Volume 250, number 2, 425–428 FEB 07307 July 1989

Functional reconstitution of rat ovarian LH/hCG receptor into proteoliposomes

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Received 8 May 1989

Rat ovarian membrane LH/hCG receptor was solubilized in various detergents and reconstituted into proteoliposomes. Upon removal of sodium cholate by active absorption on Bio-Beads SM-2, the functional interaction between receptor and adenylate cyclase was restored. Adenylate cyclase was stimulated by hCG, hCG+GTP or GppNHp and NaF. Reconstituted proteoliposomes bound more ^{125}I-hCG (528 fmol/mg protein) than membrane-bound receptors (384 fmol/mg protein). There was no difference, however, in the relative affinity of reconstituted receptor preparations for hCG.

Reconstitution; Hormone receptor; Sodium cholate; Adenylate cyclase; (Rat ovary)

1. INTRODUCTION

Reconstitution of various membrane proteins into phospholipid vesicles to form functioning membranes provides a powerful tool for studying the structure and function of biological membranes. Incorporation of receptors into model membranes has extended our knowledge on the role of membrane lipids, in coupling proteins and adenylate cyclase in the signal transducing system of β-adrenergic [1,2], nicotinic acetylcholine [3], insulin [4] and FSH receptors [5]. Occupancy of LH/hCG receptors leads to the activation of adenylate cyclase which is linked with GTP-binding protein G_s [6]. However, the functional reconstitution of LH/hCG receptor with adenylate cyclase has not been reported as yet. Here, we describe the spontaneous insertion of this receptor into vesicles with subsequent restoration of functional binding activity.

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2. MATERIALS AND METHODS

Purified hCG (CR 123; 12 780 U/mg) was generously supplied by NIAMDD, (NIH, Bethesda, MD). Na ^{125}I was purchased from the Radiochemical Centre (Amersham). Bio-Beads SM-2 were from Bio-Rad and methanol-activated prior to use [7]. Creatine phosphate, creatine kinase and GppNHp were obtained from Boehringer-Mannheim. All other chemicals were from Sigma.

Membrane preparations were obtained from superovulated rat ovaries (Wistar strain, aged 26 days) 6 days after PMSG and hCG priming [8] as described [9,10]. To solubilize gonadotropin receptor, 20 mg membrane proteins were stirred with 1 ml of 1% detergent at 4°C for 30 min in buffer A (25 mM Tris-HCl, 0.1 mM EDTA; pH 7.4). The solution was then centrifuged at 20 000 × g for 30 min. Solubilized membrane protein was applied to a Bio-Beads SM-2 column (1 × 8 cm), previously equilibrated with buffer A to remove detergent. The same buffer was used for elution. After 5-fold dilution the turbid fraction containing proteoliposomes was centrifuged at 160 000 × g for 60 min [11].

Adenylate cyclase was assayed at 30°C for 25 min [12]. The assay system contained 25 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 0.3 mM 3-isobutylmethylxanthine, 1 mM ATP, 20 mM phosphocreatine, 100 U/ml creatine kinase and 30–40 μg protein of proteoliposomes or membrane preparations in a final volume of 400 μl. When present, GTP, GppNHp, NaF and hCG were at 0.1 mM, 0.1 mM, 10 mM and 0.1 μg/assay, respectively. The assay was terminated by heating to 100°C for 3 min, followed by addition of 0.1 ml ice-cold 30% trichloroacetic acid. After centrifugation at 1500 × g for 15 min, the supernatant was extracted three times with water-saturated

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00145793/89/$3.50

diethyl ether and lyophilized. The residue was dissolved in water and the cAMP content estimated by protein binding assay as in [13].

In hCG binding assays, 0.1-ml aliquots of membrane preparations or proteoliposomes were incubated for 16 h at 24°C with 0.1 ml PBS (50 mM phosphate buffer, 15 mM NaCl; pH 7.4) + 1 mg/ml BSA with or without a 100-fold excess of unlabeled hCG and 0.1 ml ^{125}I-hCG (1–1.5 ng, spec. act. ~ 2.3 TBq/g). After incubation and centrifugation, membrane pellets were washed twice with PBS buffer [9]. Hormone-receptor complex in proteoliposomes was precipitated twice with polyethylene glycol [14]. Results are expressed as specific binding per mg protein.

