

Fluorescence Quenching Studies of the Rat Ovarian LH/hCG Receptor

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Abstract. Fluorescence quenching method providing information about the structure and dynamics of proteins, ligand-protein and protein-lipid interactions was used in a study of the rat ovarian LH/hCG receptor. The efficiency of two different quenchers, acrylamide and iodide, was tested. Acrylamide was significantly more effective in quenching of intrinsic fluorescence of ovarian membranes than iodide and therefore it was used in all of the following experiments. Both acrylamide and iodide were not effective in quenching of membranes labelled with fluorescence probe 1,6-diphenyl-1,3,5-hexatriene (DPH). In the process of desensitization of ovarian LH/hCG receptors the administration to rats of hCG modified the quenching rate of protein fluorescence and intrinsic fluorescence spectral properties of membranes. Alteration in the quenching of intrinsic fluorescence of ovarian membranes was observed after chemical modification of LH/hCG receptors by 2-hydroxy-5-nitrobenzyl bromide (HNB-B1). The accessibility of tryptophan fluorophores was increased in HNB-B1-treated membranes. Delipidation of the LH/hCG receptor modified the quenching of protein fluorescence characteristic for control proteoliposomes. These results demonstrate that fluorescence quenching technique can be successfully applied in the study of the LH/hCG receptor.

Key words: Fluorescence quenching – LH/hCG receptor – Tryptophan – Acrylamide

Introduction

Intrinsic fluorescence of proteins is a valuable tool to monitor protein conformational reorganization, ligand-protein and protein-lipid interactions. This fluorescence derives from the fluorescence properties of the aromatic aminoacids trypto-

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phan and tyrosine. The changing properties of tryptophan fluorescence, such as lifetime and blue shift of emission maximum, have been used to determine the polarity of tryptophan environment (Langner and Hui 1991) and so the position of proteins could be estimated. Studies of the quenching of tryptophan fluorescence by extrinsic quenching agents (iodide, molecular oxygen, acrylamide, trichloroethanol or other small molecules) provide information concerning exposure and micro-environment of tryptophan residues and dynamics of proteins (Eftink and Ghiron 1981). The quenching technique has been successfully used in monitoring conformational changes of human serum albumin (Chang and Lee 1984), human luteinizing hormone (Sanyal et al 1987), bovine growth hormone (Havel et al 1988), melittin (Kaszycki and Wasylewski 1990), spectrin (Kahana et al 1992) and many other proteins. The ability of brominated lipids to partially quench the intrinsic fluorescence of the acetylcholine receptor has been used to monitor interactions between the protein and surrounding lipids (Jones and McNamee 1988).

The purpose of this paper is to present the application of the fluorescence quenching method in the study of the LH/hCG receptor in rat ovarian membranes during the process of desensitization, chemical modification and delipidation.

Materials and Methods

Materials

Purified hCG (CR 123, 12 780 U/mg) was generously supplied by NIAMDD, NIH, Bethesda. Na^{125}I was purchased from the Radiochemical Centre, Amersham. Pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG Praedyn) were from Spofa, Prague. 1,6-diphenyl-1,3,5-hexatriene (DPH) was obtained from SERVA. Acrylamide, potassium iodide (KI), 2-hydroxy-5-nitrobenzyl bromide (HNB-B1), phosphatidylcholine (PC) type V-E from egg yolk and all other chemicals were purchased from Sigma.

Methods

Preparation of membranes. Luteinized ovaries were produced in 25-day-old rats (Wistar strain) by sc administration of 50 IU PMSG followed 56 h later by 30 IU hCG (Koleva et al 1992b). Rats were killed 6 days after hCG administration. Homogenates of ovaries in PBS buffer (50 mmol l⁻¹ phosphate buffer and 15 mmol l⁻¹ sodium chloride, pH 7.4) or in buffer A (25 mmol l⁻¹ NaH₂PO₄, 1 mmol l⁻¹ EDTA, 40 mmol l⁻¹ NaCl, pH 7.4) were filtered through six layers of surgical gauze, centrifuged at 1000 × g for 15 min, and the supernatant was further centrifuged at 20,000 × g for 30 min. The final membrane preparations were resuspended in the same buffer.

