Effect of Neutral Lipids on Fluidity of Corpora Lutea Plasma Membranes and the Accessibility of LH/hCG Receptor

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Abstract. Porcine corpora lutea plasma membranes were treated with neutral lipids from hen egg yolk and a special mixture consisting of 70% neutral lipids, 20% phosphatidylcholine and 10% phosphatidylethanolamine. Membrane fluidity, as determined by fluorescence polarization of DPH, significantly increased already after 30 min of incubation of the membranes with lipids. The fluidization effect of neutral lipids increased with the increasing concentration of lipids up to a concentration of 1 mg.ml^{-1}. In experiments with plasma membranes labelled with [^{3}H]cholesterol the increase in membrane fluidity was found to be partially due to a decrease of membrane cholesterol concentration. The most potent concentration of neutral lipids in extracting cholesterol was 2 mg.ml^{-1}. The increase in fluidity of plasma membranes caused by neutral lipids and lipid mixture decreased the accessibility of the LH/hCG receptor.

Key words: Membrane fluidity — Lipids — Cholesterol — LH/hCG receptor

Introduction

Biological membranes are dynamic structures composed primarily of lipids and proteins capable of influencing each other. The lipids provide a fluid solvent for membrane proteins, which perform a variety of membrane functions. It is believed that some control mechanisms of membrane functions involve the lipid fluidity. The fluidity of membrane lipid domains in turn is known to be dependent on phospholipid classes, the molar cholesterol to phospholipids ratio, the type of cholesterol (free or esterified), the saturated to unsaturated fatty acids ratio, the number of carbons in the acyl chains, and the presence or absence of amphipatic compounds (Traill and Wick 1984; Yawata et al. 1984). A number of reports have indicated that aliphatic alcohols (Dave and Witorsch 1984; Ardail et al. 1990), organic solvents (benzene, toluene, ethylbenzene and styrene) (Naskali et al. 1993), and barbiturates (Mitchell et al. 1985) increase membrane fluidity, thus modulating the function of membrane proteins. Membrane fluidization can also be mediated by incorporation of external

phospholipids or by depletion of membrane cholesterol (Bakardjieva et al. 1979; Yuli et al. 1981; Lyte and Shinitzky 1985; Leibel et al. 1987). It has been shown that fluidity of membrane lipids could be increased by treatment of membranes with phosphatidylcholine (PC) (Yawata et al. 1984; Leibel et al. 1987; Kolena et al. 1994) as well as by a mixture of PC and other lipids (Lyte and Shinitzky 1985). Such membrane fluidization introduces an attractive possibility for the restoration of physiological functions in aging and drug addicted subjects, where membrane rigidification is one of the most prominent feature (Heron et al. 1982).

This paper presents the results of experiments suggesting that neutral lipids (NL) isolated from hen egg yolk and a lipid mixture consisting of NL, PC and PE increased the fluidity of porcine corpora lutea (CL) plasma membranes and that this effect may be partly due to a decreased concentration of membrane cholesterol. Furthermore, changes of membrane lipid fluidity were correlated with the accessibility of LH/hCG receptor.

Materials and Methods

Materials

Phosphatidylcholine (PC) type V-E from egg yolk and phosphatidylethanolamine (PE) type III-A from egg yolk were obtained from Sigma or isolated from hen egg yolk in our laboratory by the method of Singleton et al. (1965). Neutral lipids (NL) were isolated from egg yolk (Lyte and Shinitzky 1985). The purity of the lipids was determined by thin-layer chromatography on silica gel sheets which were activated at $110\,^{\circ}\mathrm{C}$ for 30 min. [^{3}H]cholesterol (60 Ci.mmol^{-1}) from Radiochemical Center, Amersham was purified by TLC on silica gel sheets prior to use. Purified hCG (CR 123; 12780 U.mg^{-1}) was generously supplied by NIAMDD, NIH, Bethesda, MD. Na^{125}I was from Amersham. All solvents were from Lachema, Brno, a fluorescence probe 1,6-diphenyl-1,3,5-hexatriene (DPH) and all other chemicals were purchased from Sigma.

