-protein activity appears to be a common occurrence in desensitized cells. Although it has been found that desensitization induced by either agonist or TPA decreases receptor- G_s coupling and/or G_s activity (Sibley & Lefkowitz, 1985; Dix *et al.*, 1984, 1987), an increase in G_i function has also been observed in MDCK cells (Rich *et al.*, 1984) and hepatocytes (Heyworth *et al.*, 1984) desensitized by glucagon. Where the desensitizing agonist is inhibitory, a decrease in G_i and an increase in G_s was observed (Parsons & Stiles, 1987). In the rat testis Leydig cell it appears that, although both hormone and phorbol ester cause a decrease in the apparent activity of G_s , this desensitization is not solely due to an increase in G_i . Thus, although pertussis toxin enhances cyclic AMP in LH-desensitized cells, it does not abolish desensitization, and TPA actually inhibits G_i itself.

In summary, previous reports which imply the presence of G_i in the control of testis Leydig-cell adenylate cyclase have been supported by the present study. It is concluded that an inhibitory G protein, which can be attenuated by pertussis toxin, modulates the activity of adenylate cyclase. The size of the ribosylated α subunit of this protein is compatible with its being G_i . This G_i protein is inhibited by TPA treatment, but not by LH, and is thus responsible for the differential effects of LH and TPA on responsiveness to cholera toxin.

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