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Structure-stabilizing effect of albumin on rat ovarian LH/hCG receptors

Jaroslav Kolena *, Miroslava Ježová, Janka Vranová, Soňa Scsuková

Institute of Experimental Endocrinology, Slovak Academy of Sciences, Vlarska 3, 833 06 Bratislava, Slovak Republic

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Abstract

The stabilizing effect of albumin on structure-functional alteration of LH/hCG receptors was analyzed by thermal perturbation technique. On exposing the membranes to bovine serum albumin (BSA) the heat inactivation profile of hCG-binding sites was shifted to a temperature higher by about 5°C (T_{50} values). The receptor destabilizing action of arachidonic and oleic acids incorporated into ovarian membranes and reversal of this effect when BSA was used as fatty acid scavenger, may indicate that free fatty acids are responsible for the thermal instability of hCG-binding sites. This presumption was corroborated by digestion of membranes with phospholipase A_2 (PLA_2). This enzyme exerted effects on the thermal stability of the receptor protein resembling those observed upon insertion of fatty acids. The membrane fluidization induced by arachidonic acid can be reversed by BSA. However, alterations of lipid fluidity in membranes were not found to be a necessary prerequisite for stabilization of the LH/hCG receptor structure. Fluorescence quenching studies indicated that incorporation of oleic acid or digestion of membrane phospholipids with PLA_2 elevated the accessibility of fluorophores for acrylamide. BSA scavenging of free fatty acids approached the quenching rate of control membranes. Analysis of fluorescence of membranes bound to monodansylcadaverine probe revealed that the negative surface charge derived from free fatty acids resulted in destabilization of the receptor protein. The effects of free fatty acids on membranes suggest that altered lipid-protein interactions may directly affect the stability of the LH/hCG receptor structure.

Keywords: LH/hCG receptor; Thermal inactivation; Fluorescence quenching; Fatty acid

1. Introduction

The stability of macromolecules in their native conformation has become a topic of increasing attention over the last several years. Previous studies of hormonal receptors indicated that glycerol had a stabilizing effect on detergent-solubilized ligand-binding activity. The favorable effect of glycerol on the preservation of the activity of LH/hCG receptors and on storage of adenylylcyclase system preparations is well known [1,2]. Studies using several proteins have suggested that glycerol may act by decreasing the surface tension of aqueous solvents and by causing preferential hydration of proteins [3]. We demonstrated some beneficial effects of glycerol and other osmolytes on reconstitution of LH/hCG receptors into proteoliposomes [4]. ESR experiments indicated

Abbreviations: AA, arachidonic acid; CH, cholesterol; DPH, 1,6-diphenyl-1,3,5-hexatriene; Lyso-PC, L-α-lysophosphatidylcholine palmitoyl; MDC, monodansylcadaverine; OA, oleic acid; OApalmitoyl, oleic acid palmitoyl ester; PLA_2, phospholipase A_2; PMSG, pregnant mare's serum gonadotropin; SA, stearic acid

* Corresponding author. Fax: +421 (7) 54774247;
E-mail: ueenkole@savba.sk

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that the stimulatory effect of glycerol may be related to the modified physical state of lipids in the bilayer. Thermal perturbation was used to monitor structural changes in receptors [5–7]. Heat inactivation of LH/hCG-binding sites demonstrated a significant stabilization of the LH/hCG receptor structure when proteoliposomes were reconstituted in the presence of osmolytes.

Several mammalian cells require the addition of serum to the culture medium for optimal growth and protection of cell lines. Plasma components such as albumin, immunoglobulins and fibronectin are found to interact with the bilayer, giving rise to the stabilization effect [8]. In recent years many reports have been published concerning the interaction of albumin with phospholipid vesicles used as a delivery vehicle for drugs, enzymes and antibodies [9]. The higher binding of LH/hCG, as well as of other proteohormones, to their specific receptors in buffer containing bovine serum albumin (BSA) is well known. This report presents considerable evidence that BSA stabilized the LH/hCG receptor with concomitant structural changes, analyzed by heat perturbation technique in its native membrane environment.

2. Materials and methods

2.1. Materials

Purified hCG (CR 127, 14 900 IU mg^{-1}) was generously supplied by NHPP, NIDDK (Bethesda, MD). $Na^{125}I$ and [^{14}C]arachidonic acid were purchased from the Radiochemical Center, Amersham. Pregnant mare's serum gonadotropin (PMSG) and hCG (Praedyn) were from Spofa (Prague). 1,6-Diphenyl-1,3,5-hexatriene (DPH), monodansylcadaverine (MDC), cholesterol (CH), phospholipase A_2 (PLA_2) (from porcine pancreas, 760 U mg^{-1}), oleic acid (OA), arachidonic acid (AA), stearic acid (SA), oleic acid palmitoyl ester (OApalmitoyl), L-α-lysophosphatidylcholine palmitoyl (Lyso-PC), bovine albumin, Fraction V, fatty acid free BSA and all other chemicals were from Sigma. BSA free from fatty acids was also prepared by the charcoal treatment procedure of Chen [10].

