Changes in Physical Properties of Ovarian Membranes after hCG-Induced Desensitization in Rats

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Abstract. Using pseudopregnant rat ovaries, the possibility was examined whether hCG-induced early desensitization of the LH/hCG receptor was accompanied by changes in the physical state of membranes. Thirty min after a single s.c. injection of 75 IU hCG, the hCG-responsive adenylylcyclase activity was reduced, whereas hCG binding to ovarian membranes was still normal. Membrane lipid rigidity, as determined by fluorescence polarization of DPH, decreased as early as 30 min after injection of a desensitizing dose of hCG. There was no difference in membrane rigidity when ovarian membranes were incubated 0.5 or 2 h with hCG or LH. The decrease of membrane lipid rigidity in the process of rapid desensitization of rat luteal tissue does not appear to be associated with protein synthesis. Desensitization also modified the differential scanning calorimetric profile. The results indicate that hCG-induced changes in the physical state of rat ovary membranes are preceded by the process of desensitization.

Key words: Desensitization — LH/hCG receptor — Membrane rigidity — Adenylylcyclase

Introduction

The binding of agonists to receptors leads not only to activation of the cellular response, but often also to a rapid loss of receptor responsiveness. This hormone-induced process is called desensitization. Desensitization of hormone-stimulated adenylylcyclase is a rather complex phenomenon. Although the mechanisms involved in adenylylcyclase desensitization appear to be different, two major cate-

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gories have been distinguished. First, prolonged treatment of cells with agonists causes reduction in the density of receptors. This process is termed down-regulation and represents the internalization of receptors away from the plasma membrane. The sequestration of receptors occurs slowly and can be produced by decreased receptor synthesis or increased receptor degradation (Kolena et al. 1986b; Segaloff and Ascoli 1993). Second, agonists induce a fast modification of receptors even though the receptors remain located in the plasma membrane and are detectable by ligand binding. The process of desensitization, however, results in impairment of the interaction between the receptor and G_s regulatory protein (Waldo et al. 1983; Rebois and Fishman 1986; Hausdorff et al. 1990). Much experimental evidence has accumulated indicating that receptor phosphorylation, mainly in β_2-adrenergic receptors, is an essential event connected with receptor desensitization (Hausdorff et al. 1989). Some reports suggest that the mechanism responsible for the uncoupling of LH/hCG receptors from the G_s-adenylylcyclase system may be different from that operative in desensitization of catecholamine receptors (Rebois and Fishman 1986; Segaloff and Ascoli 1993). It is generally believed that membrane structure as well as molecular order and dynamics are essential for the maintenance of membrane function and for transmission of the signal across the bilayer. Data are presented here to document that the early hCG-induced process of desensitization of luteinized rat ovaries is associated with modification of the physical state of the membrane.

Materials and Methods

Materials

Purified hCG (CR 123; 12 780 U/mg) was generously supplied by NIAMDD, NIH, Bethesda. $Na^{125}I$ was purchased from the Radiochemical Centre, Amersham. Pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG, Praedyn) were from Spofa, Prague. Creatine phosphate, creatine kinase and guanosine-5'-O-(3-thiotriphosphate) (GTP-γ-S) were obtained from Boehringer-Mannheim. All other chemicals were from Sigma.

Methods

Luteinization of ovaries was produced in 26-day-old rats (Wistar strain) by s.c. administration of 50 IU PMSG followed 56 h later by 30 IU hCG (Kolena et al. 1990). Desensitization was induced by injecting 75 IU hCG on day 5 of pseudopregnancy; control rats received saline. Homogenates of ovaries (100 $mg.ml^{-1}$) in ice-cold buffer A (25 $mmol.l^{-1}$ NaH_2PO_4, 1 $mmol.l^{-1}$ EDTA, 40 $mmol.l^{-1}$ 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 $20,000 \times g$ for 30 min (Kolena et al. 1986a). Plasma membranes were prepared on sucrose gradient (Kolena et al. 1992). Membrane preparations in buffer A were layered over a cushion of 45% (w/w) sucrose and centrifuged at $60,000 \times g$ for 60 min at 4 °C. The interface of membranes on top of the sucrose cushion was sucked off, diluted 6-fold, and centrifuged at $20,000 \times g$ for 30 min.

