Hormonal Modulation of Structural Alteration of Rat Ovarian Luteinizing/Human Chorionic Gonadotropin Receptors

J. Kolena, M. Ježová, J. Vranová and S. Scsuková

Institute of Experimental Endocrinology, Slovak Academy of Sciences, Vlárska 3, 833 06 Bratislava, Slovakia

Abstract. The structure-stabilizing effect of homologous and heterogeneous desensitization and albumin on rat ovarian LH/hCG receptors was analyzed by thermal perturbation technique. HCG-induced down-regulation shifted the heat inactivation profile of hCG-binding sites to a temperature lower by about 7 °C (T_{50} values). In heterogeneous desensitization, which also involves uncoupling of receptors from adenylyl cyclase system, only follicle stimulating hormone (FSH) changed the stability of ovarian LH/hCG receptors. Stimulation of other hormonal receptors, which belong to the family of membrane spanning G protein-linked receptors, i.e. β-adrenergic, glucagon, serotonin and prostaglandin E (PGE) had no effect on the stability of the LH/hCG receptor. Reduction of the stability of the LH/hCG receptor by about 3 °C after $PGF_{2\alpha}$ injection to luteinized rats may be connected with specific process of luteolysis. On the other hand, albumin had a stabilizing effect on the receptor. The receptor destabilizing action of oleic acid incorporated into ovarian membranes along with calcium stimulation of endogenous phospholipase A (PLA) activity and reversal of these effects when BSA was used as fatty acid scavenger, may indicate that free fatty acids are responsible for the thermal instability of hCG-binding sites. Fluorescence quenching studies indicated that extraction of free fatty acids by albumin elevated the accessibility of fluorophores for acrylamide, and suggest that modificated lipid-protein interactions may affect the stability of the LH/hCG receptor structure.

Key words: LH/hCG receptors — Thermal inactivation — Fluorescence quenching — Rat ovary

Introduction

Biological membranes are dynamic structures containing a variety of components capable of influencing each other. Gonadotropin receptors, which are responsible

Correspondence to: Jaroslav Kolena, Institute of Experimental Endocrinology, Slovak Academy of Sciences, Vlárska 3, 833 06 Bratislava, Slovakia.
E-mail: ueenkole@savba.sk

for transmembrane communication and numerous functional interactions within the plane of the membrane, are embedded in the lipid bilayer. The cell receptor for LH/hCG is though to be an integral protein containing seven transmembrane segments (Segaloff and Ascoli 1993). The response of target cells to the binding of gonadotropin is an increase in the adenylyl cyclase activity mediated by G-proteins. The stability of proteins in their native conformation has become a topic of increasing attention over the last several years. Previous studies demonstrated that glycerol and other osmolytes had beneficial effects on the preservation of the activity of LH/hCG receptors (Metsikko et al. 1990; Kolena et al. 1992). The experiments indicated that the stimulatory effects of osmolytes might be related to the modified physical state of membranes. These substances stabilized the protein structure against thermal denaturation (Arakawa and Timasheff 1985). Heat inactivation of LH/hCG-binding sites demonstrated a significant stabilization of the receptor structure in the presence of osmolytes, serum or albumin (Kolena et al. 1992, 1999). Thermal destabilization of ovarian receptors was observed to be sensitive to the presence of hCG. Occupation of receptor binding sites with the agonist before heat inactivation induced stabilization of the receptor (Kolena et al. 1994b). The process of hCG-induced desensitization results in impairment of the interaction between the receptor and G_s regulatory protein (Segaloff and Ascoli 1993). The early desensitization of luteinized rat ovaries was found to be associated with modification of the physical state of the membrane (Kolena et al. 1994a). The present paper studies the effect of albumin and the uncoupling of the receptor from G-proteins in the process of down-regulation on the stability of LH/hCG receptors analyzed by thermal perturbation techniques in its membrane environment (Kolena et al. 1994b).