2.2. Methods

2.2.1. Preparation of membranes

Luteinized ovaries were produced in 25-day-old rats (Wistar strain) by s.c. administration of 50 IU PMSG followed 56 h later by 30 IU hCG [4]. 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 $20\,000 \times g$ for 30 min. The final membrane preparations were resuspended in the same buffer [4].

2.2.2. Lipids and BSA treatments

Fatty acids and cholesterol were dissolved in dimethyl sulfoxide and after addition of buffer A with 3.5% polyvinylpyrrolidone the dispersion was briefly (15 s) sonicated [6]. Ovarian membranes were incubated at 37°C for 30 min with lipids. Membranes were washed twice and incubated at 24 or 4°C for 30 min with defatted BSA or at 37°C for 30 min with PLA_2 in the presence of 2 mM $CaCl_2$. Finally, membranes were washed twice with buffer A.

2.2.3. Thermal inactivation

Aliquots of membrane-bound receptors were heat inactivated in a 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 binding activity determination [6].

2.2.4. hCG binding assay

In hCG binding assay, 0.1 ml aliquots of ovarian membranes were incubated for 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 [6]. The results are expressed as [^{125}I]hCG specific binding per mg protein [11].

2.2.5. Fluorescence polarization

Fluorescence polarization was measured by a Perkin-Elmer LS-5 luminescence spectrometer, equipped

with a circulation bath to maintain the sample temperature at 24°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 [12].

2.2.6. Binding of fluorescence probe

Monodansylcadaverine was dissolved in methanol, added to the membranes in buffer A. Ovarian membranes were incubated at 24°C for 30 min with 10 μM MDC. Excitation and emission wavelengths were 330 and 520 nm, respectively. F_{max} was determined by extrapolating reciprocal plot to infinite protein concentration [13]. The values of the bound and free MDC were estimated from fluorescence intensities at each concentration of F_{max} as described by Azzi [14]. The dissociation constants (K_d) and the number of binding sites (N) were obtained from Scatchard plots.

2.2.7. Quenching measurements

Quenching studies were carried out at 24°C by adding small amounts of 5 M acrylamide in buffer A, pH 7.4. The fluorescence intensity was measured as a function of quencher concentration at a fixed emission wavelength of 340 nm. The excitation wavelength of 280 nm was used. The Stern-Volmer quenching constant, K_{sv}, was calculated according to the Stern-Volmer equation $F_0/F = 1+K_{sv}[Q]$, where F_0 is the fluorescence of the unquenched fluorophore and F is the fluorescence at quencher concentration [Q] [15,7]. The squares method was used to calculate K_{sv}.