Methods

Porcine corpora lutea were obtained from a local slaughterhouse. The homogenates of CL in ice-cold buffer A (10 mmol.l^{-1} Tris-HCl, 1 mmol.l^{-1} EDTA and 20% sucrose, pH 7.4) were filtered through sixlayers of surgical gauze, centrifuged at $1000 \times g$ for 15 min and the supernatants were centrifuged at $20,000 \times g$ for 30 min (Kolena et al. 1986). The pellets were resuspended in the same buffer without sucrose (200 mg tissue per ml). Plasma membranes were isolated by the method of Eskström and Hunzicker-Dunn (1989). The membrane preparations were layered over a cushion of 45% (w/w) sucrose (9 ml membrane/16 ml cushion) and centrifuged at $60,000 \times g$ for 60 min at $4\,^{\circ}\mathrm{C}$. The interface of membranes on top of sucrose was sucked off, diluted 6-fold, and centrifuged at $20,000 \times g$ for 30 min (Kolena et al. 1992). Polyvinylpyrrolidone (PVP) was used as a hydrophobic carrier in all lipid treatments. The PVP medium consisted of 3.5% PVP, 1% bovine serum albumin and 0.5% glucose in PBS buffer (50 mmol.l^{-1} phosphate buffer and 15 mmol.l^{-1} sodium chloride, pH 7.4). The lipids were diluted in ethanol and introduced into the PVP medium so as to give an ethanol concentration of 1%. The control PVP medium contained 1% ethanol. The plasma membranes of porcine corpora lutea (200

mg proteins/100 μl) were incubated with PVP medium containing various lipid mixtures for different times at 25 °C and gently shaken. After the end of incubation the treated membranes were washed once with PBS buffer, then with 1 mol.l^{-1} KCl, and once again with PBS buffer.

For labelling of membranes with [^{3}H]cholesterol, PVP medium containing 100 ng [^{3}H]cholesterol was incubated for 90 min at room temperature under gentle shaking. After the incubation and centrifugation, the membranes were washed with 1 mmol.l^{-1} KCl and with PBS buffer. Then the membranes were treated with lipid mixtures as described above.

Fluorescence polarization was measured with a Perkin-Elmer LS-5 luminescence spectrometer at 25 °C (Kolena et al. 1986). A solution of 2 mmol.l^{-1} DPH in tetrahydrofuran was dispersed by 1000-fold agitative dilution in 50 mmol.l^{-1} PBS buffer, pH 7.4. Before measurement, the plasma membranes (approx. 100 μg proteins) were incubated for 1 h at 25 °C with 2 ml of 2 μmol.l^{-1} DPH in the above buffer. The degree of fluorescence polarization was computed by the equation:

$$P = [I_{vv} - I_{vh}(I_{hv}/I_{hh})]/[I_{vv} + I_{vh}(I_{hv}/I_{hh})]$$

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

In the hCG binding assay, 0.1 ml aliquots of treated CL membrane fraction were incubated 16 hours at 20 °C with 0.1 ml PBS buffer with or without 100-fold excess of unlabelled hCG and with 0.1 ml [^{125}I]hCG (spec. act. approx. 2.3 TBq.g^{-1}). After the incubation and centrifugation the membrane pellets were washed with 2 ml of cold buffer (Kolena 1976).

Cholesterol was determined with 2,5-dimethylbenzenesulfonic acid in the presence of sulfuric acid (Bio-La-Test, Lachema) or enzymatically (Lachema). Phospholipids were measured colorimetrically in a complex with ammonium ferrothiocyanate (Stewart 1980). Total lipids were established by Bio-La-Test, Lachema (after hydrolyzation with sulfuric acid with vanillin and o-phosphorous acid). Protein was determined by the method of Lowry et al. (1951).

Student's t-test was used for statistical evaluation. Each experiment was repeated two or three times.