Materials and Methods

Materials

Purified hCG (CR 123, 12,780 IU mg^{-1}) and porcine FSH were 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. Faction V, fatty acids free BSA and all other chemicals were from Sigma.

Methods

Luteinized ovaries were produced in 25-day-old rats (Wistar strain) by s.c. administration of 50 IU PMSG followed by 30 IU hCG 56 h later (Kolena et al. 1994b). Homogenates of ovaries (100 mg ml^{-1}) in ice-cold buffer A (25 mmol/l NaH_2PO_4, 1 mmol/l EDTA, 40 mmol/l NaCl, pH 7.4) were filtered through six 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.

Thermal perturbation techniques were used to probe structural features of the LH/hCG receptor. Aliquots of membrane-bound receptor were heat inactivated in

water bath at a constant temperature of 50 °C or 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 the determination of binding activity (Kolena et al. 1994b).

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 (Kolena et al. 1992). The results are expressed as [^{125}I]hCG specific binding per mg protein (Lowry et al. 1951).

Quenching studies were carried out at 23 °C by adding small amounts of 5 mol/l acrylamide in buffer A, pH 7.4. The intrinsic fluorescence intensity was measured as a function of quencher concentration at a fixed emission wavelength of 416 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] (Efting and Ghiron 1976; Kolena et al. 1997). The least-squares method was used to calculate the K_{sv} constant.

Data were analyzed by ANOVA and Bonferroni post test. The results were confirmed in 2–3 independent experiments.

Results

Studies of heat inactivation of hCG-binding sites were carried out to monitor structural alteration of the LH/hCG receptor. 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 (Artigues et al. 1989; Kolena et al. 1994b). The T_{50} value of about 53 °C in control membranes was decreased to almost 46 °C in ovarian LH/hCG receptors 3 h after injection of desensitizing doses 75 IU of hCG to rats on day 7 of pseudopregnancy (Fig. 1A). Thermal inactivation of the receptor is a rapid process. Incubation of untreated membranes at a constant temperature of 50 °C resulted, after 5 min, in about 42% loss of binding sites, while membranes of hCG treated rats exhibited an almost 83% decrease (Fig. 1B). In heterogeneous desensitization, in *in vitro* condition, only FSH had destabilizing effect on the LH/hCG receptor. Thermal inactivation of the receptor in the presence of FSH decreased the T_{50} value by about 3 °C but addition to the membrane receptor of isoproterenol, glucagon or cAMP had, however, no effect (Fig. 2). The essential feature of $PGF_{2\alpha}$-induced functional luteolysis seems to be a lesion at the cell membrane which prevents LH stimulation of adenylyl cyclase. Fig. 3A shows that this effect may be connected with decreased thermal stability of ovarian LH/hCG receptors (T_{50} value decreased by more than 3 °C). The *in vitro* antigonadotropic action of $PGF_{2\alpha}$ did not change the stability of the receptor (Fig. 3B).