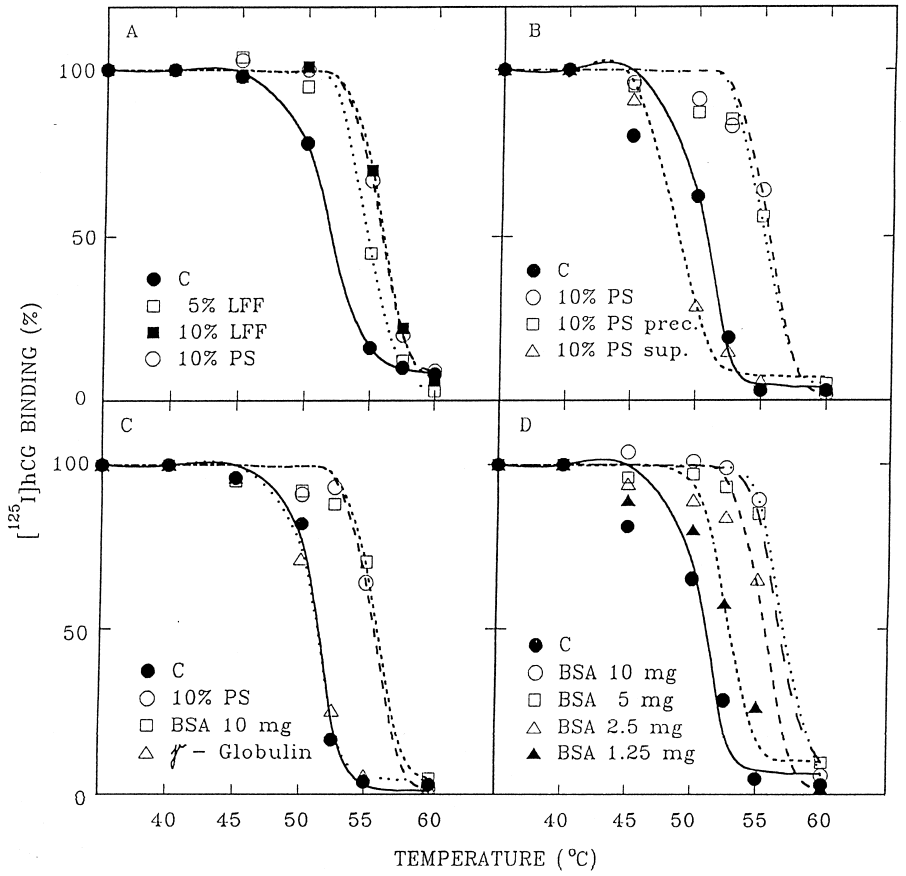

Fig. 1. Heat inactivation profile of LH/hCG receptors. The rat ovarian membrane receptor was heat inactivated in the absence (C) or presence of follicular fluid (LFF), porcine serum (PS), proteins of PS (PS prec.) or BSA. Control values of binding were about 90 fmol hCG bound per mg protein. Means of two estimations are shown. The experiments were repeated two or three times with similar results.

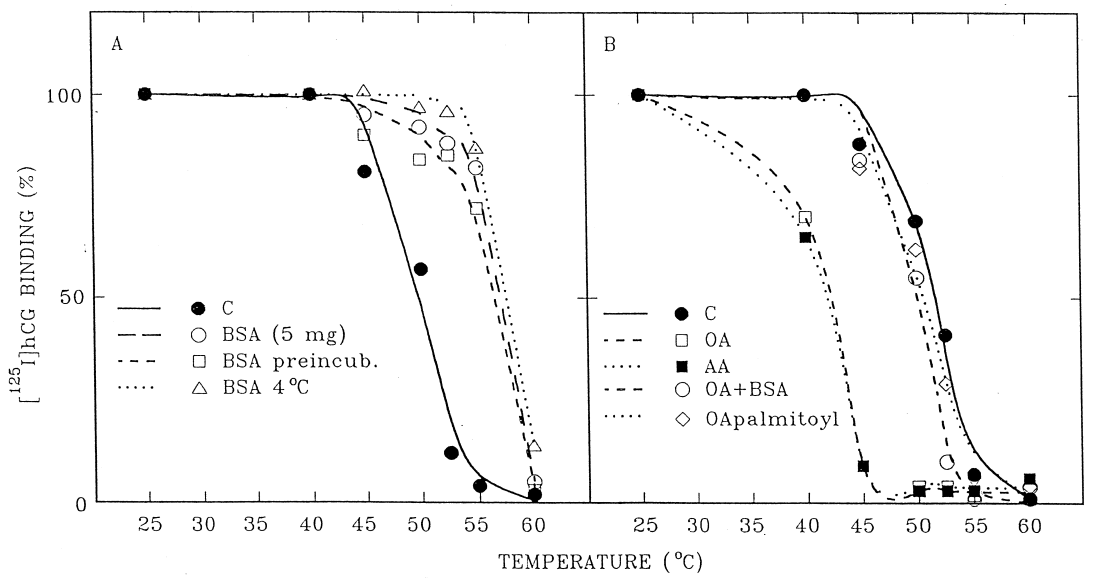

Fig. 2. Reversibility of fatty acid (2 mM) thermal destabilization of the rat ovarian LH/hCG receptor with BSA (5 mg ml^{-1}). For details, see legend to Fig. 1.

2.2.8. Data analysis

Data were analyzed by ANOVA followed by Bonferroni's multiple range test. The results were confirmed in 2–3 independent experiments.

3. Results

In our previous study, we reported that follicular fluid isolated from preovulatory follicles of porcine ovary (LFF) induced formation of LH/hCG receptors [16]. Fig. 1A shows that rat ovarian LH/hCG receptor can be stabilized in the presence of LFF during heat inactivation. The 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 is preserved [5,6]. The T_{50} value of about 52°C in control membranes increased to almost 56°C in LH/hCG receptors heat inactivated in the presence of 10% LFF. However, the same stabilizing effect was observed when ovarian membranes were treated in the presence of porcine serum or isolated proteins after ethanol precipitation (Fig. 1B). Addition of some plasma components to ovarian membranes showed that BSA had a stabilizing effect on the LH/hCG receptor structure. Thermal inactivation of the receptor with different concentrations of BSA showed that T_{50} values were higher by approx. 5°C in the presence of the maximal effective dose of 5 mg ml^{-1} (Fig. 1C,D). The stabilizing effect of BSA was not dependent on its presence in the course of thermal inactivation. The effect was similar when membranes were just preincubated for 30 min at 24 or 4°C (Fig. 2A). The binding experiments with [^{125}I]BSA documented that ovarian membranes did not exhibit specific albumin-binding sites and that albumin absorbed on membranes could be effectively washed out (data not shown).

