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Rapid report

Involvement of membrane surface charge in thermal stability of the rat ovarian LH/hCG receptor

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Abstract

Analysis of fluorescence of membrane-bound 8-anilino-1-naphthalene sulfonate and monodansylcadaverine probes revealed that a negative membrane surface charge derived from free fatty acids (FFA) resulted in destabilization of structure–functional properties of the rat ovarian LH/hCG receptor. Removal of FFA from rat luteal and porcine ovarian granulosa cells by BSA increased gonadotropin responsiveness of cells in cAMP formation.

Keywords: Luteinizing hormone/human chorionic gonadotropin receptor; Thermal inactivation; Fluorescence titration; Free fatty acid; (Rat ovary)

Intramembrane sequences of membrane receptors, including luteinizing hormone/human chorionic gonadotropin (LH/hCG) receptors, react with other proteins or with elements of a membrane lipid bilayer to stabilize the receptor in the membrane. Protein–protein and lipid–protein interactions are believed to play an essential role in cell membrane functions. A membrane electrostatic surface potential and/or membrane surface charge are another important factors controlling various physiological functions of biological membranes [1]. It is well known that the addition of serum to the culture medium is required for optimal growth and protection of several cell lines. Albumin and other plasma components were found to interact with the lipid bilayer of the cell membrane, giving rise to the stabilization effect [2]. Previously, we have demonstrated that structure–functional properties of the rat ovarian LH/hCG receptor can be stabilized in the presence of porcine serum and bovine serum albumin during heat inactivation [3]. The present findings indicate that the stabilizing effect of albumin on the LH/hCG receptor might be associated with changes in the membrane surface charge.

Luteinized ovaries were produced in 25-day-old rats (Wistar strain) by s.c. administration of 50 IU pregnant mare serum gonadotropin (PMSG) followed 56 h later by 30 IU hCG. 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) or in Tris buffer (10 mM Tris–HCl, 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 buffers [3].

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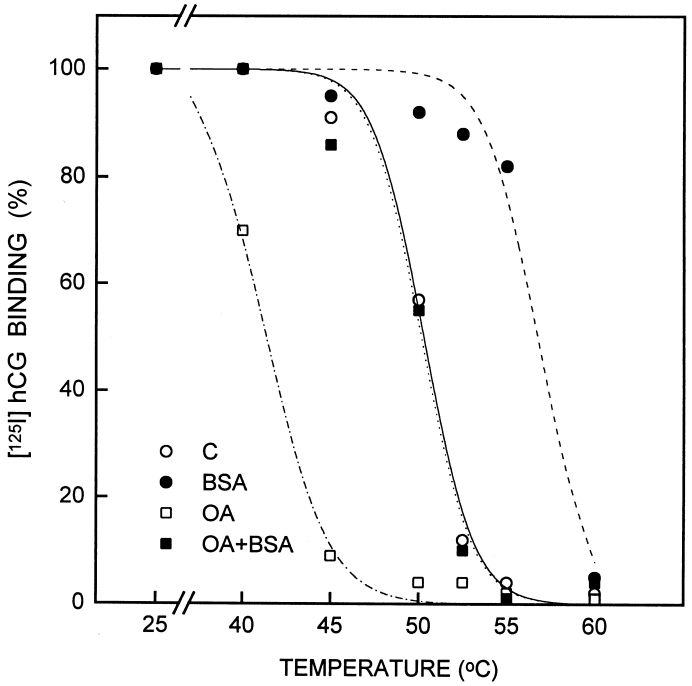

Fig. 1. Effect of BSA (5 mg ml^{-1}) and oleic acid (2 mM) on heat-inactivation profile of LH/hCG receptors. Ovarian membranes in buffer A were first incubated at 37°C for 30 min in the absence or presence of oleic acid and washed twice with buffer A. Then the membranes were incubated at 24°C for 30 min in the absence or presence of defatted BSA, washed twice with buffer A and submitted to heat inactivation. Control values of binding were about 90 fmol hCG bound per mg protein. Means of two determinations are shown. The experiment was repeated three times with similar results.

Oleic acid was dissolved in dimethyl sulfoxide (DMSO) and after addition of buffer A with 3.5% polyvinylpyrrolidone (PVP) the dispersion was briefly (15 s) sonicated. Ovarian membranes were incubated at 37°C for 30 min with oleic acid (2 mM) and washed twice with buffer A. The membranes in buffer A or in Tris buffer were incubated at 24°C for 30 min with fatty acid free bovine serum albumin (BSA) (fraction V) and washed twice with the buffers.