Preparation of proteoliposomes. Solubilized gonadotropin receptor, consisting of approximately 2.5 mg of control or delipidated membrane proteins, was stirred

with 0.5 ml of 20 mmol l⁻¹ sodium cholate in buffer A, containing 20% glycerol, at 4°C for 60 min (Kolena et al 1992b). Phosphatidylcholine was dissolved in sodium cholate with glycerol and added to solubilized membranes. The solution was then centrifuged at 105,000 × g for 60 min. The solubilized membrane protein was applied to a Bio-Beads SM-2 column (1 × 8 cm), previously equilibrated with buffer A. The same buffer was used for elution. After 5-fold dilution the turbid fraction containing proteoliposomes was centrifuged at 160,000 × g for 60 min (Kolena 1989, Kolena et al 1992b).

Membrane delipidation The membrane-bound LH/hCG receptor was delipidated by extraction using a mild non-ionic detergent Tween 20, at 23°C. A sample in 5% (v/v) Tween 20 was sonicated in a MSE Ultrasonic Dismintegrator with a small probe. The preparation was then stirred for further 30 min. After centrifugation at 20,000 × g for 30 min, the pellets were washed twice with buffer A (Kolena et al 1995).

Chemical modification Chemical modification of the membrane was carried out by incubating it with 2-hydroxy-5-nitrobenzyl bromide (HNB-B₁) (1 or 5 mmol l⁻¹) for 30 min at 25°C in PBS buffer. The agent was dissolved in DMSO the concentration of which was kept below 5%. The membranes were then centrifuged and washed twice with PBS buffer.

hCG binding assay In the hCG binding assay 0.1 ml aliquots of ovarian membranes or proteoliposomes were incubated for 16 h at 20°C with 0.1 ml PBS buffer or buffer A + 1 mg ml⁻¹ BSA with or without a 100-fold excess of unlabelled hCG and 0.1 ml [¹²⁵I]hCG (1.15 ng sp act about 2.3 TBq s⁻¹). After incubation and centrifugation the membrane pellets were washed twice with PBS buffer or buffer A. The hormone-receptor complex in proteoliposomes was precipitated twice with polyethylene glycol (Kolena et al 1992b).

Fluorescence polarization was measured and fluorescence quenching studies were performed using a Perkin-Elmer LS-5 luminescence spectrometer, equipped with a circulation bath to maintain the sample temperature at 25°C. Excitation and emission slits were 2.5 nm and 5 nm, respectively.

Fluorescence polarization A solution of 2 mmol l⁻¹ DPH in tetrahydrofuran was dispersed by 1000-fold agitative dilution in 50 mmol l⁻¹ PBS buffer. Ovarian membranes (100 µg proteins) were incubated at 25°C for 1 h with 2 ml of DPH in the above buffer (Kolena et al 1994a).

Quenching measurements The fluorescence intensity was measured as a function of quencher concentration at a fixed emission wave-length. The excitation wavelength of 280 nm was used. First, the initial fluorescence (F_0) of the membranes was measured. The membrane fluorescence was then quenched by the progressive addition of small aliquots (10 or 25 µl) of a concentrated solution of quencher (acrylamide