It is well known that BSA strongly interacts with free fatty acids. Such extraction of FFA might alter the stability of ovarian receptors. Fig. 2B shows that preincubation of ovarian membranes with 2 mM concentration of fatty acids decreased the T_{50} values by more than 9°C. Addition of OA palmitoyl ester to membrane receptors had, however, no effect. Thermal destabilization of the LH/hCG receptor with OA could be fully inverted by treatment with BSA (Fig. 2B). Removal of fatty acids from membranes was analyzed by incorporating [^{14}C]AA into membranes. After separation of [^{14}C]AA associated with membranes most of the radioactivity was removed with 5 mg ml^{-1} of BSA at 24 or 4°C (Fig. 3). Thus the interaction of albumin with ovarian membranes results in extraction of free fatty acids, leading to stabilization of LH/hCG receptors. Cholesterol is a vital

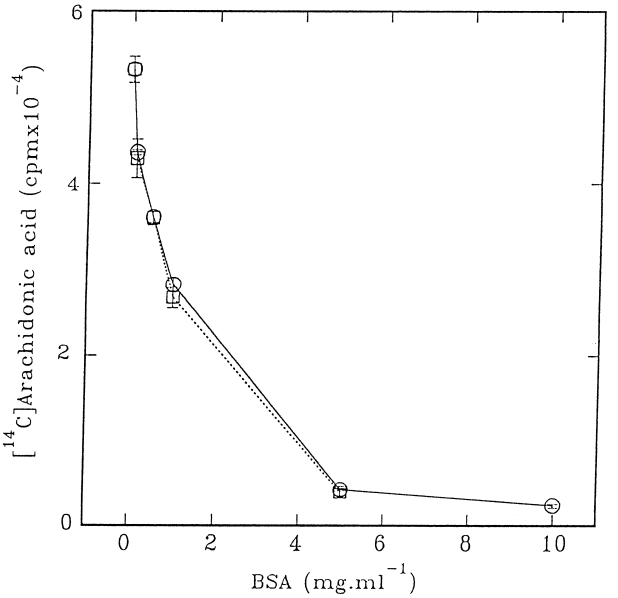

Fig. 3. Removal of AA from membranes by increasing concentrations of BSA. Ovarian membranes containing [^{14}C]AA were incubated with BSA for 30 min at 24°C (full line) or at 4°C (dotted line). After washing membranes were solubilized in Soluene-100. Means of three estimations are shown.

lipid constituent of membranes. However, cholesterol or cholesteryl-hemisuccinate had no beneficial effect on the AA heat inactivation profile of LH/hCG-binding sites (Fig. 4A). Thermal inactivation of the LH/hCG receptor is a quick process. Incubation of untreated membranes at a constant temperature of 48°C resulted after 5 min in an about 25% loss of binding sites, while membranes containing OA exhibited an almost 90% decrease. Thermal destabilization of the LH/hCG receptor was completely reversed by treatment with BSA (Fig. 4B).

Treatment of ovarian membranes with PLA_2 resulted in hydrolysis of phospholipids with a concomitant decrease of hCG-binding activity [17,18]. The hydrolysis was accompanied by formation of fatty acids and lysophosphatides. Fig. 5A shows that digestion of ovarian membranes with PLA_2 diminished in a dose dependent manner the stability of the LH/hCG receptor. Thermal destabilization of hCG-binding sites could be reversed by BSA. Albumin binds fatty acids as well as lysophosphatides [17]; however, heat inactivation of the receptor appeared to be little affected by lysophosphatide (Fig. 4A). Endogenous regulation of PLA activity is a complex mechanism, with several factors modulating its activity. Calcium is a leading candidate. Stimulation of endogenous PLA activity by 10 mM calcium caused a decrease of T_{50} by about 5°C compared to controls incubated with the calcium chelator EGTA. The presence of the same concentration of magnesium had no effect. Treatment of incubated membranes with BSA stabilized the receptor over the control values, presum-