The rat ovarian LH/hCG receptor can be stabilized in the presence of BSA

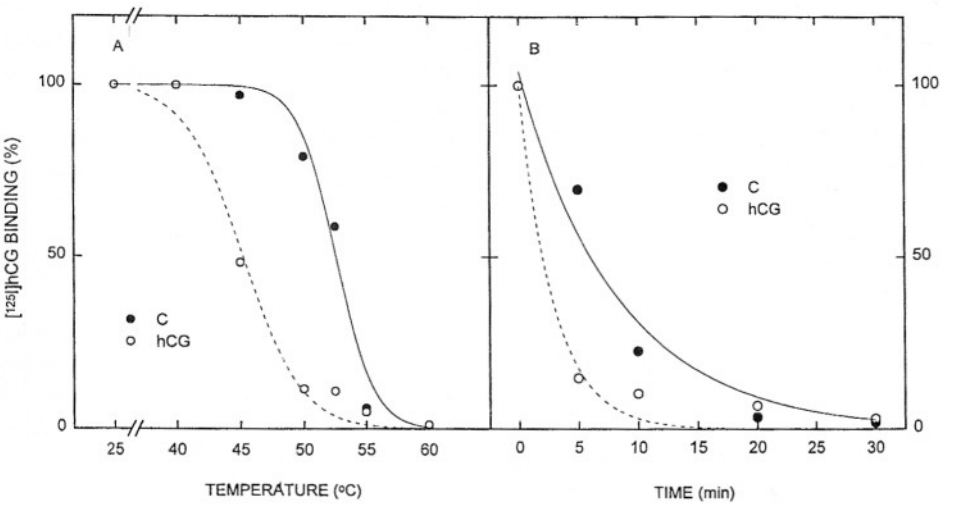

Figure 1. Effect of homologous down-regulation on thermal inactivation of LH/hCG receptors. Pseudopregnant rats were injected with 75 IU of hCG or saline (control) 3 h before they were sacrificed. Ovarian membranes were heat inactivated by raising the temperature (A) or at a constant temperature of 50 °C (B). Control values of binding were about 90 fmol hCG bound / mg protein and in hCG treated rats about 42 fmol hCG bound / mg protein. Means of two estimations are shown.

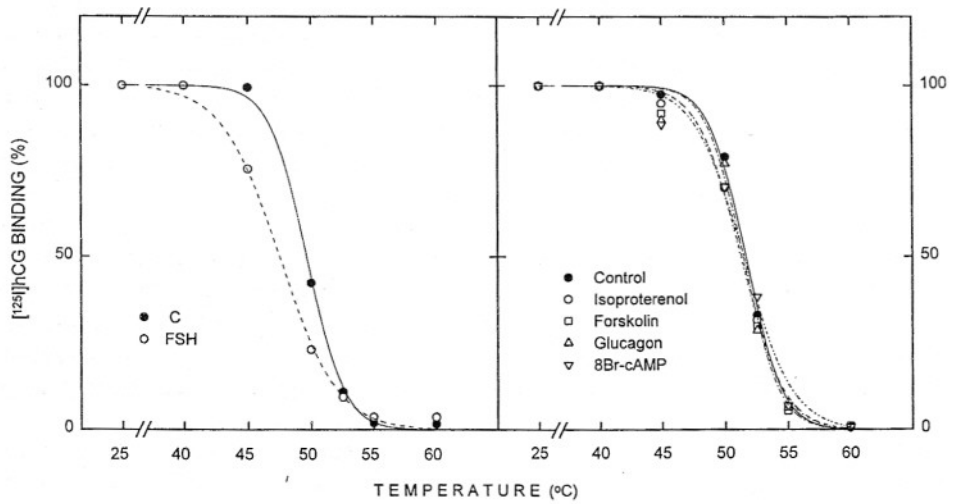

Figure 2. Heat inactivation profile of LH/hCG receptors. The rat ovarian membrane receptor was heat inactivated in the absence (C) or presence of FSH (2 μg/ml), isoproterenol (20 μg/ml), glucagon (10 μg/ml), forskolin (0.1 mmol.l^{-1}) and 8Br-cAMP (0.2 mmol.l^{-1}). For details, see legend to Fig. 1.