Results

Optimal conditions for fluidization of plasma membranes were established by incubating porcine CL plasma membranes for different periods of time with NL/PC/PE mixture (7:2:1) of 0.5 mg.ml^{-1} at temperatures of 25 °C or 37 °C (Fig. 1). The degree of fluorescence polarization decreased already after 30 min of incubation with lipids both at 25 °C and 37 °C. After 6 h of incubation the degree of fluorescence polarization did not change significantly in comparison to values obtained after 3 h of incubation (data not shown). Thus, in the following experiments CL plasma membranes were incubated with lipids for 3 h at 25 °C. As shown in Fig. 1, neutral
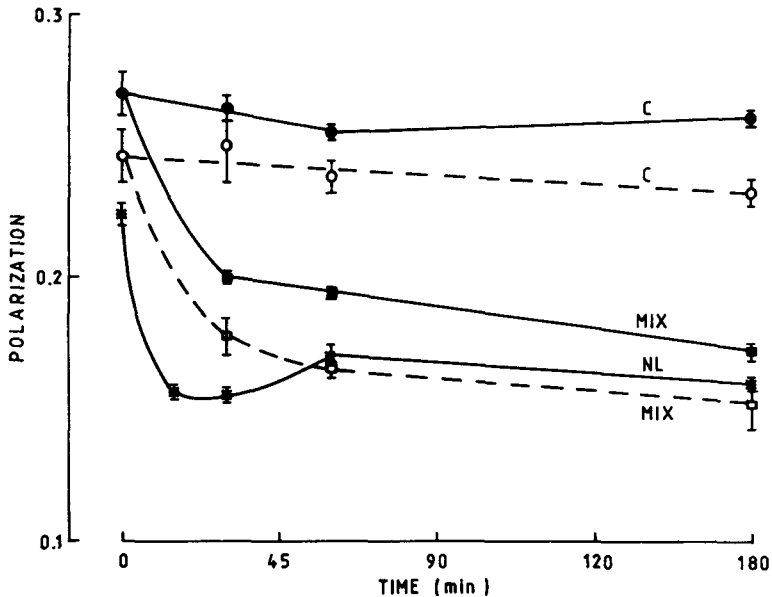
Figure 1. Fluidization of porcine corpus luteum (CL) plasma membranes incubated with NL/PC/PE mixture (0.5 mg.ml^{-1}) at 25 °C (——) or 37 °C (- – – -) and with neutral lipids (NL) (0.5 mg.ml^{-1}) at 25 °C for different periods of time. Control samples were incubated under the same conditions yet with the PVP suspension only. All measurements of fluorescence polarization of the DPH probe were performed at 25 °C. Each point represents the mean ± S.E.M. of four determinations.

lipids alone (0.5 mg.ml^{-1}) induced fluidization of CL plasma membranes already after 15 min of incubation at 25 °C. After 30 min of incubation, the fluidization potency of NL was comparable with that of the lipid mixture. The degree of fluorescence polarization decreased by 30% in the case of NL and 26% in the case of the lipid mixture.

Fig. 2 shows the membrane fluidization potency of lipid mixtures containing different concentrations of neutral lipids. The mixtures had a concentration of 0.5 mg.ml^{-1} and consisted of PC and PE (2:1) and increasing amounts of neutral lipids. The NL/PC/PE mixture containing 85% of neutral lipids was found to be the most potent membrane fluidizer. This experiment confirmed that NL at 0.5 mg.ml^{-1} concentration had a membrane fluidization potency comparable with that of the most potent NL/PC/PE mixture. After incubation with this lipid mixture the degree of fluorescence polarization decreased by 26%, and after incubation with NL by 24%.

The results presented in Fig. 3 indicate that the fluidity of CL plasma membranes increased with the increasing concentrations of NL, up to a concentration of
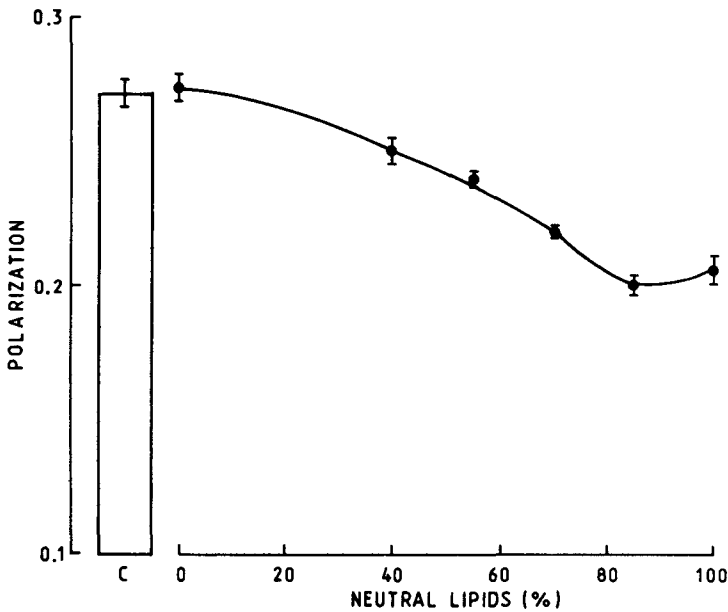
Figure 2. Increase in membrane fluidity of CL plasma membranes incubated with the mixture of PC and PE(2:1) (0.5 mg.ml^{-1}) with increasing concentrations of neutral lipids for 3 hours at 25 °C. Control membranes (C) were incubated with control medium under the same conditions. Each point represents the mean ± S.E.M. of four determinations.

