Resensitization of lutropin-desensitized tumour Leydig-cell adenylate cyclase with human erythrocyte membranes

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Purified rat tumour Leydig cells were pretreated with or without lutropin (1 h at 32°C). The plasma membranes were then isolated and the adenylate cyclase activity measured in the presence of freshly prepared or heat-inactivated (1 h at 60°C) human erythrocyte membranes. In plasma membranes from control cells in the presence of heat-inactivated human erythrocyte membranes both guanosine 5'-[β,γ -imido]triphosphate (p[NH]ppG) plus lutropin and NaF caused a 45–50-fold increase in cyclic AMP production over 30 min compared with 12–13 fold p[NH]ppG and 2–3-fold with lutropin alone. In plasma membranes isolated from lutropin-pretreated cells the NaF- and the p[NH]ppG-stimulated cyclic AMP production rates were unchanged, but no effect of lutropin could be demonstrated with or without added p[NH]ppG. However, after mixing lutropin-desensitized Leydig tumour-cell plasma membranes with freshly prepared human erythrocyte plasma membranes, the adenylate cyclase activity in the presence of lutropin, p[NH]ppG, lutropin plus p[NH]ppG and NaF were similar to those of control cell plasma membranes treated in the same manner. The possible mechanisms of this reversal of lutropin-induced desensitization by human erythrocytes are discussed.

Hormone-induced desensitization of adenylate cyclase has been reported for many systems. However, the molecular mechanisms are still not understood. We have studied the desensitizing effects of lutropin *in vitro* by using tumour Leydig cells. It has been demonstrated that lutropin causes a decrease in lutropin-stimulated cyclic AMP production, which is followed by a loss of lutropin receptors (Dix & Cooke, 1981). These effects of lutropin were prevented by addition of the protein-synthesis inhibitor, cycloheximide (Dix & Cooke, 1981). Subsequently it was demonstrated (Dix *et al.*, 1982) that initially after exposure of the tumour Leydig cells to lutropin, although the lutropin-induced cyclic AMP production was decreased, there was no decrease in the cholera toxin-stimulated cyclic AMP production or ^{125}I -labelled human chorionic gonadotropin binding. Also the p[NH]ppG-stimulated adenylate cyclase activity of plasma membranes isolated from these desensitized cells was unaltered, whereas lutropin alone or in the presence of p[NH]ppG had little or no effect. These findings led to the conclusion that the initial lutropin-induced

desensitization was due to a lesion at or proximal to the guanine nucleotide regulatory protein.

Nielsen *et al.* (1980) demonstrated that the guanine nucleotide regulatory protein activity of human erythrocyte membranes can be transferred to S49 *cyc*⁻ cell membranes without prior extraction with detergents or addition of fusogens. It was therefore of interest to see if these erythrocyte membranes could reverse the desensitization of rat tumour Leydig-cell adenylate cyclase. This was investigated by mixing plasma membranes from control and lutropin-desensitized Leydig tumour cells with human erythrocyte membranes. The adenylate cyclase activity of these mixed-membrane preparations was then determined in the presence of combinations of lutropin, p[NH]ppG and NaF.

Materials and methods

Sheep lutropin (NIH; S20 1.9 units of NIH SI/mg) was a gift from the Endocrinology Study Section, National Institute of Health, Bethesda, MD, U.S.A. Na ^{125}I (carrier-free) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Methyl-cellulose, ATP (Sigma; N. A3377), Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic

Abbreviation used: p[NH]ppG, guanosine 5'-[β,γ -imido]triphosphate.

acid], phosphocreatine, creatine phosphokinase (132 units/mg) and bovine serum albumin (fraction V) were purchased from Sigma Chemical Co. p[NH]ppG was purchased from Boehringer, Mannheim, Germany. Percoll was purchased from Pharmacia Fine Chemicals and 3-isobutyl-1-methyl-xanthine was purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A.

The 2-*O*-succinyltyrosine methyl ester derivative of cyclic AMP was radioiodinated and characterized as described by Steiner *et al.* (1972).