In the hCG binding assay, 0.1 ml of ovarian membranes was incubated for 16 h

at 20 °C with 0.1 ml buffer A + 1 mg ml^{-1} BSA with or without a 100-fold excess of unlabelled hCG and 0.1 ml [^{125}I]hCG (1–1.5 ng, specific activity about 2.3 TBq g^{-1}). After incubation and centrifugation, the membrane pellets were washed twice with buffer A (Kolena et al. 1992). The results were expressed as specific binding per mg protein (Lowry et al. 1951).

Fluorescence polarization was measured with a Perkin-Elmer LS-5 luminescence spectrometer at 25 °C. A solution of 2 mmol.l^{-1} 1,6-diphenyl-1,3,5-hexatriene (DPH) in tetrahydrofuran was dispersed by 1000-fold agitative dilution in 50 mmol.l^{-1} 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. The fluorescence polarization was computed by equation:

$$P = \frac{I_{vv} - I_{vh}(I_{hv}/I_{hh})}{I_{vv} + I_{vh}(I_{hv}/I_{hh})}$$

where I_{vv} and I_{vh} are fluorescence intensities detected through a polarizer oriented parallelly and perpendicularly to the direction of vertical polarized light. The I_{hv}/I_{hh} stands represents the ratio when the excitation is polarized horizontally and the emission observed through the analyzer oriented perpendicularly and parallelly, respectively (Kolena et al. 1986b).

Differential scanning calorimetry (DSC) measurements were performed on a Privalov DASM-4 adiabatic differential microcalorimeter with a scan rate 0.5 °C/min. The DSC experiments were carried out at pH 7.4 with a protein concentration of about 1 mg.ml^{-1} in degassed buffer A (Jasem 1986).

Adenylylcyclase activity was assayed at 30 °C for 25 min (Hwang and Ryan 1981; Kolena 1989). The assay system contained 25 mmol.l^{-1} Tris-HCl (pH 7.4), 5 mmol.l^{-1} $MgCl_2$, 1 mmol.l^{-1} EDTA, 0.3 mmol.l^{-1} 3-isobutylmethylxanthine, 1 mmol.l^{-1} ATP, 20 mmol.l^{-1} phosphocreatine, 100 U.ml^{-1} creatine kinase, and 30–40 μg plasma membrane protein in a final volume of 400 μl. When present, GTP-γ-S and hCG were at 0.1 mmol.l^{-1} and 0.3 μg.ml^{-1}, 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 3 times with water-saturated diethyl ether, and lyophilized. The residue was dissolved in water and the cAMP content determined by protein binding assay (Kolena et al. 1986a).
Student's t-test was used for statistical evaluation.

Results

Adenylylcyclase activity was determined in purified plasma membranes from ovaries of PMSG-hCG primed rats. As shown in Table 1, the addition of hCG in the absence or presence of GTP-γ-S increased the enzyme activity 2.3- and 1.9-fold, respectively, above the basal or GTP-γ-S values. The adenylylcyclase system obtained from hCG treated rats was partially desensitized. Thirty min after injection of 75 IU hCG to rats the stimulatory action of hCG was reduced to 1.7- and 1.3-fold, respectively (i.e. 26% and 32% decline in hCG-stimulated adenylylcyclase activity). Treatment of rats with desensitizing doses of hCG had no effect on the accessibility of LH/hCG receptors, whereas the degree of polarization of DPH was apparently decreased ($P < 0.001$) (Fig. 1). There was no significant difference in

Table 1. Desensitization of hCG-sensitive adenylylcyclase activity in ovarian plasma membranes. Pseudopregnant rats were injected with 75 IU of hCG or saline 30 min before they were sacrificed. Membranes were incubated with or without 0.3 $\mu g.ml^{-1}$ hCG and/or 0.1 $mmol.l^{-1}$ GTP-γ-S. Data are the means ± SE of 4 determinations. The experiments were repeated twice with comparable results. Data given in parentheses represent percentages of hCG stimulation exceeding those basal or GTP-γ-S values ($P < < 0.01$).

