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Effects of partial delipidation of rat ovarian membranes on thermal stability of LH/hCG receptors

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Abstract

The role of lipids and of possible structure-functional properties of the LH/hCG receptor were analyzed by thermal perturbation techniques in its native membrane environment. A method for the reversible removal of lipids from membranes with a mild detergent Tween 20 was developed. The receptor was reactivated with phosphatidylcholine (PC) by its reconstitution into proteoliposomes. The heat inactivation profile of LH/hCG binding sites in delipidated membranes was shifted to a temperature lower by approx. 8 C° (T_{50} values). Thermal inactivation of the delipidated LH/hCG receptor was found to be a quick process. Occupation of receptor binding sites by the agonist before thermal perturbation induced stabilization of the receptor. Thermal inactivation of the receptor by delipidation was fully reversed by treatment with soybean PC, dioleoylphosphatidylcholine (DOPC) and dipalmitoylphosphatidylcholine (DPPC), partly with phosphatidylethanolamine (PE) and sphingomyelin (SpM), but not with phosphatidylserine (PS), phosphatidylglycerol (PGl) or cholesterol. Delipidation modified the differential scanning calorimetric profile characteristic of control membranes. Delipidation of ovarian membranes also increased membrane lipid rigidity. The addition of PC, DOPC and PS to delipidated membranes decreased, that of DPPC and SpM increased, while PGl did not change the degree of fluorescence polarization of DPH, suggesting that membrane lipid fluidity was not involved in the stabilizing action of specific phospholipids against thermal inactivation of the ovarian LH/hCG receptor.

Key words: LH/hCG receptor; Delipidation; Thermal inactivation; Membrane fluidity; Reconstitution

1. Introduction

Biological membranes are dynamic structures containing a variety of components capable of influencing each other. Integral proteins, which are responsible for numerous functional interactions in the membrane, are embedded either wholly or partially in a fluid lipid bilayer. The role of lipids in membranes is not passive since the lipids may regulate many cellular functions. The cell receptor for LH/hCG is thought to be an integral protein containing seven transmembrane segments [1] and it seems likely that it interacts with lipids in the membrane. Such interaction might be required for the conformation changes of receptor after binding of hormone and transmission of the signal across the membrane. The requirement of specific lipids for a receptor system is still an area of active investigation. To gain insight into the role of lipids in receptor function in native membrane, a delipidated receptor would provide optimal conditions for studying the relevant properties after addition of specific lipids. The methods of removal of lipids from membranes include the use of organic solvents, phospholipases or detergents. Hydrolysis of the phospholipids by phospholipase C or A_2 diminished phospholipid concentration and inactivated the membrane-bound LH/hCG receptor in a dose dependent manner [2,3]. The decrease of the accessibility of LH/hCG receptor was associated

Abbreviations: Tween 20, polyoxyethylene(20)sorbitan monolaurate; DPH, 1,6-diphenyl-1,3,5-hexatriene; DSC, differential scanning calorimetry; C, control; D, delipidation; Ch, cholesterol; Ch-S, cholesteryl hemisuccinate; PC, L-α-phosphatidylcholine; DOPC, dioleoyl-L-α-phosphatidylcholine; DPPC, dipalmitoyl-L-α-phosphatidylcholine; PE, L-α-phosphatidylethanolamine; PS, L-α-phosphatidyl-L-serine; PGl, L-α-phosphatidyl-DL-glycerol; SpM, sphingomyelin.

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with changes of dynamic properties of the lipid matrix. However, removal of lipids by detergents appears to be preferable, since the cleavage product after phospholipase digestion can be toxic to cell membranes [4]. Moreover, methods involving several different detergents have been most widely and successfully applied to other types of membrane proteins [5,6].

This paper reports the effect of partial delipidation of rat ovarian membranes with a mild detergent Tween 20 on the accessibility of LH/hCG receptors. Removal of lipids produced a receptor protein in a state which can be reactivated by the addition of exogenous phospholipids. Using the given samples, we analyzed possible structure-functional relationship of the LH/hCG receptor by thermal perturbation techniques in its native membrane environment [7].