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 determinations [4]. In the hCG binding assay, 0.1-ml aliquots of ovarian membranes were incubated at 20°C for 16 h 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, specific activity 2.3 TBq g^{-1}). After incubation and centrifugation, the membrane pellets were washed twice with the buffer A [3]. The results are expressed as [^{125}I]hCG specific binding per mg protein [5].

Fluorescence measurements were performed at 22°C using a spectrofluorometer Fluorolog 3 (Jobin Yvon, USA). The excitation and emission wavelengths used for the ANS and MDC were 380 and 470 nm, and 330 and 520 nm, respectively. Blanks (without membranes or fluorescence probes) were run in parallel to correct for the emission of unbound probes and scattering. The limiting fluorescence enhancement (F_{max}) was calculated by extrapolating reciprocal plots to infinite protein concentration [6]. The concentrations of the bound and free probes were determined by comparison of fluorescence intensities at each concentration with F_{max} as described by Azzi [7]. The dissociation constants (K_d) and the number of binding sites (N) were obtained from Scatchard plots.

Isolated luteal cells were prepared by enzymatic dispersion of luteinized ovaries with collagenase [8]. Granulosa cells were aspirated from small (1–2 mm) (SGC) and large (5–8 mm) (LGC) porcine ovarian

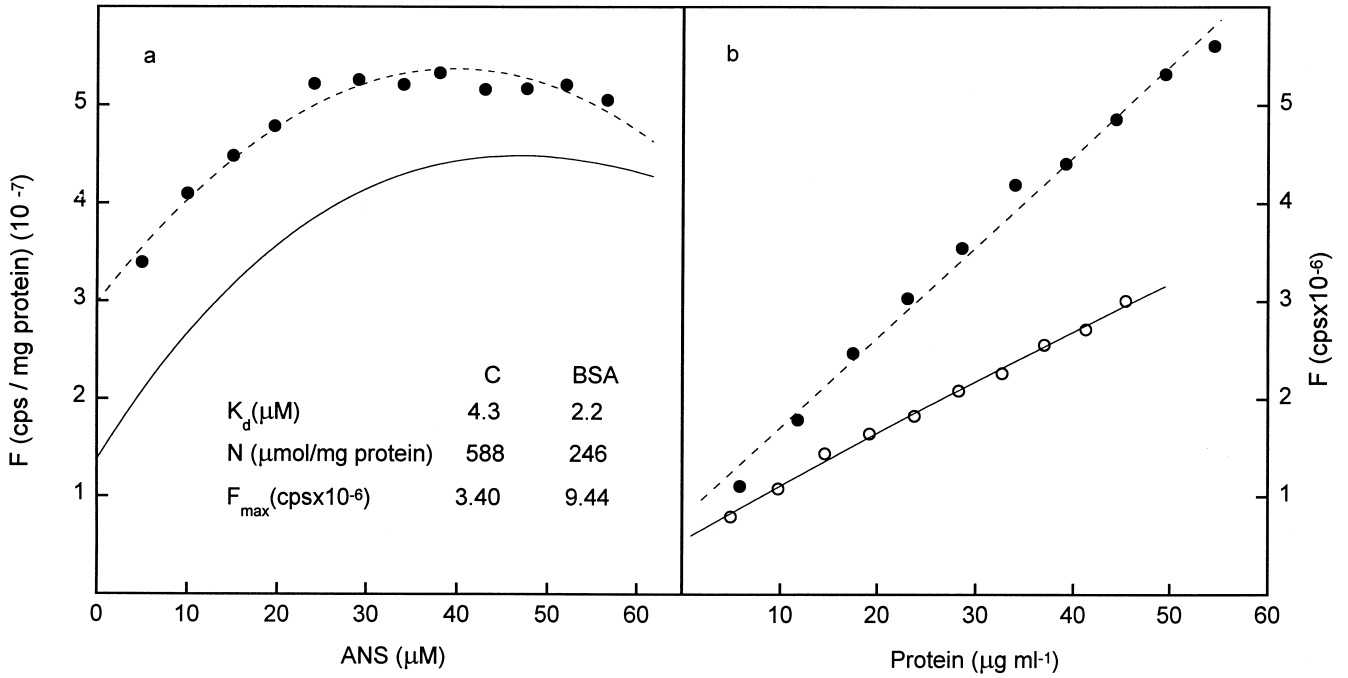

Fig. 2. ANS fluorescence enhancement after its binding to control (○) and BSA-treated (●) rat ovarian membranes as a function of the probe concentration (a) and the membrane protein concentration (fixed concentration of ANS was 20 μM) (b). The membranes in Tris buffer were incubated at 24°C for 30 min in the absence or presence of BSA (10 mg ml^{-1}), washed twice and the fluorescence intensity of ANS was measured. The limiting fluorescence enhancement at maximal binding (F_{max}), the dissociation constant (K_d) and the number of binding sites (N) were determined as described in the text. Each experimental point represents the value from three parallel samples.

follicles [9]. The cAMP concentration was determined by protein binding assay [10].