Treatment	pmol cAMP formed/min/mg protein	
	Control	Desensitized
Basal	16.4 ± 1.3	18.6 ± 1.7
hCG	37.6 ± 2.6 (+129%)	32.8 ± 2.3 (+76)
GTP-γ-S	40.0 ± 1.8	50.1 ± 1.9
GTP-γ-S + hCG	75.5 ± 2.7 (+89%)	68.6 ± 2.3 (+37)

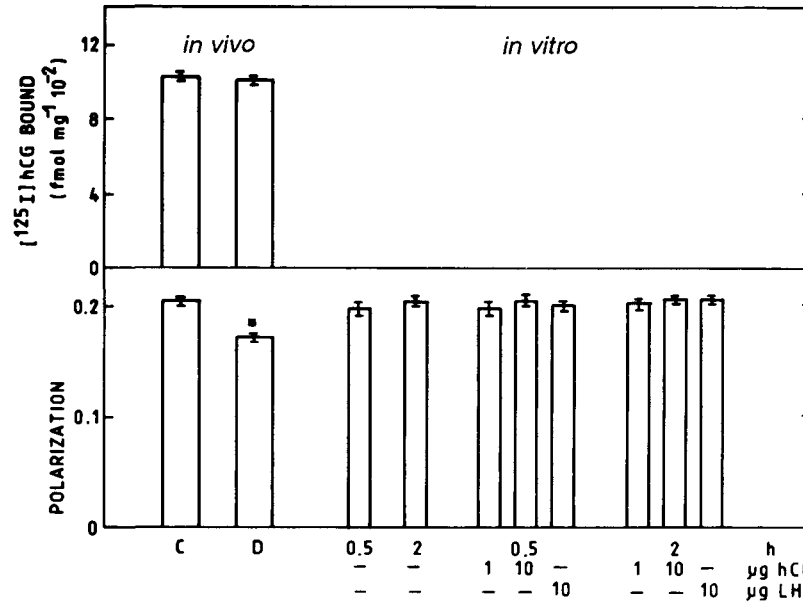

Figure 1. Effect of desensitization on the accessibility of the LH/hCG receptor and on fluorescence polarization of DPH probe in ovarian membranes. Pseudopregnant rats were treated with 75 IU of hCG (*D*) or saline (*C*) 30 min before they were sacrificed. Membranes from control rats were incubated for 0.5 or 2 h at 37 °C with 1 or 10 μg ml^{-1} hCG or LH. The values are means ± SE of 4–6 determinations. The results were confirmed in 2–3 independent experiments. Asterisk indicates differences statistically significant from control values ($P < 0.001$).

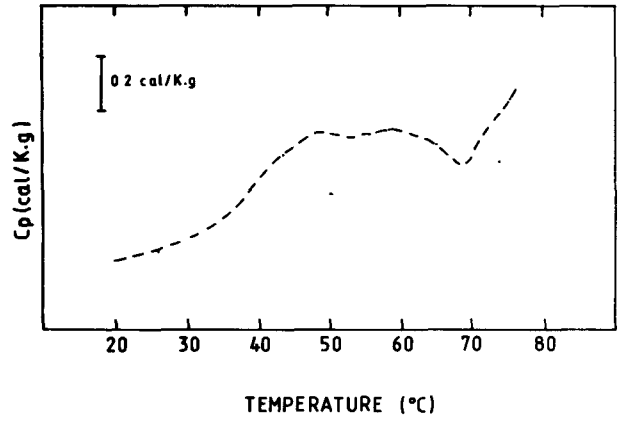

Figure 2. Differential scanning calorimetric thermogram of ovarian membranes in saline (— · — · — · —) or desensitizing hCG dose (· · ·) injected rats. The heat capacity was measured at protein concentration of about 1 mg ml^{-1}. Thermograms were highly reproducible. The rats were treated as described in the legend to Fig. 1.