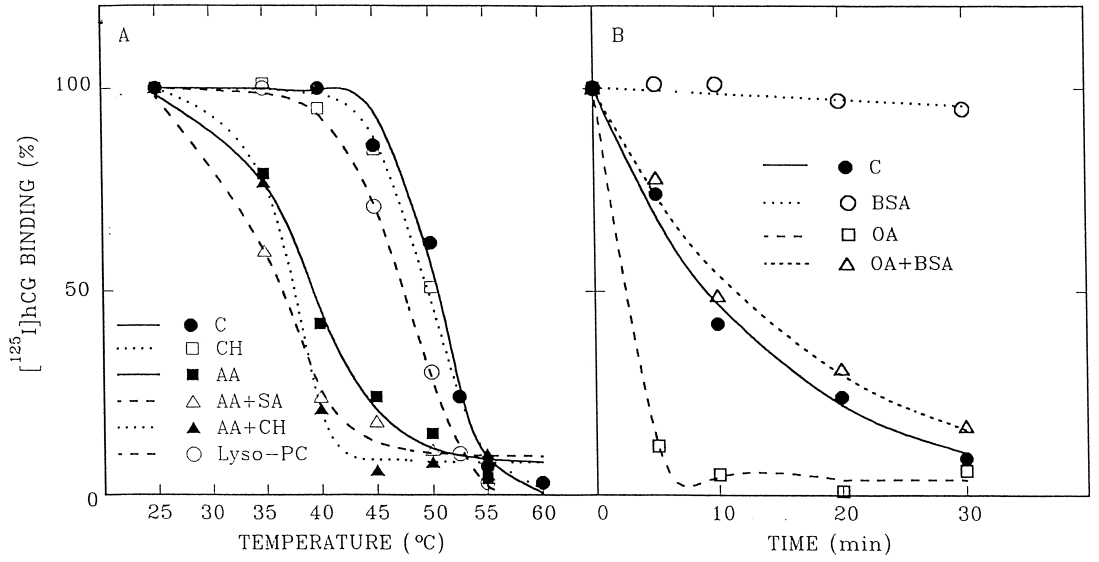

Fig. 4. Effect of fatty acids (2 mM), cholesterol (0.5 mg ml^{-1}) and lysophosphatidylcholine (1 mM) on stability of the receptor (A) and time-dependent heat inactivation of LH/hCG receptors at a constant temperature of 48°C (B). Membranes were treated as described in the legend to Fig. 1.

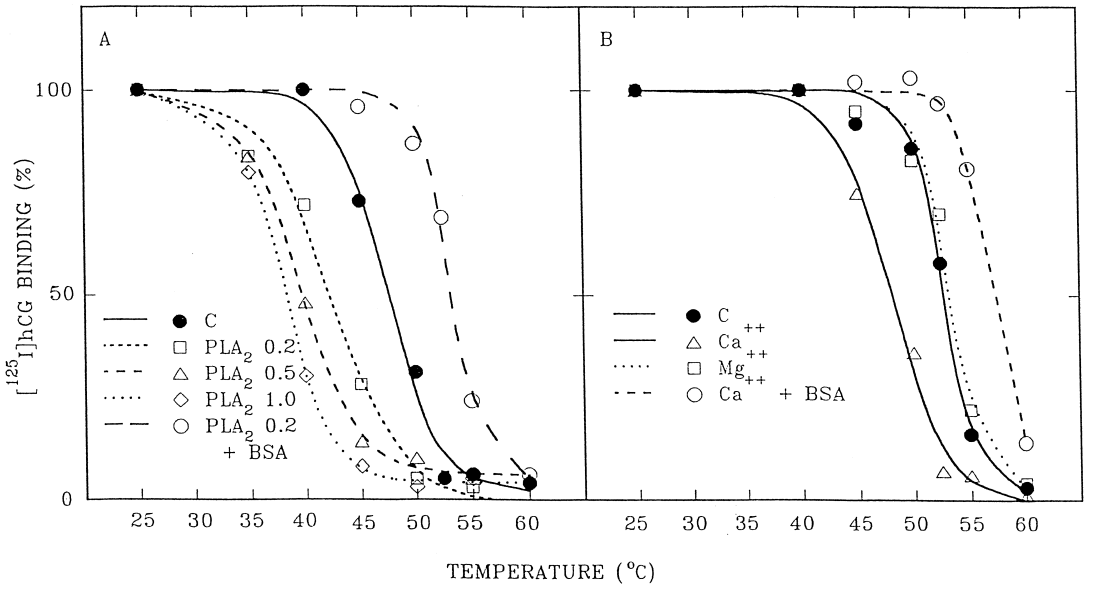

Fig. 5. Reversing effect of BSA (5 mg ml^{-1}) on changes induced by phospholipase A_2 and Ca^{2+} at thermal inactivation of LH/hCG receptors. (A) Ovarian membranes were first preincubated with 0.2–1.0 U ml^{-1} PLA_2 in buffer A containing 2 mM $CaCl_2$ for 30 min at 37°C. Membranes were then washed twice and submitted to heat inactivation. (B) Membranes were inactivated in the presence of 10 mM $CaCl_2$ or $MgCl_2$.

ably as the result of extraction of fatty acids contained in the membranes (Fig. 5B).

A series of experiments was carried out to determine whether the changes in thermal stability of LH/hCG receptors were linked with alterations in the physical state of membranes. Fig. 6 illustrates that the destabilizing effect of AA on the LH/hCG receptor is not associated with the ordering of the lipid environment in which the receptor is embedded. Insertion of AA into ovarian membranes significantly ($P < 0.001$) decreased membrane lipid rigidity and BSA reversed this effect. The same effect was found with cholesterol, in which, however, stabilization of LH/hCG receptor structure was not observed (Fig. 4A). Moreover, preincubation of membranes with cholesterol or methyl-β-cyclodextrin (20 and 60 mg ml^{-1}), which was found to selectively extract cholesterol from the plasma membrane [19], had no effect on thermal inactivation of the LH/hCG receptor (data not shown). On monitoring protein-lipid interaction, further valuable information can be obtained by quenching experiments. We used acrylamide, a neutral dynamic quencher, to see whether fatty acid modified the quenching of protein fluorescence. The Stern-Volmer quenching constant (K_{sv}) determined from the Stern-Volmer plots for control and OA-treated membranes was found to be 2.8 M^{-1} and 6.9 M^{-1}, respectively, indicating that OA increased the accessibility of fluorophores for acrylamide (Fig. 7A). Extraction of OA with BSA was comparable to the quenching of BSA-treated membranes (4.7 M^{-1} vs. 4.5 M^{-1}). Preincubation of membranes with