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

Exposure of rat ovarian membranes to defatted BSA had a stabilizing effect on rat ovarian LH/hCG receptor structure–functional properties analyzed by thermal perturbation technique (Fig. 1). This technique constitutes a valid approach to probe alterations in the receptor protein conformation in its native membrane environment [4,11]. Previously, we have shown that heat inactivation of hCG-binding sites was one of the thermal events accounting for the main thermal transition observed in differential scanning calorimetry of the membrane LH/hCG receptor [4]. The T_{50} value, i.e., the temperature at which 50% of the initial binding capacity still remains, of about 52°C in control membranes increased by approx. 5°C in the presence of the maximal effective dose of 5 mg ml^{-1} BSA. The experiments with iodinated BSA demonstrated that ovarian membranes did not exhibit specific albumin-binding sites and that BSA adsorbed on membranes could be effectively washed out (data not shown). Preincubation of ovarian membranes with oleic acid (2 mM) apparently shifted the heat-inactivation profile of hCG-binding sites to lower temperatures, the T_{50} value decreasing by more than 9°C. The receptor destabilizing action of oleic acid could be fully inverted by treatment with BSA (Fig. 1). We have shown that the fatty acid content in rat gonadal membranes proportionately increased upon incorporation of ^{14}C-labelled fatty acids [12].

Albumin, the most abundant plasma protein, is known to bind free fatty acids (FFA) and to facilitate their removal from donor cells and uptake into receptor cells [13]. It has been reported that fatty acids can alter the structure and functioning of plasma cell membranes [14] and FFA have a pronounced effect on the activity of several membrane enzymes or receptors [15]. The assumption that FFA could be responsible for the thermal instability of hCG-binding sites was strengthened by a digestion of mem-

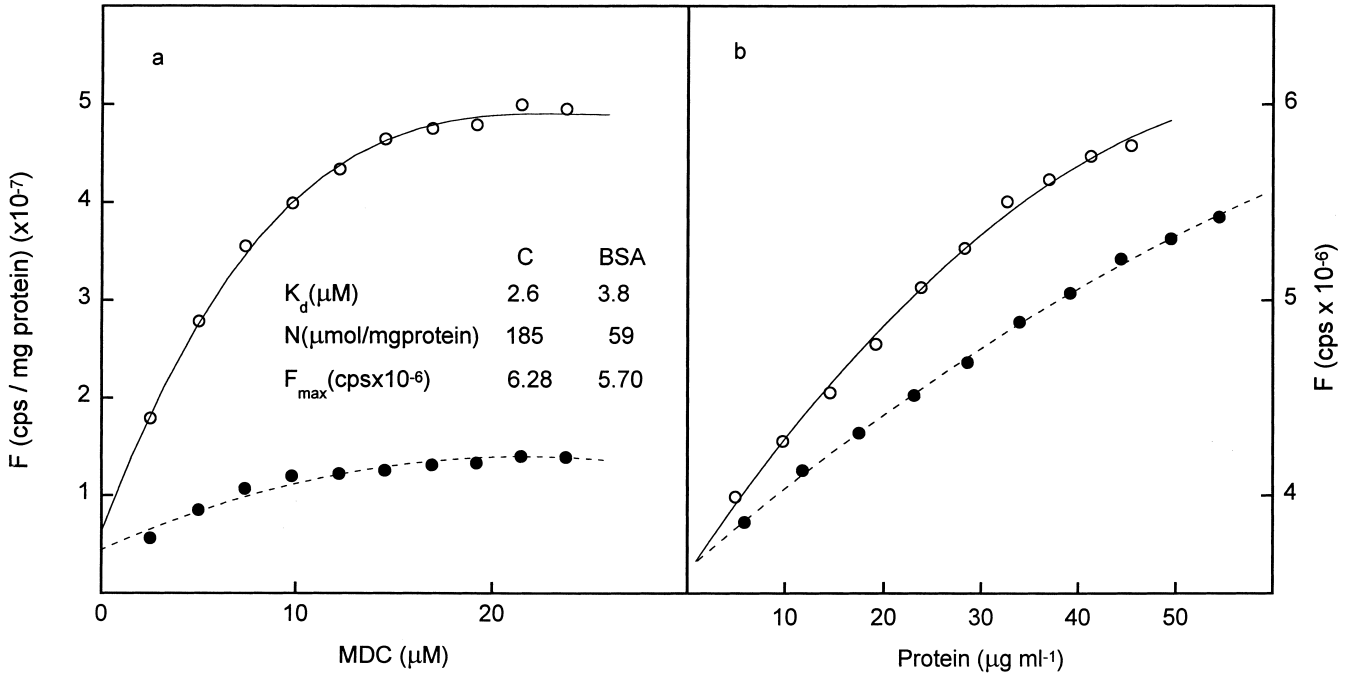

Fig. 3. MDC fluorescence after the probe binding to control (○) and BSA-treated (●) rat ovarian membranes in dependence on the probe concentration (a) and the membrane protein concentration (fixed concentration of MDC was 10 μM) (b). For details see the legend to Fig. 2.