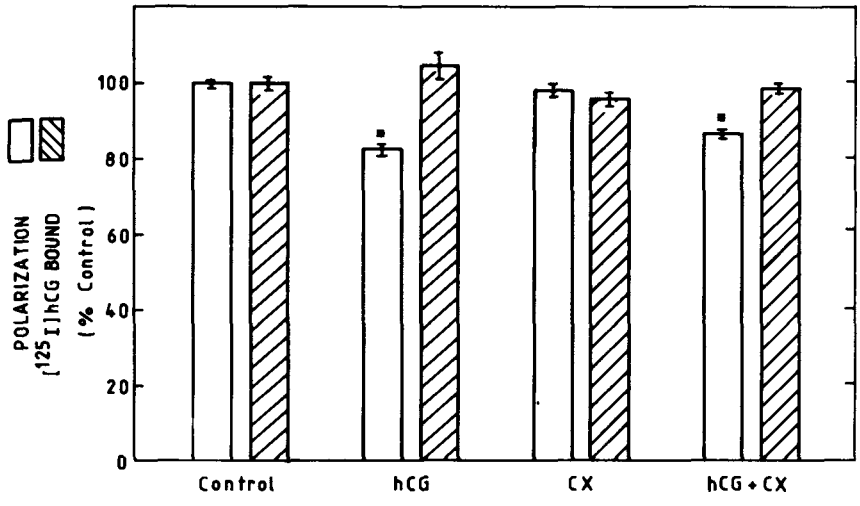

Figure 3. Effects of a single injection of hCG (75 IU), cycloheximide (CX, 800 μg/100 g) or both on the accessibility of LH/hCG receptors and the degree of polarization of DPH in ovarian membranes of pseudopregnant rats. CX and hCG were injected 60 and 30 min, respectively, before the rats were killed. Data are the means ± SE of 4 determinations (each repeated twice).

membrane lipid rigidity when ovarian membranes obtained from control rats were incubated 0.5 or 2 h with 1 or 10 μg hCG or LH. A representative DSC scan of ovarian membranes is shown in Fig. 2, indicating that desensitization modified the calorimetric profile. The maxima of thermal transition at temperatures of 50, 60 and 64 °C in the control membranes are poorly expressed. In desensitized ovarian membranes the thermal transitions of the central peaks (40–53 °C) became more expressive and cooperative and shifted to lower temperatures. Studies with cycloheximide were performed to investigate whether in the process of desensitization changes in membrane lipid rigidity may be dependent on protein synthesis. Cycloheximide was injected 1 h before sacrificing the rats. It was found that with the dose of cycloheximide used (800 μg/100 g of body weight), about 85% of protein synthesis was inhibited (Saez et al. 1978). Administration of cycloheximide alone or with the desensitizing dose of hCG had no effect either on membrane lipid rigidity or on the accessibility of LH/hCG receptors in ovarian membranes (Fig. 3).

Discussion

It has frequently been shown that treatment of the target cells with a hormone can produce desensitization, so that a second exposure to the hormone is less effective than the first. This appears to play a significant role in modulating signal transduction processes in a variety of cells (Segaloff and Ascoli 1993). Short-term exposure of cells to an agonist can induce changes in the receptor. Hormone-dependent phosphorylation of the receptor by protein kinases is involved in uncoupling of the receptor from the G_s protein and may take part in receptor sequestration. Phosphorylation and functional uncoupling has been studied in detail in β_2-adrenergic receptors (Waldo et al. 1983; Hausdorff et al. 1989, 1990). Sequestration represents a rapid translocation of receptors away from the plasma membrane to a distinct subcellular compartment where receptors are dephosphorylated and a portion is recycled back to the cell surface. At present it is not clear if the model described for the β_2-adrenergic receptor is applicable to the LH/hCG receptor. In our study the adenylylcyclase activity in plasma membranes obtained from the rat ovary was partially desensitized as early as 30 min after injection of the desensitizing dose of hCG. Such an early onset of desensitization of the adenylylcyclase system was not found in ovaries obtained from superovulated rats (Hunzicker-Dunn and Birnbaumer 1976), yet 1 h after the action of gonadotropin desensitization was observed in rabbit granulosa cells (Hunzicker-Dunn 1981). However, the partial loss of hormonal responsiveness of ovarian adenylylcyclase occurred without a reduction in the binding sites of the LH/hCG receptor. After two or more hours LH/hCG-bound receptors disappear from the cell surface and are internalized and ultimately degraded (Harwood et al. 1978; Kolena et al. 1986b). It appears unlikely that sequestration represents the major mechanism underlying rapid desensitization.