2. Materials and methods

2.1. Materials

Purified hCG (CR 123, 12780 IU mg^{-1}) was generously supplied by NIAMDD, NIH, Bethesda. $Na^{125}I$ was purchased from the Radiochemical Center, Amersham. Pregnant mare's serum gonadotropin (PMSG) and hCG (Praedyn) were from Spofa, Prague. Bio-Beads SM-2 were from Bio-Rad, and they were methanol-activated prior to use [8]. Tween 20, DPH, Ch, Ch-S, PC type III-S from soybean, PGl from egg yolk, PE type III-A from egg yolk, PS from bovine brain, SpM from bovine brain, DOPC, DPPC and all other chemicals were from Sigma.

2.2. Methods

Luteinized ovaries were produced in 25-day-old rats (Wistar strain) by sc administration of 50 IU PMSG followed 56 h later by 30 IU hCG [9]. Homogenates of ovaries (100 mg ml^{-1}) in ice-cold buffer A (25 mM NaH_2PO_4, 1 mM EDTA, 40 mM NaCl, pH 7.4) were filtered through six layers of surgical gauze, centrifuged at $1000 \times g$ for 15 min, and the supernatant was further centrifuged at $20000 \times g$ for 30 min. The final membrane preparations were resuspended in the same buffer [9].

To solubilized gonadotropin receptor, consisting of approximately 10 mg of control or delipidated membrane proteins, was stirred with 0.5 ml of 20 mM sodium cholate in buffer A, containing 20% glycerol and phospholipids, at 4°C for 60 min [8]. Phospholipids were prepared in sodium cholate with glycerol as described below. The solution was then centrifuged at $105000 \times g$ for 60 min. The solubilized membrane protein was applied to a Bio-Beads SM-2 column (1×8 cm), previously equilibrated with buffer A. The same buffer was used for elution. After 5-fold dilution the turbid fraction containing proteoliposomes was centrifuged at $160000 \times g$ for 60 min [8,10].

The membrane-bound LH/hCG receptor was delipidated by extraction using a mild non-ionic detergent Tween 20. The procedure was carried out at 23°C. A sample in 5% (v/v) Tween 20 was sonicated in a MSE Ultrasonic Desintegrator (settings: power, medium; amplitude 6) with a small probe. The power was switched on three times for 15 s, followed by 15 s cooling between treatments. The preparation was then stirred for further 30 min. After centrifugation at $20000 \times g$ for 30 min, the pellets were washed twice with buffer A.

Aliquots of membrane-bound receptor were heat inactivated in water bath by raising the temperature at a linear rate of about 1 C°/3 min. Membrane preparations were withdrawn at designated temperatures and placed on ice until determination of binding activity. Phospholipids were prepared in chloroform-methanol, dried under a stream of nitrogen, and then pumped under vacuum for more than 1 h. Lipids were then suspended in buffer A and sonicated for 10 min at amplitude 6. Ch and Ch-S were dissolved in dimethyl sulfoxide and after addition of buffer A with 3.5% polyvinilpyrrolidine the dispersion was briefly (15 s) sonicated [11].

In hCG binding assay, 0.1 ml aliquots of ovarian membranes or proteoliposomes were incubated 16 h at 20°C with 0.1 ml buffer A + 1 mg ml^{-1} BSA with or without 100-fold excess of unlabeled hCG and 0.1 ml [^{125}I]hCG (1–1.5 ng, spec. act. about 2.3 TBq g^{-1}). After incubation and centrifugation, the membrane pellets were washed twice with buffer A [12]. The hormone-receptor complex in proteoliposomes was precipitated twice with poly(ethylene glycol) [8]. Scatchard analysis [13] of the data obtained from saturation curves was carried out using a computer program. The results are expressed as [^{125}I]hCG specific binding per mg protein [14].

Fluorescence polarization was measured with Perkin-Elmer LS-5 luminescence spectrometer, equipped with a circulation bath to maintain the sample temperature at 25°C. A solution of 2 mM DPH in tetrahydrofuran was dispersed by 1000-fold agitative dilution in 50 mM phosphate buffer (pH 7.4). Ovarian membranes (100 μg protein) were incubated at 25°C for 1 h with 2 ml of DPH in the above buffer [15].