Cholesterol was assayed enzymatically [15]. Phospholipids were determined colorimetrically (as dipalmitoylphosphatidylcholine) in complexes with ammonium ferrothiocyanate [16]. Triton X-100 was estimated from the absorbance at 275 nm. Protein was determined by the method of Lowry et al. [17]. Student's t-test was used for statistical evaluation.

3. RESULTS AND DISCUSSION

This study has shown that LH/hCG receptor solubilized from rat ovaries, which contain both endogenous membrane protein and lipid, can be incorporated into proteoliposomes. Proteoliposomes were formed as soon as the detergent had been removed by absorption into Bio-Beads SM-2. The procedure of proteoliposome formation in the absence of metal ions and presence of 1 mM EDTA is known to produce unilamellar vesicles [18]. Passing a detergent through a resin column results in almost complete removal of the detergent (fig.1). ^{125}I-hCG binding activity was coeluted with protein and phospholipids in a single peak. From data reported by Moriyame et al. [19] and Rigaud et al. [20] it is clear that Bio-Beads SM-2 can absorb not only Triton X-100 but also any type of detergent, irrespective of the chemical structure of the hydrophilic and hydrophobic moieties of the detergent molecules. Although the properties of detergents, their function during membrane solubilization and behavior of the solubilized membrane proteins have been described [2,10], it appears that a suitable detergent has to be found empirically for each receptor system. As is obvious from fig.2, the highest specific binding of ^{125}I-hCG to proteoliposomes was observed with Triton X-100-solubilized receptor. As estimated from NaF stimulation, proteoliposomes prepared by solubilization of membrane protein with any detergent contain G_s protein and show adenylate cyclase activity. However, except for sodium

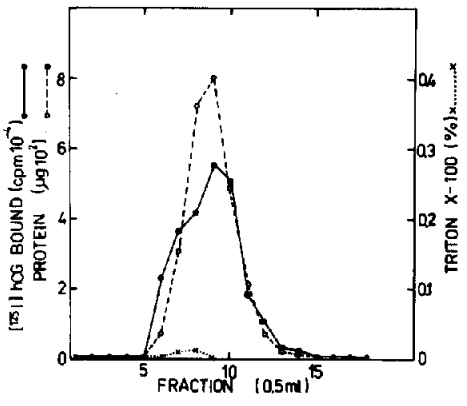

Fig.1. Elution profile of reconstituted receptor preparations. Rat ovarian membrane fraction was solubilized in 1% Triton X-100 and applied to the top of a Bio-Beads SM-2 column equilibrated in buffer A at 4°C. Fractions (0.5 ml) were collected, and ^{125}I-hCG specific binding, and protein and Triton X-100 concentrations were estimated.

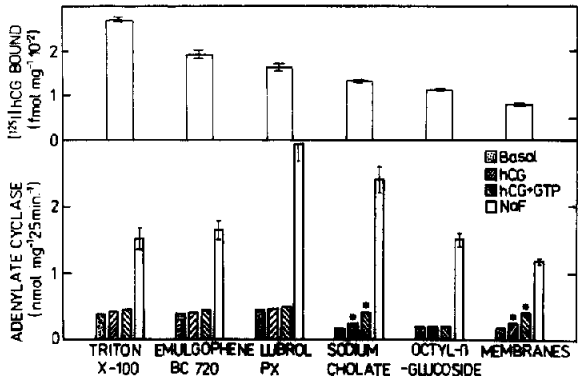

Fig.2. Effect of various detergents on the formation of proteoliposomes. Detergents were removed using Bio-Beads SM-2. Specific binding of ^{125}I-hCG and the extent of adenylate cyclase activation in proteoliposomes and membrane preparations were assayed. Asterisks indicate statistically significant differences ($p<0.01$) from basal values. Data represent means ± SE of four estimations (repeated twice).

cholate preparations ($p<0.01$), the adenylate cyclase system is not coupled to the LH/hCG receptor. This low efficiency of interaction of the receptor with adenylate cyclase may be due, at least partly, to detergent residues after reconstitution. Lubrol-PX, which is the most efficient detergent for extraction of adenylate cyclase, was found to be a strong uncoupler of the binding of β-receptor to G_s protein [11]. On the other hand, small amounts of sodium cholate do not influence

stimulation of adenylate cyclase activity [21]. Sodium cholate an unavoidable choice, since this is the only known detergent in which ovarian adenylate cyclase can be solubilized in an active form and in which it is functionally coupled with G_s protein and LH/hCG receptor in proteoliposomes (fig.3).