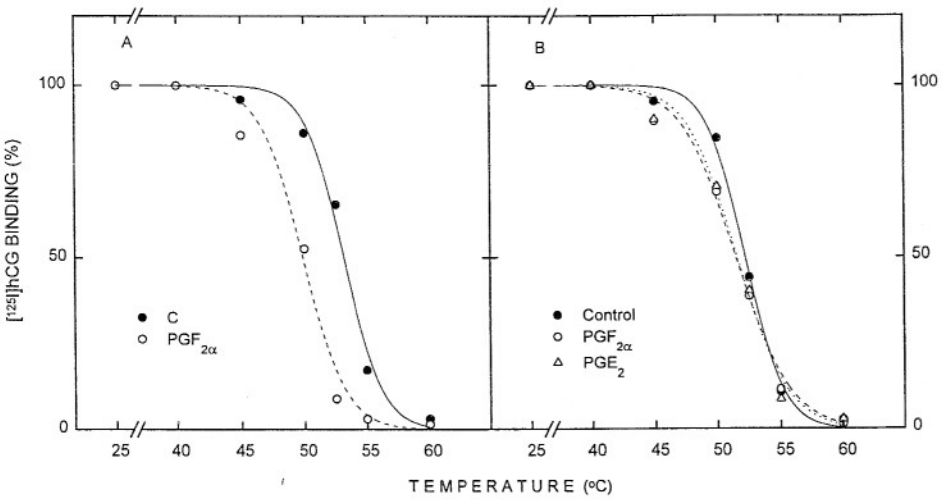

Figure 3. Effect of prostaglandins on thermal stability of ovarian LH/hCG receptors. Rats were injected with 200 μg of $PGF_{2\alpha}$ s.c. 12 h before they were sacrificed (A) or ovarian membranes were heat inactivated in the presence of $PGF_{2\alpha}$ (20 μg/ml) or PGE_2 (10 μg/ml) (B). Control and $PGF_{2\alpha}$ values of binding were about 83 fmol hCG bound / mg protein. For details, see legend to Fig. 1.

during heat inactivation or after extraction of membranes with BSA. Thermal inactivation of the receptor showed that T_{50} values were higher by approx. 6 °C when ovarian membranes were preincubated with BSA for 30 min at 23 °C (Fig. 4). It is well known that BSA interacts with free fatty acids. Fig. 4 shows that preincubation of membranes with 2 $mmol.l^{-1}$ concentration of oleic acid decreased the stability of the receptor and this effect could be fully inverted by treatment with BSA. Several factors may modulate endogenous activity of PLA_2, and calcium is a leading candidate. Stimulation of endogenous PLA activity by 10 $mmol.l^{-1}$ calcium caused a reduction of the T_{50} value by more than 4 °C compared to control membranes incubated with the calcium chelator EGTA. Treatment of membranes with BSA stabilized the LH/hCG receptor, probably as the result of extraction of fatty acids from ovarian membranes. The destabilizing effect of free fatty acids may be caused by the presence of a net negative surface charge provided by fatty acids. Fig. 4 shows that the presence of Na^+ had beneficial effects on the stability of LH/hCG receptors. On monitoring protein-lipid interaction, further valuable information can be obtained by quenching experiments. The Stern-Volmer quenching constant (K_{sv}) estimated from the Stern-Volmer plots for control and BSA-treated membranes was found to be 5.9 $mol.l^{-1}$ and 7.7 $mol.l^{-1}$, respectively, suggesting that BSA increased the accessibility of fluorophores for acrylamide (Fig. 5).