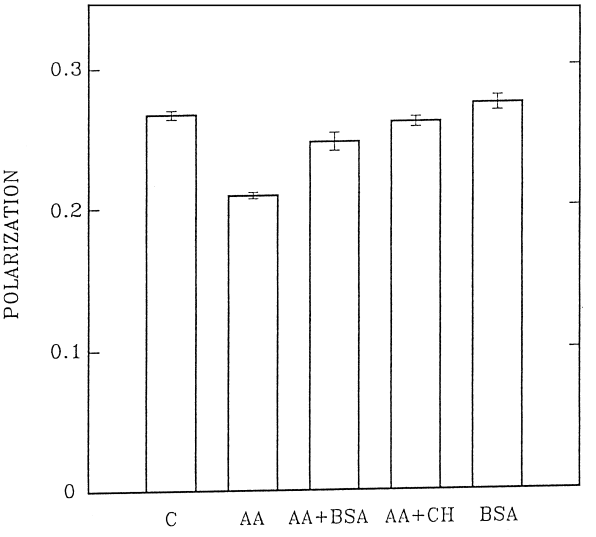

Fig. 6. Effect of lipids on fluorescence polarization of DPH probe in ovarian membranes. Membranes were treated as described in the legend to Fig. 4A.

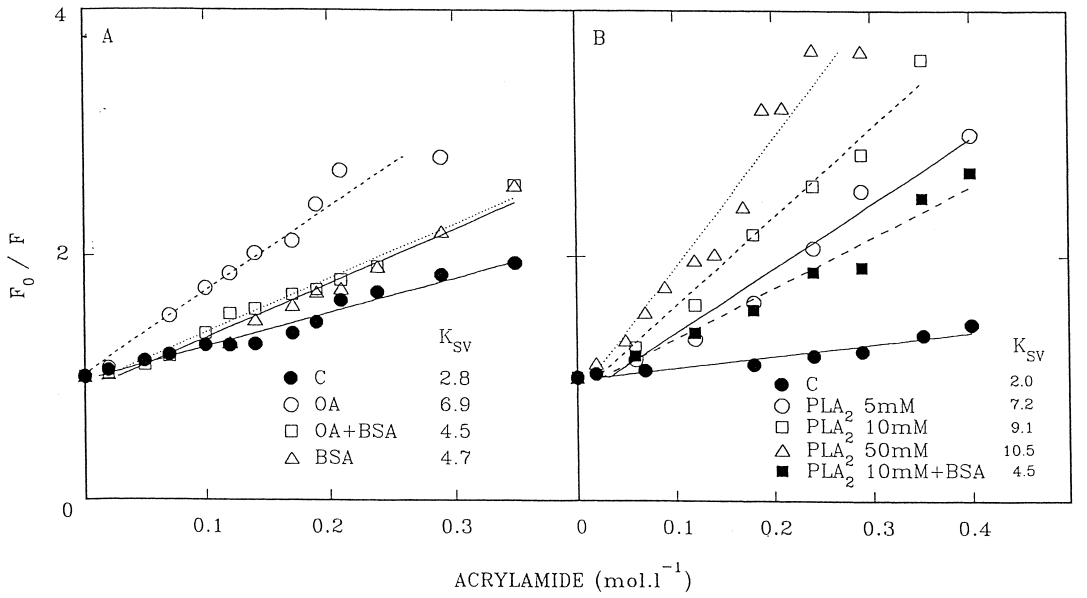

Fig. 7. Stern-Volmer plot of acrylamide quenching of membranes. Ovarian membranes were treated with oleic acid (2 mM) or PLA_2 as described in the legend to Fig. 5. After washing, the quenching of protein fluorescence was estimated. Means of two estimations are shown.

PLA_2 resulted in a similar dose-dependent increase in the accessibility of ovarian fluorophores and in a comparable BSA inverting effect (Fig. 7B).

Free fatty acids may provide a negative surface charge to the membrane bilayer [20]. Evaluation of the membrane-bound fluorescence probe MDC revealed a distinct difference in the fluorescence intensities in BSA-treated membranes compared to controls. This difference may be explained by the altered binding capacity of the ovarian membrane resulting from a decreased negative surface charge. The fluorescence of the MDC probe carrying a positive charge was decreased. The number of binding sites and receptor affinity of MDC derived from Scatchard plots were decreased after extraction of membrane free fatty acids with BSA (Fig. 8).