The gonadotropin binding properties of proteoliposomes are comparable with those of membrane preparations. A typical saturation curve for ^{125}I-hCG in both systems is shown in fig.4. Scatchard analysis of hCG specific binding to proteoliposomes indicated a single class of high-affinity binding sites with a value of $K_a = 1.5 \times 10^9$ M^{-1} similar to that calculated for membrane-bound receptors ($K_a = 1.3 \times 10^9$ M^{-1}). Reconstitution apparently is not associated with any alterations in relative affinity of the receptor for hCG; however, there are differences in the maximal number of binding sites. The hCG binding capacity of proteoliposomes was apparently higher (528 fmol/mg protein) than that of the membrane-bound receptor (384 fmol/mg protein). The increased receptor numbers in proteoliposomes suggest that receptor accessibility may be modulated to some extent by the structure of the adjacent phospholipid bilayer. In previous work on the LH/hCG receptor in gonadal membranes, the phospholipase-induced loss of phospholipids was found to decrease hCG binding to the receptor

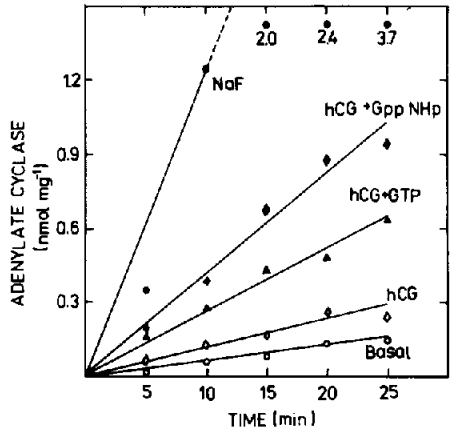

Fig.3. Time course of adenylate cyclase activity of proteoliposomes reconstituted from sodium cholate extracts. Adenylate cyclase was determined in the absence of stimulants (basal activity) and presence of hCG (0.1 μg), GTP (0.1 mM), GppNHp (0.1 mM) and NaF (10 mM). Means of three estimations (experiments repeated twice) are shown.

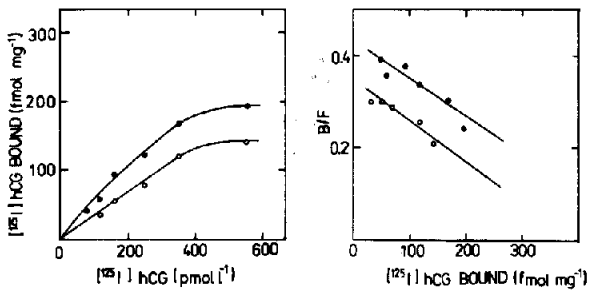

Fig.4. Scatchard analysis of binding data for proteoliposomes (●) and membrane preparations (○). Saturation curves for specific binding were obtained in the presence of increasing concentrations of ^{125}I-hCG. Experiments were run in duplicate. Results are representative of three independent experiments.

[22,23]. In these experiments the phospholipid concentrations in proteoliposomes were 3-times higher than in membrane preparations (1104.0 ± 0.02 vs 350.6 ± 0.01 nmol/mg protein, respectively; $n = 4$, $p < 0.001$). Another factor which may influence the efficiency of hCG binding is cholesterol. This is a major lipid constituent of cell membranes and significantly higher levels were observed in proteoliposomes than in membranes [total cholesterol: 424.0 ± 14.8 (proteoliposomes), 186.3 ± 5.4 nmol/mg protein (membranes); cholesterol esters: 129.2 ± 2.7 (proteoliposomes), 89.1 ± 4.4 nmol/mg protein (membranes); $n = 4$, $p < 0.001$]. Thus, an alternative explanation for the increased binding capacity in proteoliposomes would be that reconstitution restores LH/hCG receptors present in the membrane in a cryptic form. Moreover, the increased level of cholesterol may facilitate exposure of this receptor. Evidence for the stimulatory effect of cholesterol esters on the accessibility of LH/hCG receptors in gonadal membranes has been provided earlier [10].

Acknowledgement: This investigation received financial support from the WHO Special Programme of Research, Development, and Research Training in Human Reproduction.

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