Differential scanning calorimetry (DSC) measurements were performed on a Privalov DASM-4 differential adiabatic scanning microcalorimeter with a scan rate 0.5 C°/min. The accuracy of c_p measuring was less than ± 0.01 cal/K per g. The DSC experiments were carried out at pH 7.4 with protein concentration of about 1 mg/ml in degassed buffer A [16].

Cholesterol was assayed enzymatically [17]. Phospholipids were determined colorimetrically (as phosphatidylcholine dipalmitoyl) in a complex with ammonium ferrothiocyanate [18]. Total lipids were estimated by Lachema kit (Prague). Student's t-test was used for statistical evaluation.

3. Results

[^{125}I]hCG receptor binding activity of the rat ovary was very sensitive to the presence of lipids in membranes. The effect of modifying some of the possible variables of the method of delipidation are described below. Fig. 1 shows that removal of lipids from the membranes by 5% Tween 20 at 4°C or 2% Tween 20 at 23°C decreased accessibility of LH/hCG receptors but to not less than 60% of control, and that even at amplitude 10. A substantial loss of binding activity occurred after extraction of membrane lipids with 5% Tween 20 at 23°C and sonication at amplitude 6. The yield of the receptor in 16 procedures of delipidation varied in the range 22–33% of control. However, readdition of phospholipids to such delipidated receptors reconstituted the binding to the initial extent. Scatchard analysis of hCG specific binding to delipidated membranes indicated a single class of high-affinity binding sites with a value of $K_a = 1.3 \cdot 10^9\ M^{-1}$ similar to that calculated for control membranes ($K_a = 1.5 \cdot 10^9\ M^{-1}$). Decline of the accessibility of LH/hCG receptors in delipidated membranes was similar at a broad concentration range of proteins and volumes of 5% Tween 20 (Fig. 2). Under conditions employed in the course of delipidation there was no significant difference in the accessibility of receptors and total lipids whether 10.2 mg of membrane proteins was extracted with 1–4 ml of Tween 20 or 3.4–13.6 mg protein with 1 ml of the detergent. Moreover, sonication of the membrane in absence of the detergent did not affect the hCG binding properties of ovarian membranes. In view of these results delipidation of about 10 mg of membrane protein with 1 ml of 5% Tween 20 at 23°C and the procedure of sonication described in Results was routinely used in all experiments.

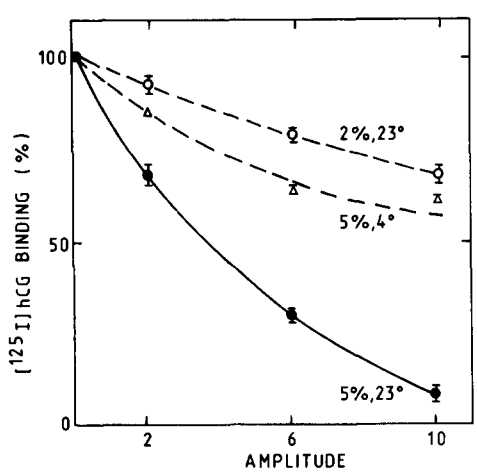

Fig. 1. Inhibitory effect of delipidation of membranes with Tween 20 on the accessibility of LH/hCG receptors. Rat ovarian membranes were extracted with 2% or 5% Tween 20 at 4°C or 23°C and sonication at different amplitudes. Each point is the mean ± S.E. of four estimations.

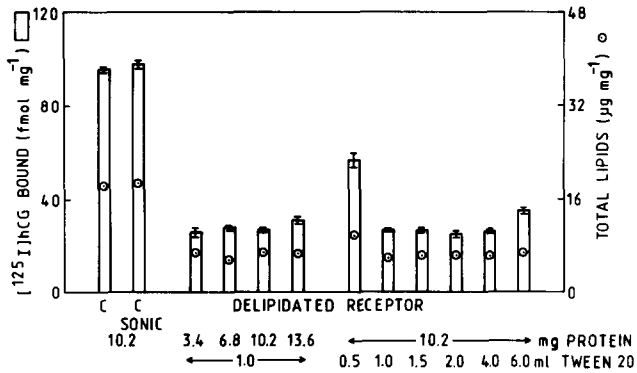

Fig. 2. Specific binding of [^{125}I]hCG to delipidated membranes and concentration of total lipids as a function of volume of 5% Tween 20 and amounts of extracted membrane proteins. SONIC, sonicated control membranes. Data are the means ± S.E. of three estimations (each repeated twice).