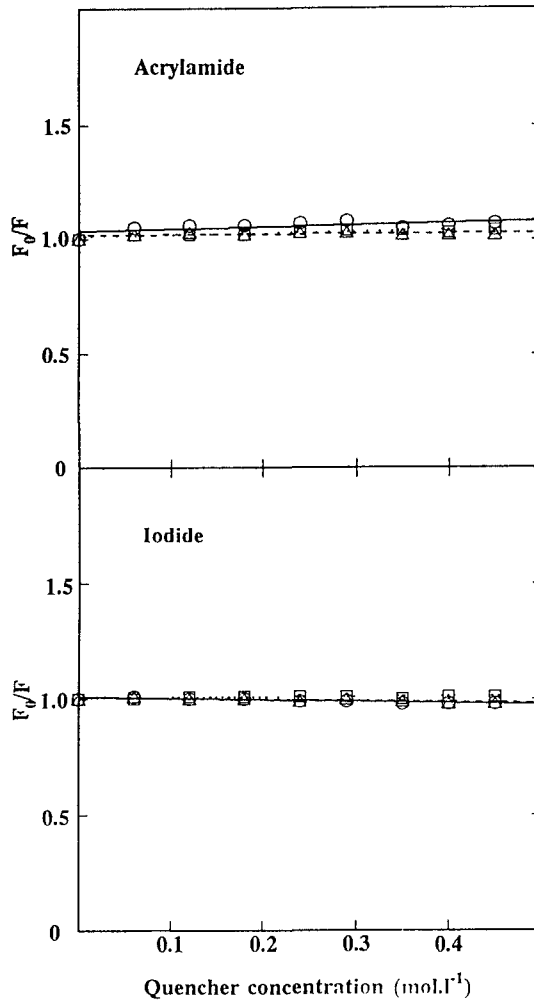


Figure 2. Stern-Volmer plots for quenching of rat ovarian membranes labelled with fluorescence probe DPH. Membranes were preincubated for 60 min at 25°C with 2 $\mu\text{mol l}^{-1}$ DPH solution. The membranes were quenched by acrylamide and iodide. The excitation and the emission wavelengths were 360 nm and 430 nm, respectively. The concentrations of membranes, see legend to Fig. 1.

for acrylamide (Fig. 3). Furthermore, desensitization apparently altered spectral properties of membrane intrinsic fluorescence. The emission maximum of 440 nm for control membranes was shifted to 420 nm for desensitized membranes (data not shown). This change of the physical properties of membranes connected with

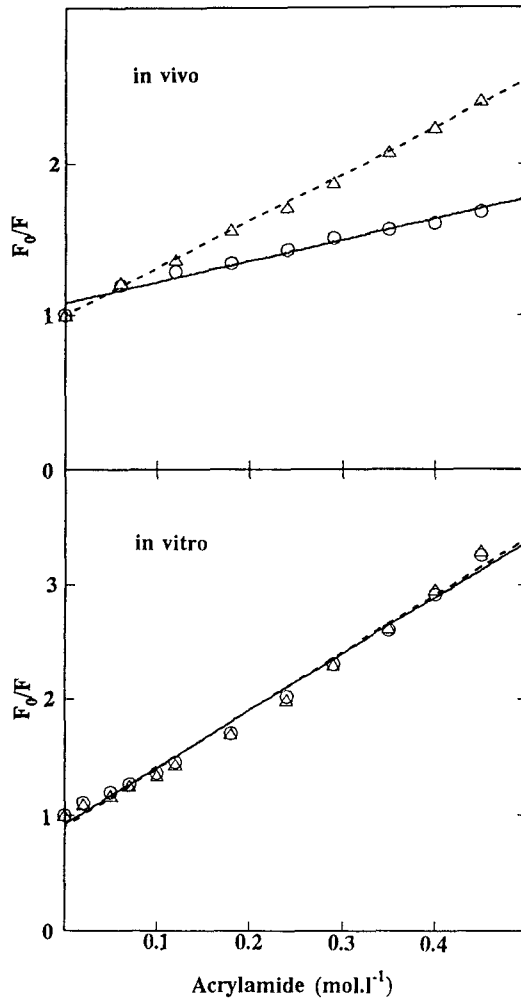


Figure 3. The acrylamide quenching of control (\circ) and desensitized rat ovarian LH/hCG receptors (Δ) ($0.4 \text{ mg protein pre ml}$). Pseudo-pregnant rats were treated with 75 IU of hCG 30 min before being sacrificed. For *in vitro* experiments, membranes from control rats were incubated for 0.5 h at 37°C with $10 \mu\text{g ml}^{-1}$ hCG. The results were confirmed in 3 independent experiments.