1 mg.ml^{-1}. At this concentration NL were the most effective, decreasing the degree of fluorescence polarization to 42% of control values. With higher concentrations the efficiency of NL was decreasing.

To examine whether the increase of membrane fluidity was not due to extraction of membrane cholesterol by neutral lipids and phospholipids, experiments were carried out with CL plasma membranes labelled with [³H]cholesterol. These membranes were incubated with NL, phospholipids and an NL/PC/PE mixture (0.5 mg.ml^{-1} each) for different periods of time (Fig. 4). The NL/PC/PE mixture was the most potent cholesterol extractor, with the radioactivity decreasing to 38% of controls after 30 min of incubation, and to 34% after 3 hours. The efficiency of NL was comparable with that of the mixture after 3 h of incubation, with the radioactivity decreasing to 47% of controls. PC alone was a less effective extractor than NL. Radioactivity decreased to 63% of controls after 3 h of incubation. PE alone was not able to extract membrane cholesterol. The potency of NL to extract membrane cholesterol increased with the increasing concentrations of NL up to 2 mg.ml^{-1}. At this concentration radioactivity decreased to about 20% of control values (Fig. 5). In further experiments the effect of NL and NL/PC/PE mixture
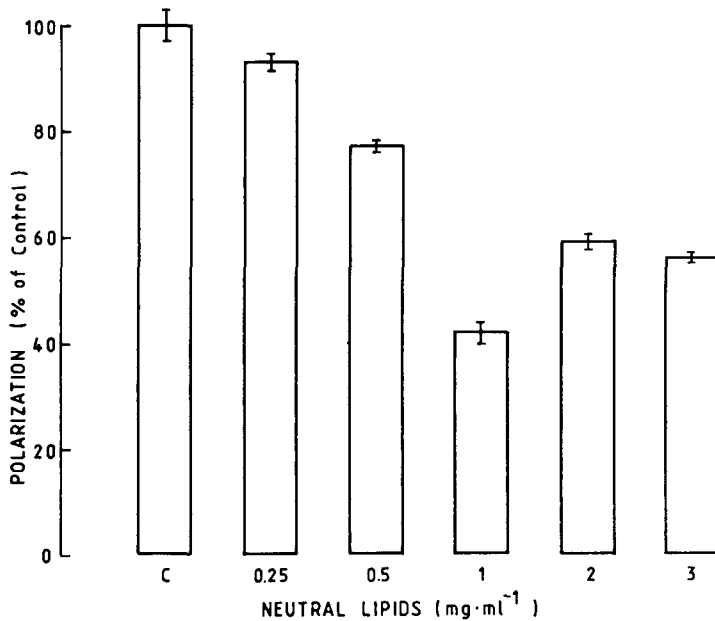
Figure 3. Changes in the degree of fluorescence polarization of the DPH probe in CL membranes treated with different concentrations of neutral lipids (NL) for 3 hours at 25 °C. Control values were 0.270 ± 0.005. Each value represents the mean ± S.E.M. of four determinations.

on the accessibility of LH/hCG receptor was examined. The results shown in Fig. 6 indicate that treatment of CL plasma membranes either with NL or the mixture (0.5 mg.ml^{-1} each) for 30 min had a significant ($P < 0.001$) inhibitory effect on specific binding of [^{125}I]hCG to membranes. The accessibility of the LH/hCG receptor decreased after incubation with NL by 42%, and after incubation with the NL/PC/PE mixture by 56%. This decrease correlated with the increase in membrane fluidity. The contents of total cholesterol, phospholipids and total lipids were determined in untreated CL plasma membranes and in membranes treated for 3 h with NL and the NL/PC/PE mixture. The cholesterol – phospholipid (C/PL) molar ratio was found to be 1.2 in untreated membranes and 0.9 or 0.5 in membranes treated with NL or the mixture, respectively.