The Leydig tumour was grown in Fischer rats and the cells were isolated and purified as previously described (Dix & Cooke, 1981).

Cell incubation and adenylate cyclase assay

The purified Leydig cells were suspended in suspension media [Dulbecco's modified Eagles minimum essential media, 0.1% (w/v) bovine serum albumin, 25 mM-Hepes and 0.1% (w/v) methyl-cellulose (pH 7.4)] and incubated for 2 h at 32°C in a shaking water bath in the presence and absence of lutropin (10 µg/ml). At the end of the incubation period the cells were finally washed twice by centrifugation (200g for 10 min). The cells were finally resuspended in bicarbonate buffer (1.0 mM-NaHCO₃/1.0 mM-MgCl₂, pH 7.0) allowed to swell for 2.5 min and homogenized with five strokes in a Dounce homogenizer with a tight-fitting pestle. After making the homogenate iso-osmotic with respect to sucrose (0.25 M) the plasma membranes were then isolated using a two-phase poly(ethylene glycol)/dextran system, which was originally described by Brunette & Till (1971). The final membrane preparation was resuspended in 10 mM-Tris/HCl buffer (pH 7.5) plus 1.0 mM-dithiothreitol. These Leydig-cell plasma membranes were then mixed with either heat-inactivated (60°C for 1 h) or freshly prepared human erythrocyte membranes at a ratio of approximately 1:10 (Leydig-cell plasma-membrane protein/human erythrocyte membrane protein). The erythrocyte membranes were prepared by the method of Hanahan & Ekholm (1974), with the modification described by Ahkong *et al.* (1980) with four centrifugation steps with subsequent resuspension in 10 mM-Tris/HCl buffer (pH 7.4). The membrane mixtures were kept at 0–4°C until the adenylate cyclase activity was assayed by the method described previously (Dix *et al.*, 1982) with the cyclic AMP radioimmunoassay described by Steiner *et al.* (1972) modified by the acetylation technique described by Harper & Brooker (1975).

Human erythrocyte membranes alone had adenylate cyclase activities of 1.71 ± 1.51 , 1.68 ± 1.37 , 13.48 ± 0.61 , 16.98 ± 1.29 and 120 ± 15.1 pmol/mg of protein per 30 min (means $\pm$ s.d., $n=3$) for basal, lutropin, p[NH]ppG, p[NH]ppG plus lutropin and NaF stimulation respectively, these activities are

relatively small compared with the reconstituted activities of the mixed membranes. These values were subtracted from the respective mixed-membrane values to assess the corresponding Leydig-cell plasma-membrane adenylate cyclase activities. In the heat-inactivated human erythrocyte membranes adenylate cyclase activity was not detectable.

Protein determination

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

Results

The activity of the adenylate cyclase of purified Leydig-cell tumour plasma membranes in the presence of a combination of lutropin, p[NH]ppG and NaF were consistent with previous results (Dix *et al.*, 1982). Lutropin alone caused a 2–3-fold increase in adenylate cyclase activity, whereas p[NH]ppG caused an approx. 45–50 fold increase (Fig. 1a).

Pre-incubation of the Leydig cells with a desensitizing concentration of lutropin (10 µg/ml for 2 h at 32°C) before plasma-membrane preparation abolished the stimulating effect of lutropin alone and its synergistic effect with p[NH]ppG on adenylate cyclase (Fig. 1b). The NaF and the p[NH]ppG responses were not significantly affected (Fig. 1b). These results are again consistent with previous observations (Dix *et al.*, 1982).

Incubation of control Leydig-cell plasma membranes in the presence of freshly prepared human erythrocyte plasma membranes caused a potentiation of lutropin- and p[NH]ppG-stimulated adenylate cyclase activity, although having no significant effect on the p[NH]ppG-plus-lutropin- or the NaF-stimulated activities (Fig. 1c). However, the lutropin-induced desensitization of Leydig-cell plasma membranes was completely reversed in the presence of human erythrocyte plasma membranes (Fig. 1d), so that the adenylate cyclase activities in the presence of lutropin and lutropin plus p[NH]ppG were similar to those of control plasma membranes treated in the same manner (Figs. 1c and 1d). These results are typical of three different experiments.