4. Discussion

The results of this study showed that albumin had a stabilizing effect on rat ovarian LH/hCG receptor structure. Thermal perturbation methods were used to monitor structural changes in the receptor. Heat inactivation of hormone-binding sites on the receptor molecule provides a direct approach to evaluate alterations of receptor conformation which occur as a

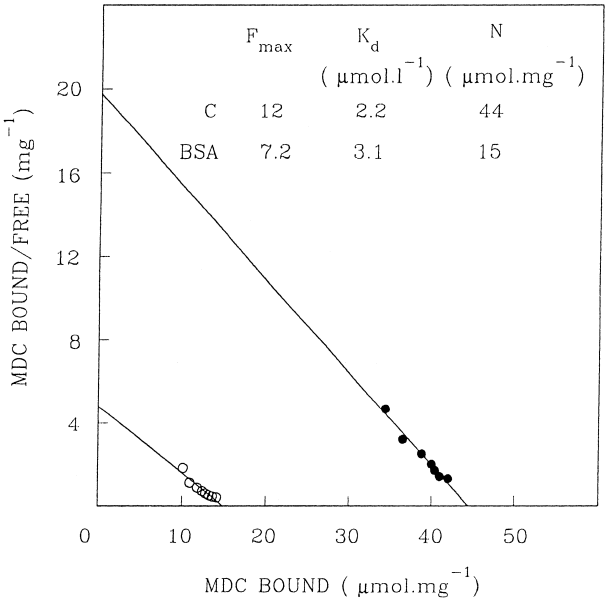

Fig. 8. Scatchard plot of the binding of monodansylcadaverine (MDC) to ovarian membranes. Membranes were incubated without or with BSA (5 mg ml^{-1}) and fluorescence intensity of MDC was estimated at various concentrations of MDC and fixed amounts of membrane protein (0.4 mg) at 24°C. The dissociation constant (K_d) and the number of binding sites (N) of MDC were obtained as described in Section 2.2.

result of hormone binding or perturbation of membrane bilayer [5–7]. Previously, we showed that the model of thermal inactivation of hCG-binding sites on the ovarian membrane receptor yielded information similar to that obtained by differential scanning calorimetry [6]. The observed thermal transition was found to be sensitive to the presence of hCG, supplying further evidence on specific structural changes of the receptor. Thermal stabilization of the LH/hCG receptor produced by BSA caused an increase in T_{50} values by about 5°C. However, the differences in binding activity were not associated with any alteration of the relative affinity of the receptor for hCG (data not shown). Albumin is known to bind free fatty acids and lysophosphatides and to remove them from the lipid bilayer. The receptor destabilizing effect of fatty acids incorporated into ovarian membranes and the reversal of this activity by defatted albumin corroborates the hypothesis that free fatty acids are responsible for the thermal instability of hCG-binding sites. The stability of the receptor was only slightly affected after insertion of lysophosphatides. The role of fatty acids was further strengthened by experiments with phospholipase A_2. PLA_2 cleaves acyl chains from the *sn*-2 position of the glycerol backbone of the phospholipid, yielding free fatty acids (predominantly arachidonic acid) and lysophosphatides. The resulting lysophospholipid may then be further degraded by a lysophospholipase to fatty acids. The results reported here indicate that the hydrolysis of *sn*-2 ester bonds of phospholipids by PLA_2 produced effects on the thermal stability of hCG-binding sites resembling those observed upon insertion of free fatty acids. The inhibitory effect of PLA_2 was reversed upon removal of phospholipid by-products from the membranes with BSA [17]. However, free fatty acids in membranes may be produced not only by PLA, but also by other hydrolases, such as triacylglycerol lipase, cholesterol esterase and acylcarnitine hydrolase. The free fatty acid content in the membrane actually represents a balance between the release of acids by hydrolysis and their reesterification by acyl transferases. It is highly conceivable that free fatty acids released from membrane phospholipids have physiological roles, distinct from their role as precursors in the formation of prostaglandins.

There have been many attempts to show that modulation of the physical state of the lipid matrix in membranes may affect the accessibility and function of receptors. Thus the activities of serotonin, insulin, prolactin and LH/hCG receptors were found to be affected by changes in the order of membrane lipids [21,22,12]. Free fatty acids have a pronounced effect also on the activity of several integral membrane proteins, such as enzymes or receptors [12]. The fluorescence polarization results of this study show that the membrane fluidization induced by arachidonic acid can be reversed by BSA. However, the rigidifying effect of cholesterol, similar to that of BSA, did not change the destabilizing activity of arachidonic acid on the receptor. The results suggest that alterations of lipid fluidity in ovarian membranes may not be a necessary prerequisite for stabilization of the LH/hCG receptor structure. The cholesterol-LH/hCG interaction is especially interesting. As a structural membrane component, cholesterol is involved in the preservation of cell integrity and membrane fluidity. And yet, despite the widely accepted assumption that cholesterol plays an important role in maintaining the functional state of receptors [5], our results showed that neither addition nor removal of cholesterol from ovarian membranes had any effect on the thermal stability of the LH/hCG receptor.