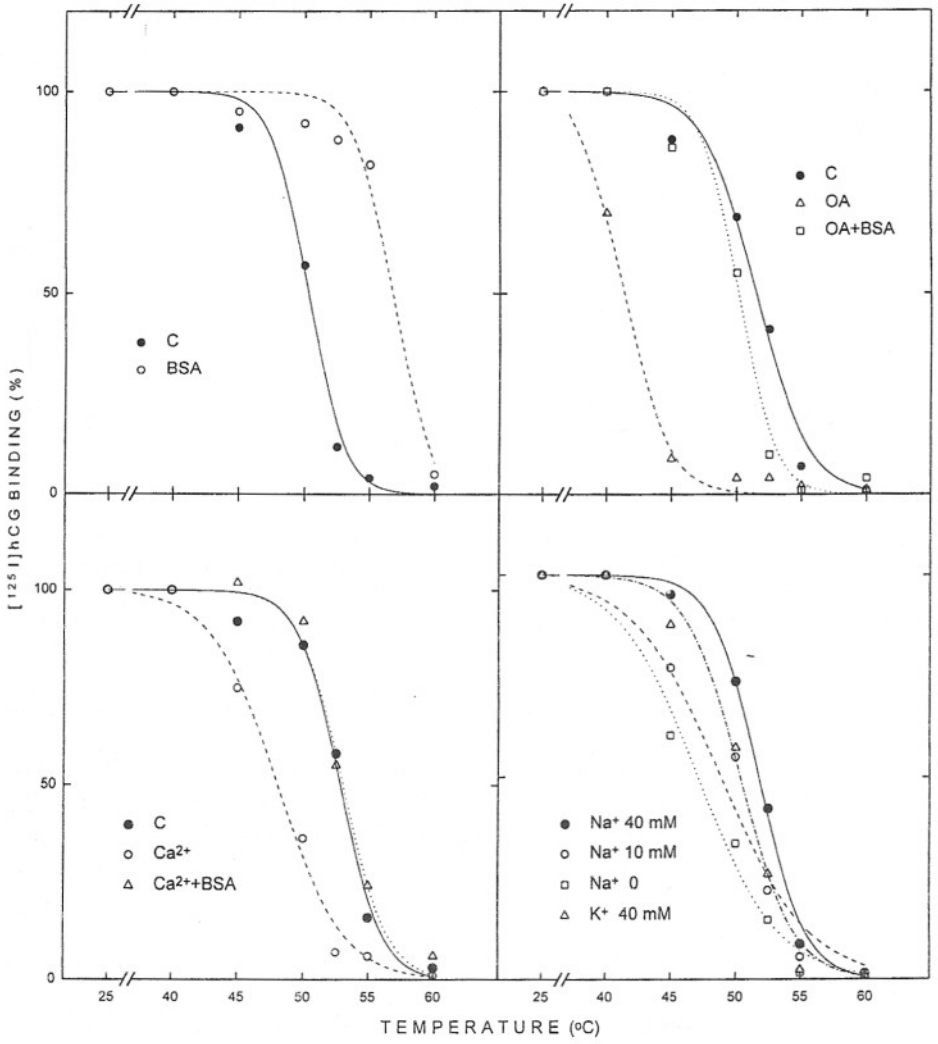

Figure 4. Reversing effect of BSA on changes induced by fatty acid (OA) and Ca^{++}; and effect of Na^{+} on thermal inactivation of LH/hCG receptors. Ovarian membranes were first preincubated with oleic acid (2 mmol.l^{-1}) or Ca^{++} (10 mmol.l^{-1}) for 30 min at 23 °C. Membranes were then washed twice and treated with BSA (5 mg/ml). Ovarian membranes were heat inactivated by raising the temperature as described in legend to Fig. 1.

Discussion

It has repeatedly been shown that treatment of target cells with a hormone can produce desensitization, so that a second exposure to the hormone is less effective than

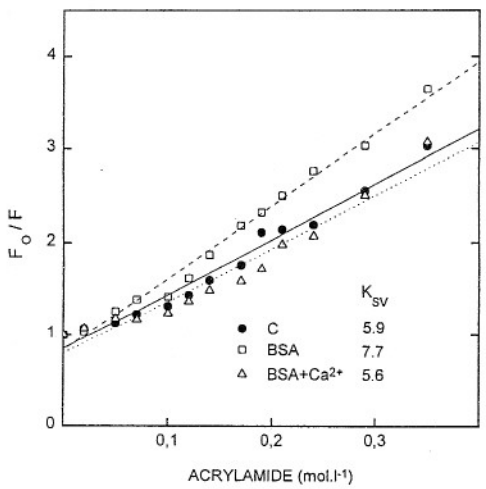

Figure 5. Effects of BSA and Ca^{++} on Stern-Volmer plots with constants (K_{sv}) of acrylamide quenching for ovarian membranes. Experiments were done as described in the legend to Fig. 4.