Analysis of heat inactivation of LH/hCG binding sites is shown in Fig. 3. Heat inactivation procedure represents a temperature-dependent loss of LH/hCG binding sites that can be expressed in terms of their T_{50} value, i.e., the temperature at which 50% of initial binding capacity remains [19]. The T_{50} value at about 49°C in control membranes decreased to 41°C in delipidated ovarian membranes. Decline of thermal stability of LH/hCG receptors is probably caused by partial extraction of membrane cholesterol and phospholipids. The amount of total cholesterol dropped to 51% and

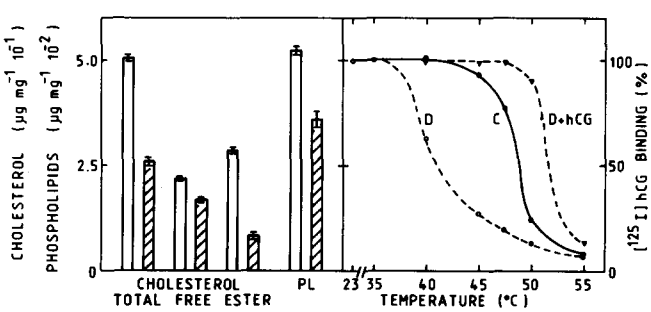

Fig. 3. Effects of delipidation of membranes with Tween 20 on concentration of cholesterol and phospholipids (PL) and heat inactivation of LH/hCG binding sites in control (C), delipidated (D) and delipidated + hCG bound ovarian membranes ($n = 2$). Each value of lipid concentration in control (open column) and delipidated (hatched column) membranes is the mean ± S.E. of 12 estimations. The results were confirmed in three independent experiments.

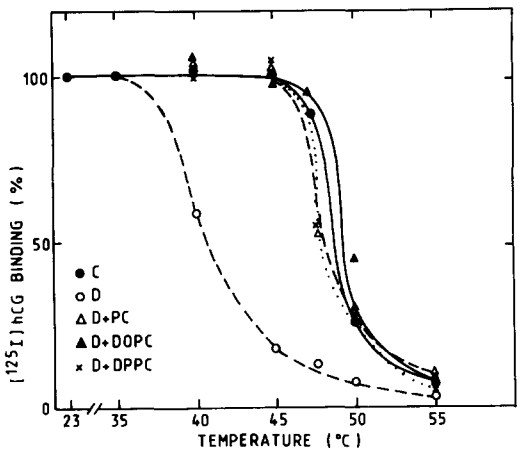

Fig. 4. Reversibility of thermal destabilization of delipidated LH/hCG receptor with phosphatidylcholine. Rat ovarian membrane lipids were extracted with Tween 20 and receptor was heat inactivated in the absence or presence of 1.5 mg ml^{-1} of phospholipids. Control values of binding were about 95 fmol hCG bound per mg protein. Means of two estimations are shown. The experiments were repeated two or three times with similar results.

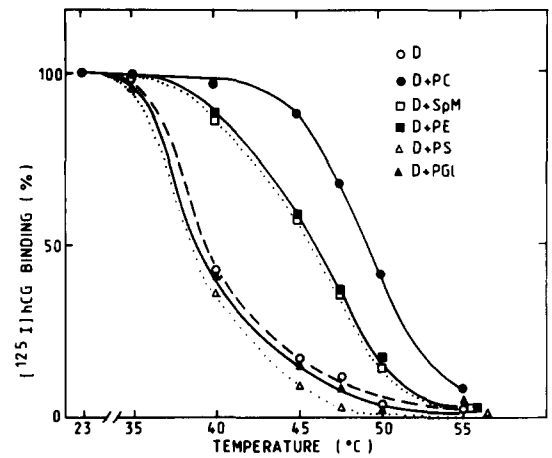

Fig. 5. Effect of various phospholipids on heat inactivation profile of delipidated LH/hCG binding sites. For details, see legend to Fig. 4.