Sequestered receptors are generally already uncoupled from G_s proteins and the onset of ligand-induced desensitization was observed before receptor sequestration. Moreover, blocking of sequestration by concanavalin A or phenylarsene oxide had little effect on early desensitization of adenylylcyclase (Waldo et al. 1983; Hausdorff et al. 1990). A similar molecular mechanism of uncoupling β_2-adrenergic receptors from stimulatory G_s protein involving phosphorylation of the receptor by protein kinase was suggested for the LH/hCG receptor (Sanchéz-Yague et al. 1992). Some evidence however indicates that the uncoupling phenomenon involving phosphorylation is rather unlikely for the LH/hCG receptor and supports the notion that uncoupling may not be the only mechanism involved in the process of desensitization (Rebois and Fishman 1986; Segaloff and Ascoli 1993).

The change of the physical state of membrane lipids can influence not only the accessibility of the receptor for LH/hCG, but also transmission of the signal across the membrane (Kolena et al. 1986a, 1990). The events involved in the process of rapid desensitization may include changes in the order and dynamics of membrane lipids. The present data showed a decrease in ovarian membrane lipid rigidity as early as 30 min after injection of a desensitizing dose of hCG to rats. The changes of the physical state of the membrane were confirmed also by differential scanning calorimetry. Desensitization modified the calorimetric profile characteristic of the control membranes. The dominant 40–50 °C thermal transitions became more expressive and cooperative. It is likely that these thermal transitions reflect structural transitions of membrane phospholipids. The alteration of the physical state of the membrane was connected specifically with the process of desensitization of luteal tissue and with hCG treatment *in vivo*. Incubation of luteal membranes with hCG and LH or injection of male rats with hCG (Kolena et al. 1986b) had no effect on gonadal membrane rigidity. In a process of down-regulation of the insulin receptor, Santini et al. (1990) reported transient decrease of membrane order in human erythrocytes. These changes in the order of membrane lipids during receptor internalization under *in vitro* conditions are the apparent result of an alteration of the cytoskeletal components during concentrative endocytosis of the insulin receptor. Hormone mediated down-regulation is preceded by desensitization of adenylylcyclase. Hormone induced loss of receptors requires protein synthesis as cycloheximide inhibited down-regulation (Saez et al. 1978; Fishman et al. 1985). However, the decrease in membrane lipid rigidity observed in the process of rapid desensitization of rat luteal tissue does not appear to be accompanied by protein synthesis. We reported earlier that a rise in the order of rat luteal membrane lipids was associated with an increase of the accessibility of LH/hCG receptors and steroidogenesis (Kolena et al. 1990). However, when such corpora lutea undergo rapid desensitization, membrane lipid rigidity is decreased. A reduction of membrane rigidity may be a requirement for the induction of changes which lead to the phenomenon of desensitization. Ethanol, a well-known lipid-

fluidizing agent, potentiates the extent of hCG-induced desensitization of porcine luteal adenylylcyclase. This effect was found to be linked to the coupling efficiency of the LH/CG receptor with stimulatory G_s protein rather than to the functional state of G_s protein or adenylylcyclase (Ekstrom and Hunzicker-Dunn 1990). Supported desensitization of the GnRH receptor by ethanol was ascribed to the ability of ethanol to increase the fluidity of membrane lipids (Gorospe and Conn 1987). Therefore, a decrease of membrane lipid rigidity in rat luteal tissue, as an early effect of desensitization, may facilitate the transformation of the LH/hCG receptor system to its desensitized state.

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