desensitization was not observed in the treatment of membranes with hCG *in vitro*. There was no difference in the degree of polarization of DPH when ovarian membranes were incubated for 0.5 or 2 h with hCG (Kolena et al 1994a). The Stern-Volmer constants, K_{SV} , for control and hCG-treated membranes were found to be almost the same, 4.9 and 5.0 mol^{-1} , respectively (Fig 3). Also, fluorescence spectral

properties of membranes were not significantly changed. The emission maximum of 400 nm for controls was shifted to 394 nm for hCG treated membranes (data not shown).

Chemical modification of the gonadotropin receptor was used to obtain direct information on the importance of tryptophan residues for ligand binding to the receptor. Treatment of ovarian membranes with 1 mmol l^{-1} and 5 mmol l^{-1} 2-hydroxy-5 nitrobenzyl bromide (HNB-Br) for 30 min at 25°C decreased the binding activity of LH/hCG receptor to 30% and 14% of control values, respectively (Fig. 4A). HNB-Br, that has specificity toward tryptophan, increased the accessibility of tryptophan residues to acrylamide. Ovarian membranes were incubated with 5 mmol l^{-1} HNB-Br for 30 min at 25°C . Stern-Volmer constants, K_{SV} , for control and HNB-Br treated membranes were found to be 3.1 and 5.4 mol^{-1} , respectively (Fig. 4B). HNB-Br also modified spectral properties of membrane fluorescence.

Properties of biological membranes are dependent on lipid composition of the membrane. $[^{125}\text{I}]\text{hCG}$ receptor binding activity in proteoliposomes was sensitive to the presence of lipids in membranes. Binding activity of partially delipidated LH/hCG receptor reconstituted into proteoliposomes was reduced to 32% of controls. Re-addition of PC (2.5 mg) to the delipidated receptor reconstituted the bind-

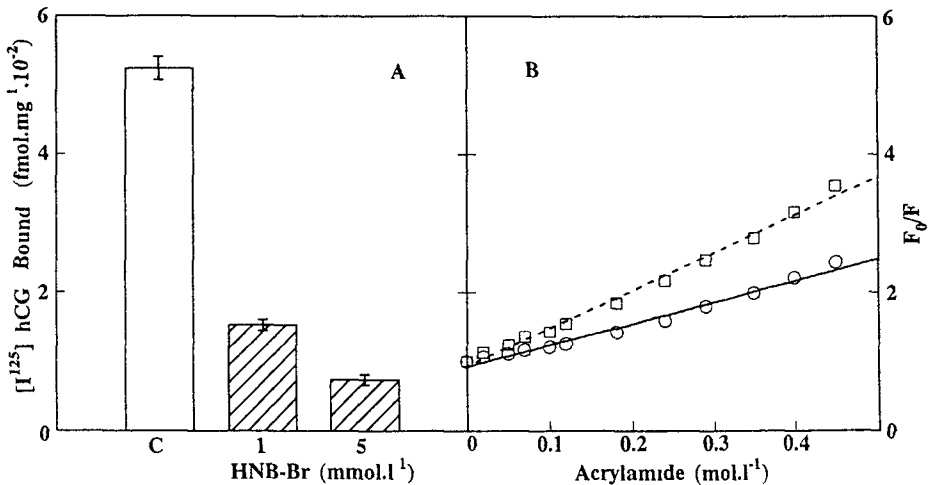


Figure 4. Effect of protein-modifying reagent HNB-Br (1 and 5 mmol l^{-1}) on the accessibility of rat ovarian LH/hCG receptor (A) and the acrylamide quenching (5 mmol l^{-1} HNB-Br) of ovarian membranes (B). Membranes were incubated for 30 min at 25°C with HNB-Br and then washed twice with phosphate buffer. The excitation and the emission wavelengths were 280 nm and 380 nm for control (\circ) and 340 nm for HNB-Br-treated membranes (\square) respectively. (A) Data are means \pm SE of 3 determinations (each repeated twice). (B) The experiments were repeated 3 times with comparable results.