branes with phospholipase A_2. This enzyme exerted effects on the thermal stability of LH/hCG receptors resembling those observed upon insertion of fatty acids [3]. We suppose that the destabilizing effect of FFA on the receptor protein might be caused by the presence of a negative surface charge provided by FFA to the membrane bilayer. This hypothesis is supported by the analysis of the fluorescence of membrane-bound fluorescence probes 8-anilino-1-naphthalene sulfonate (ANS) and monodansylcadaverine (MDC). These probes carry an opposite charge and therefore are believed to occupy different binding sites on the membrane. The fluorescence of anionic probe ANS was increased after its binding to BSA-treated membranes (Fig. 2), while the fluorescence of positively charged MDC bound to BSA-treated membranes was decreased (Fig. 3) when compared to untreated membranes. These changes can reflect the altered binding capacity of ovarian membranes caused by a decrease in the negative surface charge after FFA extraction from the membranes with BSA. The observed increase of F_{max} for ANS

Table 1
Effect of BSA on basal and gonadotropin-stimulated cAMP formation by rat luteal and porcine granulosa cells

		cAMP C	BSA (pmol/10^6 cells)	*P*
Luteal cells	C	0.70 ± 0.14	0.43 ± 0.06	n.s.
	hCG	12.1 ± 1.4	16.5 ± 1.0	< 0.05
	LH	9.1 ± 0.4	15.1 ± 1.4	< 0.01
SGC	C	0.002 ± 0.001	0.001 ± 0.0001	n.s.
	FSH	17.08 ± 2.6	53.3 ± 12.9	< 0.05
LGC	C	0.019 ± 0.003	0.061 ± 0.009	n.s.
	LH	78.8 ± 18.9	146.8 ± 12.0	< 0.05

Luteal cells isolated from rat luteinized ovaries or porcine granulosa cells from small (SGC) and large (LGC) ovarian follicles were incubated at 37°C for 3 h with gonadotropins (10 μg ml^{-1}) in the absence or presence of BSA (10 mg ml^{-1}). Data represent mean ± S.E. for four determinations. *P*, comparison between BSA-treated and control cells.

bound to modified membranes was probably due to an increase in the binding affinity to ANS (ANS dissociation constant (K_d) decreased), since the number of ANS binding sites (N) decreased when compared to controls (Fig. 2). ANS fluorescence is extremely sensitive to changes in the probe environment, as the probe binds noncovalently to both membrane proteins and lipids. The number of ANS molecules bound to the membrane is strongly influenced by the surface charge of the membrane. Therefore, changes in the membrane surface charge might increase the proportion of ANS molecules bound in the protein domain of the membrane and, although there are fewer binding sites in this domain, their affinity to ANS is higher [16]. Preincubation of ovarian membranes with BSA decreased the number of MDC binding sites (N) and F_{max} as well as the affinity of membrane binding sites to MDC (K_d increased) (Fig. 3). MDC is known to occupy preferentially anionic binding sites [17] and the observed changes in K_d and N may reflect the reduction of negative charged groups in the vicinity of MDC binding sites.

In order to find out whether the stabilizing effect of BSA on the LH/hCG receptor is associated with the functional state of ovaries, the effect of BSA on hormonal responsiveness of ovarian cells in cAMP formation was estimated. BSA failed to change basal but significantly enhanced LH- and hCG-stimulated cAMP production by rat luteal cells (Table 1). The similar effect of BSA on cAMP synthesis by granulosa cells isolated from porcine ovaries was observed. It is well documented that granulosa cells isolated from small follicles (SGC) possess mostly follicle-stimulating hormone receptors, while granulosa cells from large follicles (LGC) were shown to contain increased amounts of LH receptors [18]. Melsert et al. [19] demonstrated that fatty acid free BSA and purified serum albumins enhanced LH-induced pregnenolone production in immature rat Leydig cells. They have shown that the stimulatory effect of albumin species depends on their fatty acid content, and the authors have suggested that fatty acids can act as inhibitors of Leydig cell steroidogenesis in rats. The results of this study indicate that the removal of FFA with BSA might reduce the negative surface charge of ovarian membranes and thus stabilize the rat ovarian LH/hCG receptor.

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