phospholipids to 68% if they were expressed per mg of protein ($P < 0.001$). The concentration of proteins also decreased after delipidation by about 30%. On comparing the lipid level in identical samples before and after lipid depletion, the content of total cholesterol and phospholipids in the delipidated membrane were 34% and 46% of controls, respectively. The results in Fig. 3 further suggest that occupation of binding sites by hCG before thermal perturbation caused a significant stabilization of the LH/hCG receptor. Thermal destabilization of the LH/hCG receptor by delipidation of the membrane with Tween 20 can be fully inverted by treatment with phospholipids. Addition of 1.5 mg ml^{-1} PC, DOPC or DPPC to delipidated membranes increased T_{50} values so as to reach virtually the values observed in control membranes (Fig. 4). The presence of the negatively charged phospholipids PS or PGl had no effect on the thermal stability of the LH/hCG binding sites of the delipidated receptor protein. Moreover, heat inactivation of receptors could not be fully reversed upon the addition of PE and SpM. T_{50} values were lower at approx. 3°C than the characteristic T_{50} values for PC membranes (Fig. 5). The method for lipid depletion used in these studies extracts both phospholipids and cholesterol from membranes. However, addition of cholesterol or cholesterol-hemisuccinate to the delipidated membrane-bound receptor had no effect on stabilizing the LH/hCG receptor structure. Fig. 6 shows that heat inactivation profiles of LH/hCG binding sites in the presence of 0.2 mg ml^{-1} Ch or Ch-S are identical with that demonstrated in delipidated as well as PC stabilized membranes. Similar results were obtained with cholesterol concentration of 1 mg ml^{-1} (data not shown). Thermal inactivation of the delipidated LH/hCG receptor is a quick process. While loss of binding sites during incubation of untreated membranes at a constant temperature of 47°C was explicitly manifested at about 20 min, delipidated membranes exhibited large inactivation of receptors as early as at 2 min of incubation. However, in the presence of PC the rate of heat inactivation of LH/hCG binding sites in delipidated membranes was significant decreased (Fig. 7).

Several studies have suggested that modifying the physical state of the surrounding lipid matrix in membranes might affect the functioning of receptors. Therefore, series of experiments were performed to determine whether the changes in the accessibility of receptors were linked with alterations in membrane lipid fluidity. As seen Fig. 8, delipidation of ovarian membranes significantly ($P < 0.001$) increased membrane lipid rigidity, as determined by fluorescence po-

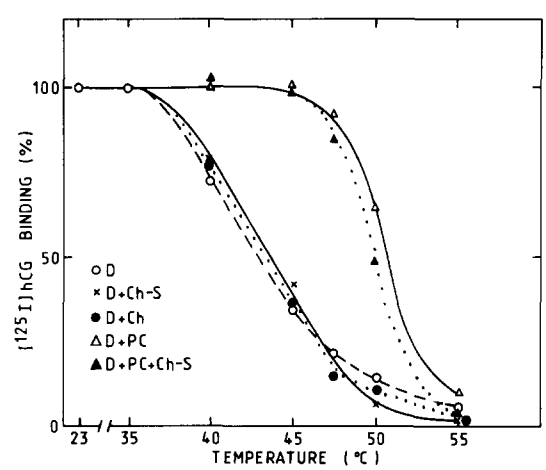

Fig. 6. Profile of heat inactivation of delipidated LH/hCG receptor in the presence of 0.2 mg ml^{-1} cholesterol or cholesteryl hemisuccinate. Experiments were done as described in the legend to Fig. 4.

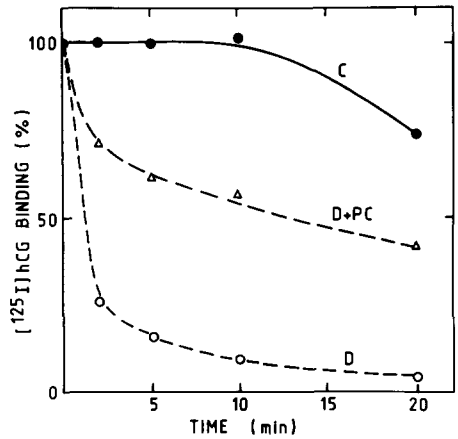

Fig. 7. Time-dependent heat inactivation of control or Tween 20 delipidated LH/hCG receptors at a constant temperature of 47°C. Membranes were treated as described in the legend to Fig. 4.