Discussion

In a previous study (Dix *et al.*, 1982) it was demonstrated that the coupling between the adenylate cyclase moiety and the guanine nucleotide regulatory protein of the lutropin-desensitized Leydig-cell adenylate cyclase was intact and that the lesion lies at or proximal to the coupling of the guanine nucleotide regulatory protein to the lutropin receptor. These conclusions are in agreement with

Fig. 1. Effect of human erythrocyte membranes on the lutropin-desensitized tumour Leydig-cell plasma-membrane adenylate cyclase activity

Purified rat tumour Leydig cells were incubated for 2 h at 32°C in the presence of suspension media (a and b) or lutropin (10 µg/ml; a and b). Cells were then washed and plasma membranes prepared as previously described (Dix *et al.*, 1982). The purified membranes were then mixed with either heat-inactivated (1 h at 60°C) human erythrocyte membranes (a and b) or freshly prepared human erythrocyte membranes (c and d) and the basal (▨) and lutropin-(1 µg/ml) (■), p[NH]ppG-(0.1 mM) (▤), lutropin-(1 µg/ml) plus p[NH]ppG-(0.1 mM) (□) and NaF-(10 mM) (□) stimulated cyclic AMP production determined as described in the Materials and methods section. Results are means ± S.D. for triplicate determinations from one of three similar experiments.

but contain similar amounts of nucleotide regulatory protein activity to other erythrocyte membranes (Nielsen *et al.*, 1980). The activity of the latter can be transferred to S49 *cyc*⁻ membranes, which lack their own regulatory unit (Nielsen *et al.*, 1980). The resensitization of Leydig-cell plasma membranes by human erythrocytes could therefore also be due to a transfer of human erythrocyte guanine nucleotide regulatory protein, which is then able to couple to the lutropin receptor and the catalytic moiety of adenylate cyclase. Such a mechanism is only plausible if the guanine nucleotide regulatory protein is embedded in the cytoplasmic half of the membrane bilayer, but not if it is a transmembrane protein. However, the guanine nucleotide regulatory protein has a small hydrophobic domain (Ross & Gilman, 1980), which is consistent with the suggestion that it is embedded in the cytoplasmic half of the membrane bilayer (Houslay *et al.*, 1981). These physical properties may allow this protein to transfer between membranes. In support of this explanation Enoch *et al.* (1977) have demonstrated that proteins of a similar nature to the guanine nucleotide-binding protein, NADH-cytochrome *b₅* reductase and cytochrome *b₅*, can be transferred between phospholipid vesicles. If such a mechanism is responsible for resensitization, then the lesion in the desensitized membranes would be at the level of the regulatory protein and not the lutropin receptor.

Hunzicker-Dunn *et al.* (1979) have reported that phosphoprotein phosphatase can reverse lutropin-induced desensitization of follicular adenylate cyclase. It is possible that human erythrocyte membranes contain phosphoprotein phosphatase (Imhof *et al.*, 1980). If a phosphorylation of the lutropin receptor or the guanine nucleotide regulatory protein is involved in hormone-induced desensitization, as has been suggested by other workers (Hunzicker-Dunn *et al.*, 1979; Ezra & Salomon, 1980), human erythrocyte membranes may be supplying a phosphoprotein phosphatase, which dephosphorylates either the lutropin receptor or the guanine nucleotide regulatory protein.

Whether one or even both of these mechanisms are correct remains to be elucidated, but whatever the mechanism the present work shows that human erythrocytes can resensitize lutropin-desensitized Leydig-cell adenylate cyclase. These studies also provide another useful approach to the understanding of the molecular events of desensitization.

those of other workers investigating isoprenaline-induced desensitization of S49 lymphoma cells (Iyengar *et al.*, 1981; Green & Clark, 1981).

In the present study it has been demonstrated that human erythrocyte membranes can reverse the lutropin-induced desensitization in purified tumour Leydig-cell plasma membranes. Human erythrocyte membranes have very low levels of catalytic activity,

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