the first one (Segaloff and Ascoli 1993). Changes in the functional properties of receptors and of the effector system may account for the attenuation of the hormone action. Even though the mechanisms implicated in desensitization appear to be different, two major agonist-triggered changes in the properties of receptors could presumably be involved: first, agonists induce a quick modification of receptors even if the receptors remain located in the plasma membrane, and the second, prolonged treatment of cells with agonists causes a decrease in the density of receptors. This process is termed down-regulation and represents the uncoupling and internalization of receptors away from the plasma membrane. The results of this study showed that homologous desensitization with hCG had a destabilizing effect on rat ovarian LH/hCG receptor structure. Heat inactivation of hCG-binding sites provides a direct approach to evaluate changes of receptor conformation which appear as a result of hormone binding or perturbation of the membrane bilayer (Artigues et al. 1989; Kolena et al. 1994b). Previously, we showed that the model of thermal inactivation of the receptor provided information comparable to that obtained by differential scanning calorimetry (Kolena et al. 1994a). Desensitization of LH/hCG receptors modified the calorimetric profile representative of control membranes. The dominant 45–53 °C thermal transition became more expressive and cooperative and was shifted toward lower temperatures (Kolena et al. 1994b). Another phenomenon is known as heterogeneous desensitization, in which treatment of a cell with a hormone can make the cell less sensitive to another hormone that works through a different receptor. The mechanisms of heterogeneous desensitization are relatively poorly understood, however, they are not associated with a receptor down-regulation but involve uncoupling of the receptor and other components of the adenylyl cyclase system. Multihormonal desensitization is probably associated with functional alternation of G-proteins and the cAMP-dependent protein kinase

phosphorylation of hormone receptors (Segaloff and Ascoli 1993). Results of heat inactivation experiments of this study showed that only FSH changed the stability of ovarian LH/hCG receptors. Other hormonal receptors which belong to the family of membrane spanning G protein-linked receptors, i.e. β-adrenergic, glucagon, serotonin and prostaglandin E had no effect on the stability of the LH/hCG receptor. The resistance in the receptor suggests that there are differences between LH/hCG receptor and other receptors coupled to adenylyl cyclase in factors controlling their structures or interactions with G proteins. The reduction of LH/hCG receptors stability after injection of $PGF_{2\alpha}$ to luteinized rats may be connected with specific process of luteolysis. Long-term effects of $PGF_{2\alpha}$ on corpus luteum function have been postulated to cause luteolysis either by diminishing of LH receptors, uncoupling of LH stimulation of cAMP synthesis or inhibition of LH stimulation of protein kinase A activity (Lahav et al. 1989).

Albumin had a stabilizing effect on ovarian LH/hCG receptor structure (Kolena et al. 1999). Albumin is known to bind free fatty acids and to remove them from membranes. The destabilizing effect of oleic acid incorporated into membranes as well as calcium stimulation of endogenous phospholipase A activity and the reversal of this action by defeated albumin corroborates the hypothesis that free fatty acids are responsible for the heat instability of the receptor. The role of fatty acids was substantiated by experiments with phospholipase A_2. This enzyme cleaves acyl chains of the glycerol backbone of the phospholipid, yielding mainly arachidonic acid. The inhibitory effect of phospholipase A_2 was reversed upon removal of fatty acids from ovarian membranes (Kolena et al. 1999). This destabilizing effect may be caused by the presence of a net negative surface charge provided by free fatty acids. The presence of Na^+ had beneficial effects on the stability of ovarian LH/hCG receptors. Na^+ has been suggested to affect agonist binding. The binding affinity of hCG to rat luteal membranes increased with the increasing Na^+ concentrations, to reach a maximum at 40 $mmol.l^{-1}$ (McIlroy 1988). Other investigators reported that the affinity of the LH/hCG receptors for LH is lower in buffers containing NaCl than in buffers containing isoosmolar concentrations of an appropriate substitute (Quintana et al. 1993). A net negative charge provided by free fatty acids may modify lipid-protein interactions and affect the stability of the LH/hCG receptor structure. Quenching experiments provided support to this assumption. The intrinsic fluorescence of protein appears to be a valuable probe in monitoring protein conformation and protein-lipid interaction. Our experiments indicated that extraction of free fatty acids by albumin elevated the accessibility of fluorophores for acrylamide. An elevated quenching rate suggests exposure of tryptophanyl residues and alterations in the dynamics of the proteins neighboring such residues (Effting and Ghiron 1976). The rat luteal LH/hCG receptor contains two tryptophan residues (Segaloff and Ascoli 1993) and recently, we demonstrated that modification of tryptophan residues was associated with destabilization of the LH/hCG receptor structure (Kolena et al. 1997). Fatty acids present the principal properties of endogenous modulators. Their plasma concentrations vary with nutritional and metabolic status, and their local content can change promptly in re-

