Original article

Pseudopregnancy-dependent changes in rat ovarian LH/hCG receptors in relation to membrane lipid fluidity

J Kolena [1], K Matejčíková [1], A Danišová [1], Z Virsík

[1] Institute of Experimental Endocrinology Centre of Physiological Sciences,
Slovak Academy of Sciences, Vlárska 3, 833 06 Bratislava;
[2] Research Institute of Preventive Medicine, 831 01 Bratislava, Czechoslovakia

Summary — The specific binding of [^{125}I] hCG to ovarian membrane preparations as well as membrane fluidity have been investigated in immature rats during hormonally-induced pseudopregnancy. Membrane fluidity was monitored either by fluorescence polarization analysis of 1,6-diphenyl-1,3,5-hexatriene or by electron spin resonance of 16-, 12-, 5-doxyl stearic acid and CAT 16. A significant positive correlation was found between membrane lipid rigidity and the number of LH/hCG receptors. Luteinization of the ovary induced mobility of molecules in the hydrophobic membrane part at about the C_{16} carbon level. The changes in rigidity of membrane lipid were the apparent result of alterations in the cholesterol to phospholipids ratio. The results suggest that the increased rigidity of membrane lipid during pseudopregnancy may maximally expose ovarian LH/hCG receptors maintained in a cryptic form.

LH/hCG receptor / rat ovary / membrane fluidity / spin probes / cholesterol / phospholipid

Résumé — Fluidité des lipides membranaires et accessibilité des récepteurs ovariens de LH/hCG pendant la pseudo-gestation chez la rate. *La liaison spécifique de [^{125}I] hCG des préparations membranaires d'ovaires et la fluidité de ces membranes ont été étudiées pendant la pseudo-gestation induite par PMSG et hCG chez les rates immatures. La fluidité a été étudiée par polarisation de fluoresence du DPH, ou par résonance paramagnétique électronique des sondes 16-DSA, 12-DSA, 5-DSA et CAT-16. Une corrélation positive significative a été observée entre la rigidité des lipides membranaires et l'accessibilité des récepteurs LH/hCG. La lutéinisation des ovaires influence la mobilité des molécules lipidiques dans la partie hydrophobe de la membrane au niveau du carbone C_{16}. Les changements de la rigidité des lipides membranaires résultent apparemment de l'altération du rapport cholestérol/phospholipides. Les résultats montrent que l'augmentation de la rigidité des lipides membranaires pendant la pseudo-gestation facilite l'accessibilité des récepteurs LH/hCG dont parallèlement le nombre augmente.*

récepteur de LH/hCG / ovaire / rate / fluidité membranaire / sonde de résonances / cholestérol / phospholipide

INTRODUCTION

The cell membrane is a dynamic matrix which responds to various physiological conditions by changing its physical state. Receptors are a heterogeneous population of molecules interacting with membrane components, and their activity may be modified by alterations in membrane lipid fluidity. The functions of serotonin, insulin and prolactin receptors were found to be affected by changes in membrane lipid fluidity (Heron *et al*, 1980; Ginsberg *et al*, 1981; Dave and Witorsch, 1983). We reported earlier that in rat testes during post-natal development (Kolena and Ondriaš, 1984) and in maturing granulosa cells (Kolena *et al*, 1986c) the levels of LH/hCG receptors were associated with changes in membrane lipid fluidity. Further studies have demonstrated that after incorporation of cholesteryl-hemisuccinate into rat testicular membranes, the accessibility of LH/hCG receptors and membrane lipid rigidity is increased in a dose-dependent manner (Kolena *et al*, 1986a; Kolena and Kasal, 1989).

Induction of pseudopregnancy with PMSG and hCG in immature rats has proved to be a suitable model for the study of the regulation of ovarian steroidogenesis (Rothchild, 1981). Both ovarian hCG binding and progesterone concentration undergo variations depending on the functional state of the luteinization of rat ovaries (Midgley Jr *et al*, 1974; Lee *et al*, 1975, Straalen and Zeilmaker, 1982). Since development of a pseudopregnancy state of the rat ovary can be induced in connection with alterations of membrane structure, the relationship of ovarian LH/hCG receptors and membrane lipid fluidity was investigated.