Discussion

The results presented in this study not only suggested the ability of the mixture of lipids but also the ability of neutral lipids themselves to fluidize porcine CL plasma membranes *in vitro*. Neutral lipids isolated from hen egg yolk consist almost
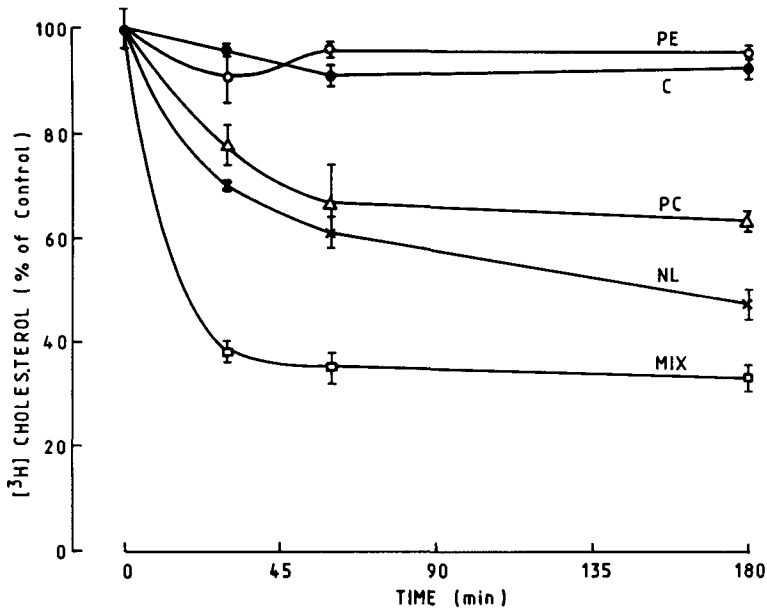
Figure 4. Changes in cholesterol content of CL plasma membranes labelled with [³H]cholesterol after treatment with NL/PC/PE mixture (MIX), neutral lipids (NL), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (0.5 mg.ml⁻¹ each) for different periods of time at 25 °C. Control samples were incubated with control PVP medium under the same conditions (C). Control values at time 0 were approx. 296.000 cpm per mg protein. Each value represents the mean ± S.E.M. of three determinations.
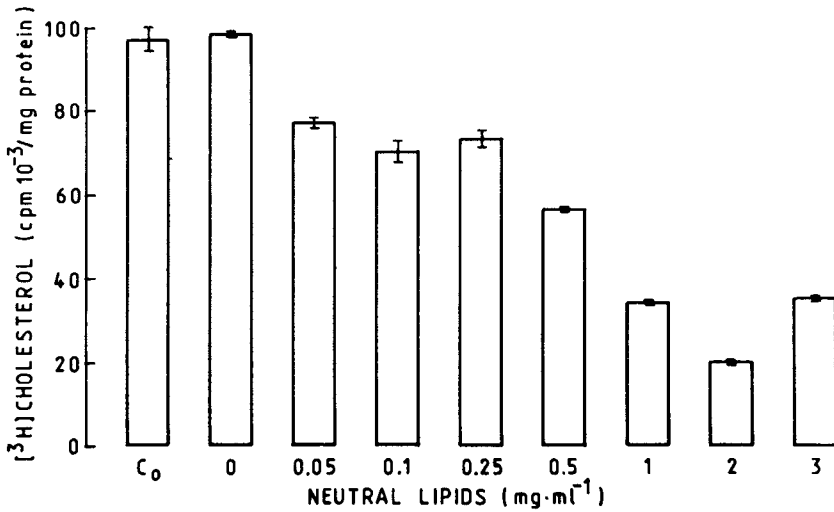

Figure 5. Effect of neutral lipids (NL) on cholesterol content of CL plasma membranes. The membranes labelled with [³H]cholesterol were incubated with different concentrations of NL for 3 hours at 25 °C. C_0 represents radioactivity of [³H]cholesterol incorporated in membranes before incubation with NL. Each value represents the mean ± S.E.M. of three determinations.