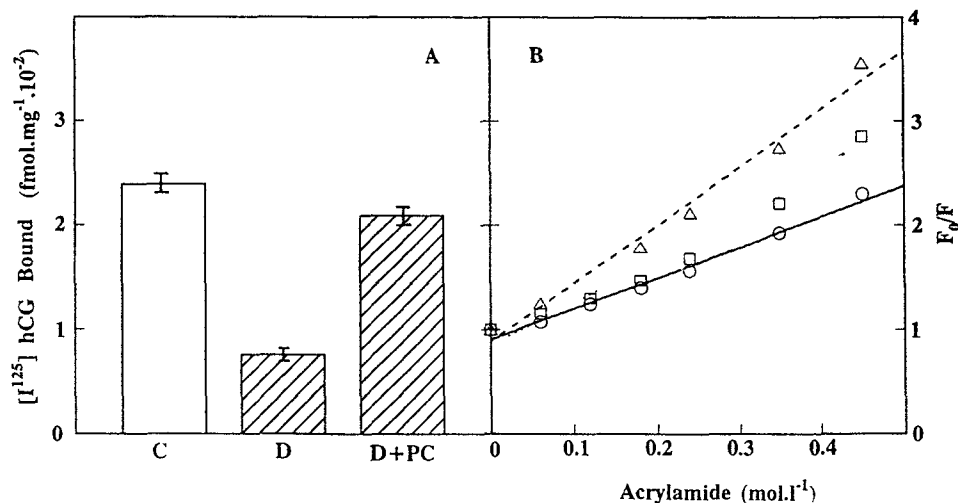


Figure 5. Specific binding of [¹²⁵I]hCG and Stern-Volmer plots for acrylamide quenching of control (○), delipidated (△) and delipidated + phosphatidylcholine (PC) (□) reconstituted receptor into proteoliposomes. Soybean PC (2.5 mg) was added during solubilization of hCG binding activity with 20 mmol l⁻¹ sodium cholate in buffer A containing 20% glycerol. The detergent was removed using Bio-Beads SM-2 (1). Data are means ± S.E. of 4 determinations (each repeated twice). (B) The results were confirmed in 3 independent experiments.

ing sites to the initial extent (Fig. 5A). On comparing the lipid level in identical samples before and after lipid depletion, the contents of total cholesterol and phospholipids in the delipidated membrane were 34% and 46% of controls, respectively (data not shown) (Kolena et al. 1994b). The possible structure-functional alteration of delipidated LH/hCG receptor reconstituted into proteoliposomes was analyzed by the quenching method. The Stern-Volmer plots for this experiment are shown in Fig. 5B. The corresponding Stern-Volmer constants determined from the slopes for control, delipidated and delipidated + phosphatidylcholine (PC) proteoliposomes with incorporated LH/hCG receptor (Kolena et al. 1995) were found to be 2.9, 5.6 and 4.0 mol⁻¹, respectively. The results indicated altered accessibility of fluorophores for acrylamide. An increase of quenching after delipidation was observed but the addition of PC to delipidated proteoliposomes made quenching to approach to control proteoliposomes. Delipidation altered spectral properties of proteoliposome intrinsic fluorescence, a blue shift of emission maximum from 450 nm for control to 424 nm for delipidated proteoliposomes was observed (data not shown).

