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Rapid report

Luteinizing hormone receptors are associated with non-receptor plasma membrane proteins on bovine luteal cell membranes

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Abstract

Biophysical studies of the bovine luteinizing hormone (LH) receptor on luteal cell membranes suggest that this receptor may be part of a larger molecular weight structure. We have used 5-iodonaphthyl-1-azide (INA) to identify plasma membrane proteins near LH receptors on plasma membranes from bovine corpora lutea. Following binding of eosin isothiocyanate-derivatized ovine LH or human chorionic gonadotropin (hCG), five proteins with molecular weights of 71, 57, 55, 49 and 36 kDa were selectively derivatized with [^{125}I]-INA following 2 h exposure at 22°C to 514 nm light. However, there was no fluorescence energy transfer between LH receptors occupied by ovine LH or hCG indicating that LH receptors were not self-associated in these membrane preparations. Together these results suggest that, following hormone binding, single copies of the LH receptor may exist in large molecular weight structures that include non-receptor proteins.

Keywords: Plasma membrane protein; Corpus luteum; Receptor

The luteinizing hormone (LH) receptor has been well characterized in a number of species including rat [1], mouse [2], man [3] and pig [4]. The receptor is a 90 kDa protein with seven transmembrane segments and is structurally similar to other G protein-coupled receptors [1]. While the receptor itself appears to be a monomeric protein, there is little information on how this receptor interacts with other plasma membrane components or with proteins involved in signal transduction. To address this question we have examined the rotational diffusion of LH receptors in bovine corpora lutea membranes [5] where, following binding of either LH or human chorionic gonadotropin (hCG), the LH receptor is rotationally immobile on the time scale of our experiments. These results suggest physically large, and thus slowly rotating, plasma membrane complexes containing the LH receptor.

Rotational diffusion measurements do not provide information on the composition of protein complexes. Therefore, a method developed by Raviv et al. [6,7] and modified by Meiklejohn et al. [8] was used to determine whether the LH receptor complex contained other non-receptor proteins. Fluorescence energy transfer techniques were then used to determine

Abbreviations: LH, luteinizing hormone; hCG, human chorionic gonadotropin; INA, 5-iodonaphthyl-1-azide; TRITC, tetramethylrhodamine isothiocyanate; FITC, fluorescein isothiocyanate

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whether receptor complexes contained more than one LH receptor.

For these studies we prepared plasma membranes from mature, non-regressing bovine corpora lutea that were collected from ovaries obtained at slaughter as has been previously described [5]. Protease inhibitors were added to the buffers used during corpora lutea homogenization and membrane storage to eliminate protease activity in membrane samples [5]. Membrane preparations were tested for endogenous proteolytic enzyme activity using an endoproteinase test kit (Boehringer Mannheim, Indianapolis, IN).

To determine whether non-receptor membrane proteins were near the LH receptor following binding of LH or hCG, we incubated plasma membranes with 5-iodonaphthyl-1-azide (INA), a lipophilic molecule that inserts readily into the plasma membrane and can be photosensitized by energy transfer from a triplet-forming chromophore. INA will covalently derivatize proteins and lipids situated within perhaps 100 Å of the binding site of the eosin-labeled hormone [8]. Experimentally, 10 ml of bovine plasma membranes were treated with 10 μl of 26 mM [^{I25}I]-INA in ethanol for 2 h with gentle mixing. This and all subsequent steps were performed in the dark. [^{125}I]-INA was prepared as described by Bercovici and Gitler [9]. Membranes were then labeled with 1 nM eosin isothiocyanate (EITC)-derivatized oLH or EITC-hCG for 1 h at room temperature, EITC-LH (NIH ovine LH S-24) or EITC-hCG (NIH hCG CR-121) conjugates having been prepared using previously published methods [10]. The molar ratios for derivatized hormones and the concentration of protein in solution were determined spectrophotometrically. The hormone preparations used in these experiments had 1–1.5 mol of EITC per mol of oLH or hCG. Immediately prior to use, hormone preparations were centrifuged at 30,000 $\times$ g for 5 min in a Beckman Airfuge to remove any protein aggregates which formed during storage at 4°C. Following hormone labeling, membrane samples were then deoxygenated by bubbling with N_2 and by 30 min exposure to an oxygen scavenging medium containing 5 mM D-glucose (Sigma, St. Louis, MO), 50 units/ml catalase (Sigma, St. Louis, MO) and 10^4 units/ml glucose oxidase (Sigma, St. Louis, MO) [11]. Samples were exposed to 400 mW of 514 nm light from a Coherent Radiation 100-10 argon ion laser for 2 h.