sponse to extracellular or intracellular signals. Direct interaction of free fatty acids with the receptor or with protein matrix surrounding the receptor may apparently influence the stability of the LH/hCG receptor structure.

Acknowledgements. This work was supported, in part, by the Slovak Grant Agency for Sciences, VEGA 2/7179/20

References

Arakawa T., Timasheff S. N. (1985): The stabilization of proteins by osmolytes. Biophys. J. **47**, 411—414

Artigues A., Villar M. T., Fernandez A. M., Ferragut J. A.,Gonzales-Ross J. M. (1989): Cholesterol stabilizes the structure of the nicotinic acetylcholine receptor reconstituted in lipid vesicles. Biochim. Biophys. Acta **985**, 325—330

Efting M. R., Ghiron C. A. (1976): Exposure of tryptophanyl residues in proteins quantitative determination by fluorescence quenching studies. Biochemistry USA **15**, 672—680

Kolena J., Matejčíková K., Šrenkelová G. (1992): Osmolytes improve the reconstitution of luteinizing hormone/human chorionic gonadotropin receptors into proteoliposomes. Mol. Cell. Endocrinol. **83**, 201—209

Kolena J., Scsuková S., Jasem P.
(1994a): Changes in physical properties of ovarian membranes after hCG-induced desensitization in rats. Gen. Physiol. Biophys. **13**, 299—307

Kolena J., Scsuková S., Tatara M., Jasem P. (1994b): Effect of partial delipidation of rat ovarian membranes on thermal stability of LH/hCG receptors. Biochim. Biophys. Acta **1193**, 293—300

Kolena J., Scsuková S., Tatara M., Vranová J., Ježová M. (1997): Involvement of tryptophan in the structural alterations of the rat ovarian LH/hCG receptor. Exp. Clin. Endocrinol. Diabetes **105**, 304—307

Kolena J., Ježová M., Vranová J., Scsuková S. (1999): Structure-stabilizing effect of albumin on rat ovarian LH/hCG receptors. Biochim. Biophys. Acta **1416**, 208—216

Lahav M., Davis J. S., Rennert H. (1989): Mechanism of the luteolytic action of prostaglandin $F_{2\alpha}$ in the rat. J. Reprod. Fertil. **37**, 233—240

Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J. (1951): Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**, 265—275

McIlroy P. J. (1988): Effects of cations on binding of human choriogonadotropin. Biochem. Cell Biol. **66**, 1258—1264

Metsikko M. K., Petaja—Repo U. E., Lakkakorpi J. T., Rajaniemi H. J. (1990): Structural features of the LH/CG receptor. Acta Endocrinol. **122**, 545—552

Quintana J., Wang H., Ascoli M. (1993): The regulation of the binding affinity of the luteinizing hormone/choriogonadotropin receptor by sodium ions is mediated by a highly conserved aspartate located in the second transmembrane domain of G protein-coupled receptors. Mol. Endocrinol. **7**, 767—775

Segaloff D. L., Ascoli M. (1993): The lutropin/choriogonadotropin receptor... 4 years later. Endocrine Rev. **14**, 324—347

Final version accepted September 3, 2000