Discussion

The results presented in this paper show the possibility of an application of the fluorescence quenching method in the study of gonadotropin receptor properties in relation to the physical state of the ovarian membranes. Quenching studies can provide valuable information concerning the exposure of tryptophanyl residues and the dynamics of the protein matrix surrounding such residues. This technique may be used to investigate protein dynamics and ligand induced changes in conformation of proteins (Eftink and Ghiron 1981). Quenching of the intrinsic fluorescence of proteins by the addition of extrinsic agents is governed by the ability of quencher to get into close contact with the fluorophore. The access can be restricted by either steric or chemical barriers to quencher penetration. The steric barriers are not rigid and act to limit the penetration of large quenching agents through the protein matrix. Chemical barriers are due to the chemical environment in which the fluorophore is located (Havel et al 1988). Such steric or chemical barriers can be involved in the restriction of the iodide quenching as observed in our experiments as the accessibility of tryptophan fluorophores for iodide was not changed. Our results showed that acrylamide is a very effective quencher of intrinsic fluorescence of ovarian membranes, this might be taken as an indication that these barriers are not involved in acrylamide quenching. One of the most attractive features of acrylamide as a quencher has been the fact that it does not significantly interact with protein (Eftink and Ghiron 1981). The rate constant of the quenching reaction between the quencher and a fluorophore (tryptophan residue) is known to be dependent on diffusion-controlled process. Therefore an increased quenching rate generally suggests an increase in the proximity of quencher molecules to the fluorophore.

Desensitization modified the quenching rate of protein fluorescence and spectral properties of membrane intrinsic fluorescence indicating that conformational properties of the receptor were changed. These results suggest that the hCG-induced alteration of the physical state of luteal membranes may be a requirement for the induction of changes that lead to desensitization. Such alteration of the physical state of the luteal membrane may facilitate the transformation of the receptor to its desensitized state (Kolena et al 1994a).

The amino acids of receptors in contact with ligands at the binding site may play an important role. Chemical modification studies of protein hormone receptors have been used in order to understand the nature of their structure and binding domains. Most of these studies have been limited to membrane receptor preparations. Treatment of rabbit mammary prolactin receptor with HNB-B₁ resulted in a loss of specific binding activity of the receptor. Based on the relative specificity of HNB-B₁ to tryptophan, it would appear that tryptophan may be critical for prolactin binding to the receptor (Mahajan and Ebner 1986). In a previous study

it was demonstrated that disulfide, histidyl and tyrosyl groups may be involved in the interaction of hCG with its receptor (Kolena and Šebokova 1987). In these experiments HNB-B1 modified the quenching of tryptophan as well as tyrosine residues of LH/hCG receptor from rat ovarian membranes.

Reconstitution of delipidated LH/hCG receptor into proteoliposomes enhanced the accessibility of fluorophores for acylamide, but its reconstitution in the presence of phosphatidylcholine turned them into the same physical state as control proteoliposomes. The role of lipids in membranes is not passive since the lipids may regulate numerous cellular functions. The presence of a lipid environment is known to be essential for the maintaining of the receptor function. The LH/hCG receptor is an integral protein containing 7 transmembrane segments (McFarland et al 1989) and it seems likely that it interacts with lipids in the membrane. Lipid-receptor interactions may be required for conformation changes of the receptor and signal transmission across the membrane. Delipidation of native membranes was observed to reduce the function of LH/hCG receptor from porcine and rat corpora lutea (Kolena 1992, Kolena et al 1992a). The requirement of specific lipids for a receptor system is still an area of active investigation. Phosphatidylcholine meets the criteria for reconstitution of the receptor into proteoliposomes in its functional state (Kolena 1989, Kolena et al 1992b). PC forming a bilayer structure may be the most suitable species for preserving receptor activity during reconstitution. There are probably more important requirements for the polar group than for acyl side chains of phospholipids. The reacting abilities and thermal stability of PC with different hydrocarbon chains (soybeanPC, dioleoylPC, dipalmitoylPC) were essentially the same (Kolena et al 1995).

The mechanism of the quenching reaction is not known in detail yet, but may involve internal conversion due to electron exchange, enhanced intersystem crossing due to spin-orbital coupling or electron spin exchange, or electronic energy transfer (Eftink and Ghiron 1981). Although the technique of fluorescence quenching is experimentally a simple one, the analysis and interpretation of the data can be more complex in membrane preparations than in a particular protein. Despite some difficulties, fluorescence quenching is a useful method in the study of the functional state of gonadotropin receptors.

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