MATERIALS AND METHODS

Purified hCG (CR 123; 12 780 U mg^{-1}) was generously supplied by NIAMDD, NIH, Bethesda, MD. Na^{125}I, [1,2,6,7–^{3}H] progesterone, [1,2–^{3}H] testosterone and [2,4,6,7–^{3}H] estradiol were purchased from the Radiochemical Centre, Amersham. PMSG (Gestyl) a product of Organon, Oss and hCG (Praedyn) was from Spofa, Prague. The spin probe I-oxyl-2,2,6, 6-tetramethyl-4-dimethyl-aminopiperidinecetyl bromide (CAT 16) was obtained from the Institute of Organic Chemistry, Sofia. 1,6-diphenyl-1,3,5-hexatriene (DPH), spin probes with a dimethyl-oxyzolidinyl group (16 DSA, 12 DSA and 5 DSA) and all other chemicals were from Sigma.

Luteinized ovaries were produced in 26-day-old rats (Wistar strain) by sc administration of 50 IU PMSG followed 56 h later by 30 IU hCG (Parlow, 1961). Homogenates of ovaries in ice-cold 50 mM TRIS–HCl (pH 7.4) were filtered through 6 layers of surgical gauze, centrifuged at 1 000 g for 15 min, and the supernatant was further centrifuged at 20 000 g for 30 min (Šebököva and Kolena, 1984; Kolena *et al*, 1986a). The final membrane preparations were resuspended in the same buffer (100 mg tissue per ml).

Fluorescence polarization (P) was measured with a 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 1 000-fold agitative dilution in 50 mM TRIS-HCl buffer. Crude ovarian membranes (100 μg protein) were incubated for 1 h at 25 °C with 2 ml of DPH in TRIS-buffered saline (Shinitzky *et al*, 1976; Kolena *et al*, 1986a, b).

Samples for electron spin resonance (ESR) measurements were prepared as follows : 10 μg of spin probe were mixed with 0.06 ml of membrane preparation (3 mg protein). ESR spectra were recorded using an ERS-230 X-band spectrometer (ZWG AdW, Berlin) with a microwave power output of 4 mW, frequency 9.5 GHz, and a magnetic field 3 300 GS (Kolena and Ondriaš, 1984). Since the outer splitting in most of our spectra could not be measured due to a low signal, noise ratio (the value of $A_\perp$) was used as a

parameter characterizing the spin label ordering. The order parameter decreased linearly with the increasing $A_\perp$ for $S > 0.1$ and $A_\perp < 1.1$ mT (Gaffney, 1976). At $S < 0.1$ and $A_\perp > 1.1$ mT the spin label motion was nearly isotropic and its rate of molecular reorientation could be described by rotational correlation time τ calculated by Schreier et al (1978). Membranes were labeled with an experimentally determined low probe/membrane protein concentration to avoid spin-spin interaction effects on the hyperfine splittings.

In hCG binding assays, 0.1 ml aliquots of membrane preparations were incubated 16 h at 24 °C with 0.1 ml PBS (50 mM phosphate buffer and 15 mM sodium chloride, pH 7.4) + 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 $\approx$ 2.3 TBq g^{-1}). After incubation and centrifugation, the membrane pellets were washed twice with PBS buffer (Kolena, 1976). The results were expressed as specific binding per mg protein.

RIA or protein binding methods were used for the estimation of progesterone (Kolena and Channing, 1985), estradiol (Kolena et al, 1977) and testosterone (Šebököva and Kolena, 1978) concentrations in plasma and ovarian membranes. Cholesterol was assayed enzymatically (Satè, 1984). Phospholipids were determined colorimetrically (as dipalmitoyl phosphatidylcholine) in a complex with ammonium ferrothiocyanate (Stewart, 1980). Protein was determined by the method of Lowry et al (1951). Student's t-test was used for statistical evaluation.

RESULTS

Figure 1 shows changes of ovarian LH/hCG receptors and plasma and ovarian hormones occurring during pseudopregnancy. It can be seen that until day 5 after hCG ovulatory injection, specific binding of [^{125}I] hCG to ovarian membranes rose and subsequently decreased to the end of pseudopregnancy. The pattern of plasma progesterone concentrations correlated well with that of the binding activity ($r = 0.98$, $P < 0.001$). The concentration of

ovarian progesterone rose of a maximum on day 8; the high level was maintained until day 22 and did not correlate with the plasma progesterone level. Estradiol and testosterone concentrations increased by day 2 after hCG and then fell to control values.