larization of probe DPH. On the other hand, incubation of delipidated membranes with PC considerably increased membrane lipid fluidity beyond that of the control membranes. Thermal inactivation of membranes had no effect on the degree of fluorescence polarization when mobility of probe DPH was measured at 23°C. The addition of PC, DOPC and PS to delipidated membranes significantly ($P < 0.001$) decreased, of DPPC and SpM increased and that of PGI did not change the fluorescence polarization of DPH (Fig. 9). These different actions of phospholipids on membrane lipid fluidity and receptor inactivation suggest that lipid fluidity is not related to the stabilizing action of phospholipid in the process of thermal inactivation of LH/hCG receptors.

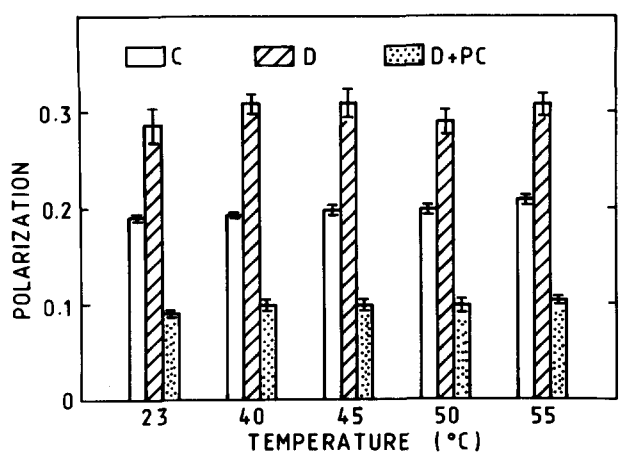

Fig. 8. Effects of thermal perturbation on fluorescence polarization of DPH probe in control, delipidated and delipidated+PC membranes. Membranes were submitted to rising temperatures and at selected temperatures samples were removed, cooled and mobility of probe DPH was measured at 23°C. Results are representative of three independent experiments. The values are means ± S.E. of four estimations.

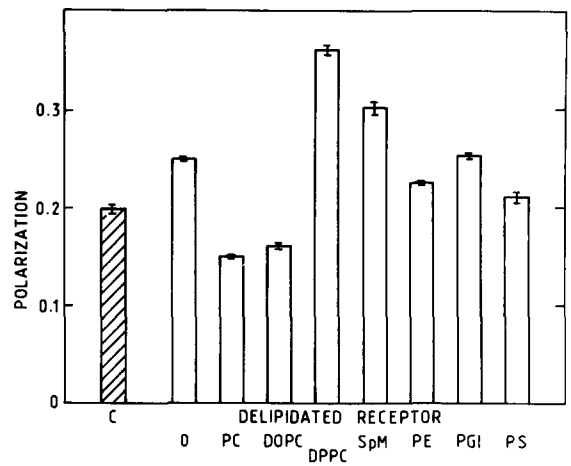

Fig. 9. Effect of phospholipids on fluorescence polarization of DPH probe in delipidated membrane. Tween 20 extracted ovarian membranes were incubated 60 min at 23°C with different phospholipids. The data represent the means ± S.E. of four estimations. The results were confirmed in a further experiment.

A representative DSC scan of LH/hCG receptor containing ovarian membranes is displayed in Fig. 10, indicating that delipidation and PC modify the calorimetric profile characteristic for control membranes. At the sites of T_{50} values for 41°C and 49°C changes occur at the level of the first two structural transitions in membrane lipids. In delipidated membranes the maxima are expressed poorly, suggesting that their structures were perturbed. Thermal transition of the central peak (58.5°C) was shifted to higher temperatures by 0.7 C° and the next two transitions were practically completely disturbed. Treatment of delipidated membranes with PC stabilized structural transitions and increased the cooperativity of the system.