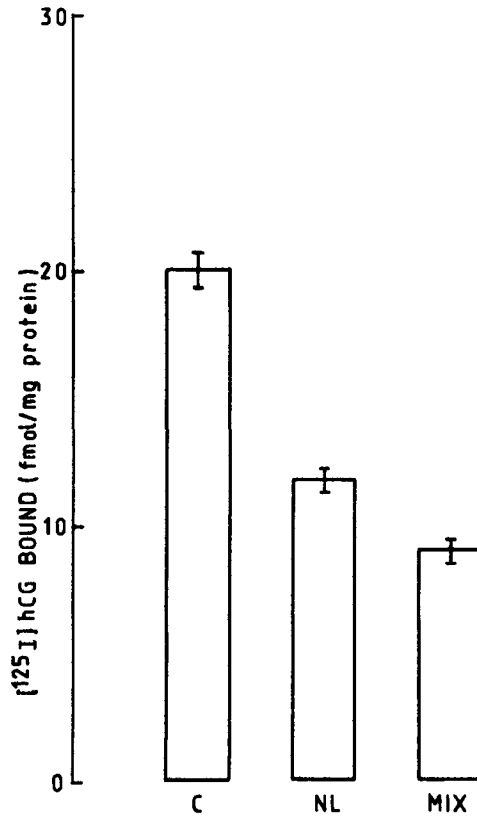**Figure 6.** Specific binding of [^{125}I]hCG to porcine CL plasma membranes incubated with neutral lipids (NL) (0.5 mg.ml^{-1}) and NL/PC/PE mixture (0.5 mg.ml^{-1}) for 30 minutes at 25 °C. Each value represents the mean ± S.E.M. of five determinations.

exclusively of triacylglycelors. Neutral lipids were found to increase the fluidity of CL plasma membranes already after a short time (15 min), and this effect was partially due to the depletion of cholesterol in membranes. The fluidization effect of NL on membranes was shown to be associated with a decrease of the accessibility of the LH/hCG receptor.

Depending on the system used, the activity of the membrane hormonal receptors can be modified by alterating membrane lipid composition and thus by changing membrane fluidity. It is generally believed that the ordering of the lipid environment in which the receptor is embedded can affect its accessibility. Membrane receptors can be differently affected by changes of lipid fluidity in different cells and after different treatments. When lipid fluidity is increased, prolactin receptors from the membranes of the ventral prostate (Dave and Witorsch 1983)

and beta-adrenergic receptors in liver cell membranes (Bakardjieva et al. 1979) became more exposed. On the other hand, a decrease of fluidity enhanced exposition of beta-adrenergic receptors in rabbit reticulocytes (Strittmatter et al. 1979) and of serotonin receptors in mouse brain membranes (Heron et al. 1980). A previous study (Kolena et al. 1990) showed that increase of rigidity of membrane lipids was positively correlated with the accessibility of LH/hCG receptors during pseudopregnancy. The changes in rigidity of membrane lipids observed during the development of pseudopregnancy were evidently a result of an alteration in C/PL molar ratio. Under physiological conditions and with naturally occurring phospholipids, a rise in the C/PL ratio will be associated with a decrease in membrane fluidity (Shinitzky and Inbar 1976). 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 upon increasing membrane rigidity. Also, incubation of rat testicular membranes with cholesterol-hemisuccinate resulted in an increase in both membrane lipid rigidity and the accessibility of the LH/hCG receptor (Kolena et al. 1986). Cholesterol is one of the major lipid constituents of most biological membranes. As a structural membrane component, it is involved in maintaining the cell integrity and membrane fluidity. Cholesterol fluidizes the gel phase and has a condensing effect on the liquid-crystalline phase of membrane phospholipids. However, the rigidifying action of cholesterol on membranes may not be the sole cause of the increased accessibility of the LH/hCG receptor (Kolena and Kasal 1989). It has been shown that in aqueous dispersion neutral lipids (mostly acylglycerols) with or without phospholipids can form chylomicron-like assemblies which are supposed to facilitate the translocation of membrane cholesterol and along with phospholipids to serve as a source for monomeric phospholipids (Lyte and Shinitzky 1985.)

In the present study, the treatment of porcine CL plasma membranes with neutral lipids and with the mixture of neutral lipids and phospholipids, operating mainly by extracting membrane cholesterol, resulted in an increase of membrane fluidity associated with a decreased accessibility of the LH/hCG receptor. These results suggest a crucial role of optimum fluidity of membrane lipids and of the presence of cholesterol during the interaction of gonadotropins with the receptor.

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