As shown in figure 2, ovarian membrane lipid rigidity, as determined by fluorescence polarization of DPH increased
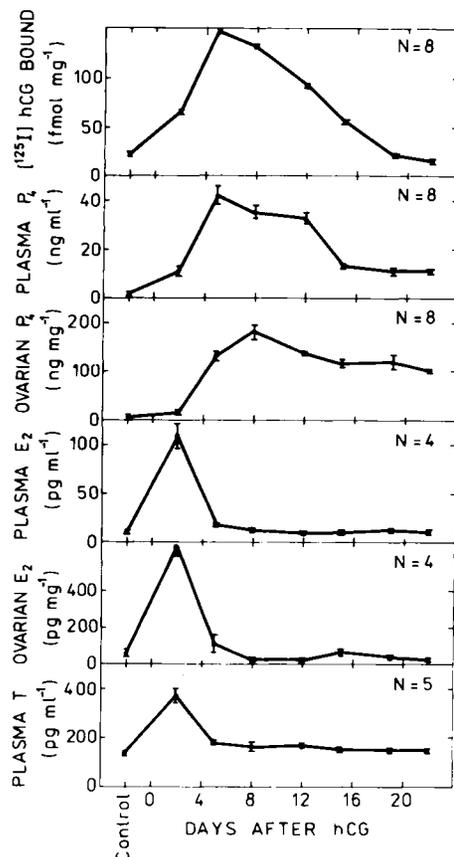

Fig 1. Specific binding of [^{125}I] hCG to ovarian membrane preparations and concentrations of progesterone (P$_4$), estradiol (E$_2$) and testosterone (T) during pseudopregnancy in rats. Each point represents data ($\pm$ SE) obtained from 4–8 animals. Control rats were 30 days old.
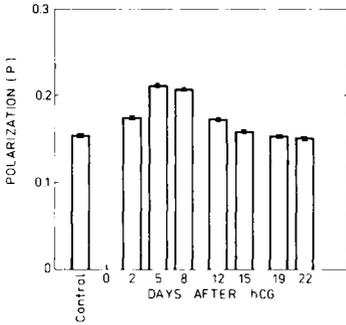
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Fig 2. Development of changes in fluorescence polarization of DPH probe in ovarian membranes of rats treated with PMSG and hCG. Each value is the mean ± SE of 4 estimations. The results were confirmed in 2 independent experiments.

cantly ($P < 0.005$) higher correlation times than did control membranes ($1.376 \pm 0.0214.10^{-9}$ s and $1.280 \pm 0.0150.10^{-9}$ s, respectively at 26 °C and $1.054 \pm 0.0295.10^{-9}$ s and $0.902 \pm 0.0420.10^{-9}$ s, respectively at 37 °C). This effect was not observed with spin probes 12 DSA and 5 DSA on parameter $A_\perp$ or CAT 16 correlation times.

Further experiments were performed to determine whether increased membrane rigidity of luteinized ovaries was connected with a rise in membrane content of cholesterol. As can be seen from figure 4, the profiles in the ovarian molar ratios of total, esterified and free cholesterol to phospholipids throughout pseudopregnancy was
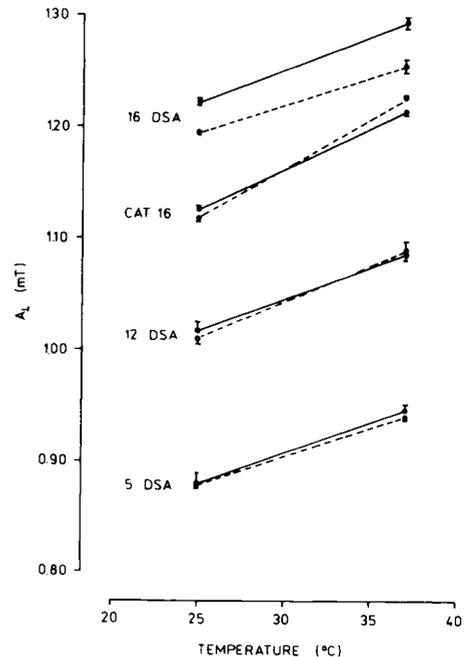
significantly until day 5 after hCG and then returned to control values on d 19. A significant positive correlation was found ($r = 0.68$, $P < 0.01$) between the degree of fluorescence polarization and the accessibility of LH/hCG receptors in ovarian membranes during pseudopregnancy. An even more detailed evaluation of ovarian membrane rigidity was carried out by electron spin resonance. The decrease in $A_\perp$ was interpreted as an increase in membrane rigidity at the given depth within the membrane. Figure 3 shows that the parameter $A_\perp$ for ovarian membranes was lowest with 5 DSA, intermediate with 12 DSA and highest with 16 DSA, reflecting the known mobility of the acyl chain in membranes (McConnel and McFarland, 1972). Increasing the temperature was followed by an increase in parameter $A_\perp$ for each of the spin probes. However, the values of $A_\perp$ for CAT 16 and 16 DSA were outside the range of the linear relationship between the order parameter and $A_\perp$. We therefore evaluated correlation times from our spectra for these 2 spin labels. Membranes isolated from luteinized ovaries on d 6 after hCG and labeled with 16 DSA had signifi-