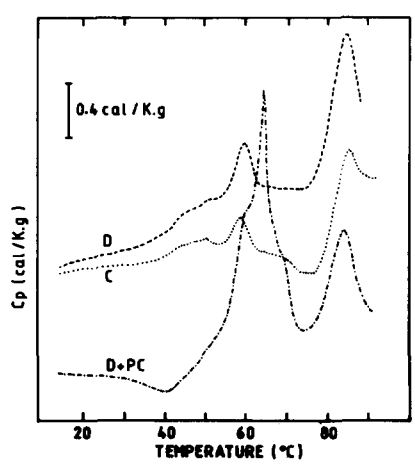

Fig. 10. Differential scanning calorimetric thermogram of control, delipidated and delipidated+PC ovarian membranes. The heat capacity was measured at protein concentration of about 1 mg ml^{-1} in buffer A. Thermograms were highly reproducible.

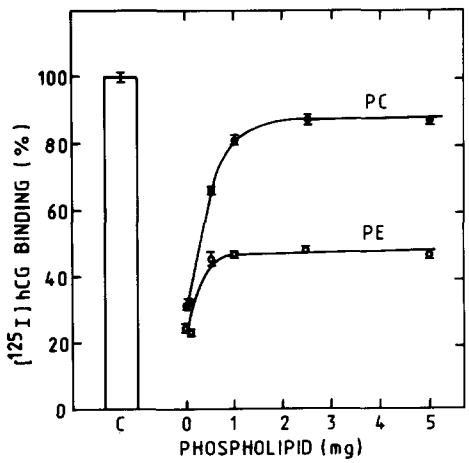

Fig. 11. The stimulatory action of phospholipids on the reconstitution of Tween 20 delipidated LH/hCG receptor into proteoliposomes. Increasing concentrations of PC and PE were added during solubilization of hCG binding activity with 20 mM sodium cholate detergent. The detergent was removed using Bio-Beads SM-2. Binding activity of proteoliposomes in control was approx. 280 fmol hCG bound per mg protein. The experiments were repeated twice. Values are the means ± S.E. of three estimations.

Further experiments documented that delipidated LH/hCG receptors reconstituted into proteoliposomes can be reactivated by exogenous phospholipids. Fig. 11 shows that detergent delipidation results in loss of receptor activity, which can be restored by readdition of PC. Addition of 2.5 mg of PC before reconstitution increased hCG binding to proteoliposomes to 90% of control values. Within the DOPC and DPPC, the ability to restore binding activity was at the level of control values (data not presented). PE provided significant restoration of activity, though only to 50% of LH/hCG receptor found in control proteoliposomes.

4. Discussion

The results in this study showed that the LH/hCG receptor from rat ovarian membranes can be partially delipidated by extraction with a mild detergent Tween 20, removing a large fraction of both phospholipids and cholesterol. The ability of the receptor to bind specifically hCG is lost to a great extent by such treatment. It is known that the presence of a lipid environment is essential for preserving receptor function. Extensive delipidation of native membranes was found to diminish the function of bacteriorhodopsin in purple membrane [6], β-adrenergic receptor in turkey erythrocyte membrane [19], nicotinic acetylcholine receptor from *Torpedo california* [20,21] and LH/hCG receptor from porcine corpora lutea [2,3]. However, a loss of LH/hCG binding sites after digestion with phospholipase C and A_2 is irreversible (data not shown). Delipidation of membranes by the procedure developed in this study provides a receptor whose binding activity can be almost fully restored with the proper phospholipid. Phosphatidylcholine fulfils the criteria for reconstitution of the delipidated LH/hCG receptor into proteoliposomes in its functional state [8]. Reconstitution of the receptor with PE was much less effective than that with PC. The results suggest that membrane phospholipids are essential for the stability of LH/hCG binding domains and that PC forming a bilayer structure may be the most suitable species for maintaining receptor activity during reconstitution.