Specific lipid-protein and lipid-lipid interactions result in a precisely controlled dynamic architecture of membrane components. The intrinsic fluorescence of protein appears to be a valuable probe in monitoring protein conformation and protein-lipid interaction. Quenching experiments indicated that free fatty acids elevated the accessibility of fluorophores for acrylamide. An increased quenching rate generally suggests exposure of tryptophanyl residues and changes in the dynamics of the protein matrix surrounding such residues [15]. The extracellular domain in the rat luteal LH/hCG receptor contains a tryptophan residue (amino acid 297). The second tryptophan molecule is located at the first connecting loop [23]. In addition to extracellular regions of the receptor, Roche et al. [24] identified an extracellular connecting loop as an additional potential hormone contact site. Recently, we demonstrated that modification of tryptophan residues was associated with destabilization of the LH/hCG receptor structure [25]. The clean-cut effect of free fatty acids on ovarian membranes suggests that the altered lipid-protein

interactions are directly affecting the stability of the LH/hCG receptor structure. This effect may be caused by the presence of a net negative surface charge provided by free fatty acids [20]. This assumption is supported by binding studies with the fluorescence probe monodansylcadaverine. The reduced fluorescence intensity of MDC in membranes induced by free fatty acids was caused by an increase in the dissociation constant as well as a decrease in the number of binding sites of MDC. These results correspond with the established finding that the MDC probe occupies preferentially anionic binding sites. Removal of free fatty acids with BSA would cause the membranes to lose their negative surface charge, thus promoting stabilization of the LH/hCG receptor structure.

Acknowledgements

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References

[1] J.A. Dias, J.S. Huston, L.E. Reichert Jr., Endocrinology 109 (1981) 736–742.
[2] L. Birnbaumer, P.C. Yang, M. Hunzicker-Dunn, J. Bockaert, J.M. Duran, Endocrinology 99 (1976) 163–184.
[3] K. Gekko, S.H. Timasheff, Biochemistry 20 (1981) 4667–4676.
[4] J. Kolena, K. Matejčíková, G. Šrenkelová, Mol. Cell. Endocrinol. 83 (1992) 201–209.
[5] A. Artigues, M.T. Villar, A.M. Fernandez, J.A. Ferragut, J.M. Gonzales-Ros, Biochim. Biophys. Acta 985 (1989) 325–330.
[6] J. Kolena, S. Scsuková, M. Tatara, P. Jasem, Biochim. Biophys. Acta 1193 (1994) 293–300.
[7] J. Kolena, S. Scsuková, M. Ježová, J. Furdová, M. Tatara, P. Jasem, Mol. Cell. Endocrinol. 113 (1995) 53–60.
[8] D. Liu, L. Huang, Biochemistry 28 (1989) 7700–7707.
[9] G. Gregoriadis, Liposomes as Drug Carriers: Recent Trends and Progress, J. Wiley, Chichester, 1988.
[10] R.F. Chen, J. Biol. Chem. 242 (1967) 173–181.
[11] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265–275.
[12] J. Kolena, P. Blažíček, Š. Horkovics-Kováts, K. Ondriaš, E. Šeboková, Mol. Cell. Endocrinol. 44 (1986) 69–76.
[13] W. Witt, G. Gercken, Biochim. Biophys. Acta 862 (1986) 100–110.
[14] A. Azzi, Methods Enzymol. 32B (1974) 234–246.
[15] M.R. Efting, C.A. Ghiron, Biochemistry 15 (1976) 672–680.
[16] J. Kolena, C.P. Channing, Hormone Res. 21 (1985) 185–198.
[17] S. Azhar, A.K. Hajra, K.M.J. Menon, J. Biol. Chem. 251 (1976) 7405–7412.
[18] J. Kolena, A. Danišová, K. Matejčíková, Endocr. Regul. 26 (1992) 171–174.
[19] U. Klein, G. Gimpl, F. Fahrenholz, Biochemistry 34 (1995) 13784–13793.
[20] R.M. Straubinger, N. Duzgunes, D. Papahadjopoulos, FEBS Lett. 179 (1985) 148–154.
[21] D.S. Heron, M. Shinitzky, M. Hershkowitz, D. Samuel, Proc. Natl. Acad. Sci. USA 77 (1980) 7463–7467.
[22] J.R. Dave, R.J. Witorsch, Biochem. Biophys. Res. Comunn. 113 (1983) 220–228.
[23] D.L. Segaloff, M. Ascoli, Endocr. Rev. 14 (1993) 324–347.
[24] P.C. Roche, R.J. Ryan, D.J. McCormick, Endocrinology 131 (1992) 268–274.
[25] J. Kolena, S. Scsuková, M. Tatara, J. Vranová, M. Ježová, Exp. Clin. Endocrinol. Diabetes 105 (1997) 304–307.