Fig 3. Temperature dependence of the hyperfine splitting parameter $A_\perp$ of spin probes 16 DSA ($N = 8$), CAT 16 ($N = 3$), 12 DSA ($N = 2$) and 5 DSA ($N = 6$) in ovarian membranes of control rats (full line) or rats 6 days after hCG treatment (dashed line). Shown are the mean ± SE values.
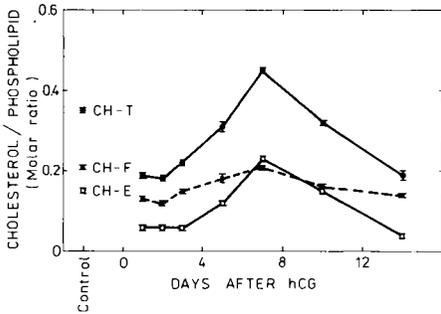
Fig 4. Total (Ch-T), free (CH-F) and esterified (CH-E) cholesterol to phospholipids molar ratios in rat ovarian membrane preparations during pseudopregnancy. Values are the mean ± SE of 4 determinations (each repeated twice).

similar to the dynamics of changes of the degree of polarization of membrane lipid with DPH probe ($r = 0.81$–0.84, $P < 0.02$). However, molar ratios of all cholesterol fractions to phospholipids were significantly ($P < 0.001$) higher in control ovaries (30-day-old rats) than during the first days of pseudopregnancy.

DISCUSSION

Parallel changes in LH/hCG receptor levels and progesterone secretion occurred during pseudopregnancy in rats. Similar changes in receptors and steroidogenesis were observed earlier in prepubertal superovulated rats (Migley Jr *et al*, 1974; Lee *et al*, 1975; Straalen and Zilmaker, 1982). Three periods can be distinguished during the existence of corpora lutea: rising phase, plateau, and regression (Rothchild, 1981). During formation of corpora lutea, the affinity of binding sites for hCG was not affected, and the marked increase of hCG binding was associated with alterations in receptor number (Lee and Ryan, 1973). As luteolysis proceeded, there was a decrease in both LH/hCG receptor and pro-

gesterone levels. A similar relation between corpus luteum LH/hCG receptors and progesterone secretion appears to exist throughout pregnancy in the rat (Kolena *et al*, 1977). The drop in estradiol and testosterone secretion after an initial increase is probably related to the extent to which thecal cells take part in the composition of luteal tissue.

In the present studies, correlation was observed between LH/hCG receptors and membrane lipid rigidity during pseudopregnancy, indicating that the ordering of the lipid environment in which the receptor is embedded can affect the accessibility of the latter. Although diphenylhexatriene is an almost ideal probe for measuring fluidity, since it is hydrophobic and occupies the region near the centre of the bilayer (Engel and Predergast, 1981), more information on the order of membrane lipid can be derived using ESR spin probes. The spin probes 5-, 12- and 16-doxyl stearic acids reflect the mobility of the hydrophobic membrane part at the C_5, C_{12} and C_{16} carbon membrane depth (Griffith and Jost, 1976), and CAT 16 reflects the mobility of the polar part of the membranes (Lassmann and Hermann, 1984). Our experiments indicate that luteinization of the ovary affects motion in the hydrophobic membrane part at about the C_{16} carbon level. Cholesterol in rat testicular membranes also produces a similar ordering effect; this steroid nucleus occupies the outer portion of the membrane bilayer, but the strongest ordering effect is at the deepest location of the gonadal membrane (Kolena *et al*, 1986a). The changes in rigidity of membrane lipid during development of pseudopregnancy are the apparent result of an alteration in the cholesterol to phospholipids ratio. Under physiological conditions and with naturally occurring phospholipids, a rise in the cholesterol/phospholipid ratio will be associated with an increase in membrane rigidity (Shinitzky and Inbar,

1976). It is obvious that the increase of LH/hCG receptors in luteinized ovaries reflects the synthesis of new receptor molecules; however, increased rigidity of membrane lipid may maximally expose receptors maintained in a cryptic form. The increased accessibility of LH/hCG receptors in luteal membranes appears to be in agreement with the concept of vertical displacement of membrane proteins (Borochov and Shinitzky, 1976). According to this concept, the bulk of membrane proteins becomes more exposed to the aqueous medium by increasing membrane rigidity. Our present results, along with previous studies on the action of various cholesteryl esters on membrane lipid fluidity, and the accessibility of LH/hCG receptors in rat testicular membranes (Kolena et al, 1989) are consistent with this presumption.

ACKNOWLEDGMENTS

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