We used thermal perturbation techniques to monitor structural changes in the LH/hCG receptor induced by action of hCG and lipid-receptor interaction in its native membrane environment [19]. Heat inactivation of hCG-binding sites demonstrated that there was a significant destabilization of the LH/hCG receptor structure when membranes were subjected to delipidation. Thermal destabilization of receptors produced by delipidation caused a decrease in T_{50} values by about 8 C°. The changes observed in the delipidated membrane receptor during heat inactivation were reflected in the information obtained by DSC. Delipidation modified the calorimetric profile characteristic for the control sample. In the location of T_{50} values for 41°C and 49°C there were changes at the level of the first two structural transitions of membrane lipids. In delipidated membranes the maxima were expressed poorly, suggesting that their structures were perturbed. Thermal destabilization was found to be sensitive to the presence of hCG, supplying a further evidence on specific structural changes of the LH/hCG receptor. Occupation of receptor binding sites with the agonist before heat inactivation induced considerable stabilization of the receptor, which is in line with our previous results with water-soluble LH/hCG receptor [22]. The hormone-receptor complex was found to be denatured at a temperature lower by about 10 C° than the free receptor. Moreover, this study shows that specific phospholipids have to be added to the delipidated receptor to restore its binding activity. The experiments testing several phospholipids revealed that only phosphatidylcholine was able to shift the heat inactivation profile to control membrane values. There is probably a more stringent requirement for a specific polar group than for hydrocarbon chains. In spite of different hydrocarbon chains of soybean PC, DPPC and DOPC, the effectiveness of these phospholipids was essentially identical. The specificity may be related to the choline group of phospholipids, because despite structural similarities of the headgroup of PC, PS and PE (choline is *N,N,N*-trimethylethanolamine, serine is 2-carboxyethanolamine and ethanolamine), the different phospholipid polar heads had different effects on the thermal stabilization of the LH/hCG receptor. Considering these facts, direct interaction of the choline

group of PC with the LH/hCG receptor cannot be ruled out. It was postulated that the actual peptide-receptor associated form of the ligand may be its complex with phospholipid [23]. In general, PC represents a phospholipid with low selectivity. Preferential selectivities are usually found for negatively charged lipids [24]. Several membrane receptors require negatively charged phospholipids, or mixtures thereof, which provide the proper physicochemical environment for the receptor system [25]. As described above, the presence of phospholipids whose polar group bears a net negative charge (PS and PGl) had no stabilizing effect in the course of heat inactivation of the LH/hCG receptor in delipidated membranes.

A further vital lipid constituent of membranes is cholesterol. As a structural membrane component, cholesterol is involved in maintaining cell integrity and membrane fluidity. Cholesterol has a condensing effect on membrane lipids in the liquid-crystalline state. We reported earlier that the accessibility of the LH/hCG receptor was increased in a dose-dependent manner after incorporation of cholesteryl hemisuccinate into rat testicular membranes [15,26]. The effect of cholesterol on the structure of receptors may be attributed to modification of the physical state of the membrane or to a more direct interaction of cholesterol with the receptor protein. In the rat testicular membrane we postulated that the rigidifying action of cholesterol is unlikely to be the sole cause for the increased the accessibility of the LH/hCG receptor, but its influence may be connected with specific chemical properties of cholesteryl hemisuccinate [11]. Artigues et al. [27] arrived at similar conclusions. They reported that cholesterol stabilized the acetylcholine receptor structure through direct interaction with the receptor protein and not by modification of physical properties of the membrane. However, in spite of the widely accepted assumption that cholesterol plays an important role in maintaining the functional state of receptors [25,27], the results of this study showed that neither cholesterol nor cholesterol hemisuccinate had any effect on thermal stabilization of the LH/hCG receptor in delipidated membranes.

It is generally believed that membrane structure and molecular order and dynamics are essential for the maintenance of membrane function. The ordering of the lipid environment in which the receptor is embedded can affect the accessibility of the latter [15,26]. A positive correlation was found between the order of membrane lipids and the number of LH/hCG receptors during the formation of rat corpora lutea [9]. However, when membranes isolated from such corpora lutea were delipidated, membrane lipid rigidity was further elevated, but both the accessibility of LH/hCG receptors as well as their thermal stability were considerably diminished. Likewise, the beneficial action of phospholipids on heat inactivation of the LH/hCG receptor may not be associated with changes of the apparent membrane fluidity. Both DOPC and DPPC had a stabilizing effect on the LH/hCG receptor, but DPPC increased and DOPC decreases membrane lipid rigidity. Similarly, there were differences between the identical action of the couple SpM and PE or PS and PGl on thermal stability of the delipidated membrane LH/hCG receptor and they exerted different influences on fluidity of membrane lipids. We therefore suggest that changes of the order of the lipid matrix in delipidated membranes are not a necessary prerequisite for the stabilizing action of some phospholipids on thermal perturbation of the receptor protein structure.

Acknowledgement

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