Electrophoresis of solubilized membrane proteins was performed as described by Laemmli [12]. Membranes were pelleted by centrifugation at 20,000 $\times$ g, the supernatant was decanted and the pellet was resuspended in 300 μl of 1% Triton X-100 in the dark. Bovine plasma membranes were sonicated for 1 h at which time, to complete solubilization of membrane proteins, 300 μl of SDS sample buffer was added to the membranes. The proteins were then separated on a 9% polyacrylamide gel at 100 V for 5 h in the dark. The gel was fixed for 1 h in 10% glutaraldehyde and washed for 24 h in double-distilled water. The washed gel was silver stained and placed in a plastic bag for autoradiography. The gel,

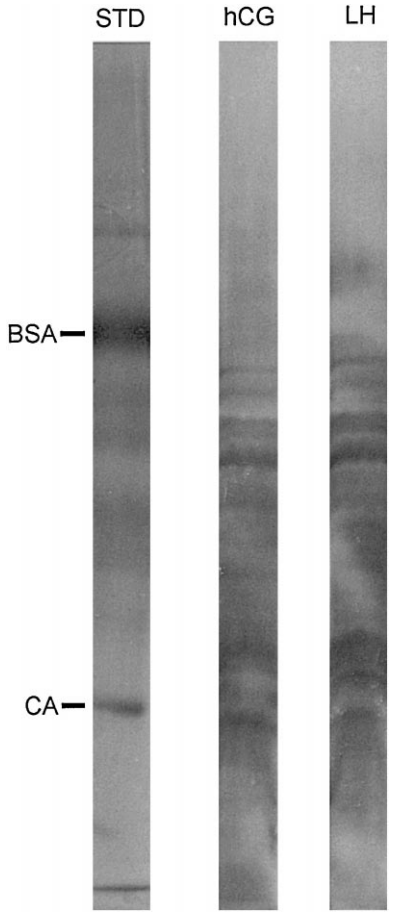

Fig. 1. Silver stain of the bovine corpora lutea proteins. The molecular weight standards (STD), bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa), are shown in the left lane. In the middle lane and right lane are membrane proteins from bovine corpora lutea treated with EITC-hCG and EITC-oLH, respectively.

Dupont Cronex Lighting Plus intensifying screens, and Kodak X-OMAT AR film in a tray exposure cassette were placed in a −70°C freezer. The film was developed 2 weeks later and scanned at 300 dot per inch resolution on an Hewlett Packard Scan Jet Plus white light scanner interfaced to a Microscan 2000 video image analyzer (Technology Resources, Nashville, TN).

A silver stain of proteins separated by gel electrophoresis (Fig. 1) and an autoradiograph of that gel (Fig. 2) showed that the plasma membrane preparations used in these experiments contained numerous proteins of which only a small subset were reproducibly derivatized with [^{125}I]-INA. Densitometric scans of the autoradiograph shown in Fig. 3, revealed at least five iodinated proteins with molecular weights of approximately 71, 57, 55, 49, and 36 kDa. Interestingly, the relative abundance of the 49 kDa INA-derivatized protein differed when EITC-oLH vs. EITC-hCG was bound to receptors on membrane preparations. There was approximately two-fold more 49 kDa protein on EITC-oLH labeled preparations compared to membrane preparations labeled with EITC-hCG. We did not detect any membrane proteins with the molecular weight of the LH receptor [1]. Such a result would be obtained if LH receptors were not accessible to [^{125}I]-INA.

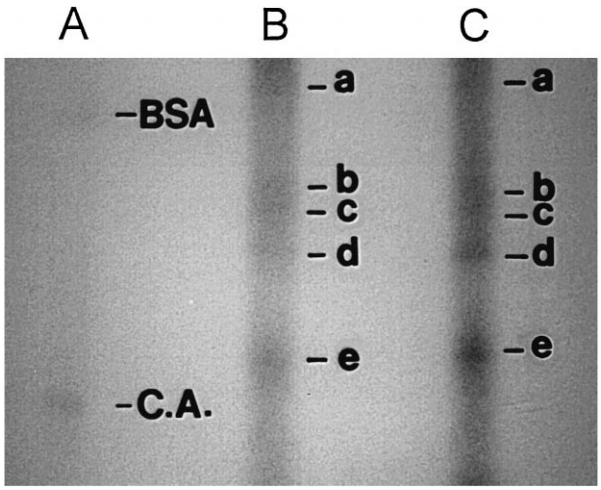

Fig. 2. Autoradiograph of the silver stained gel shown in Fig. 1. Lane A shows the two molecular weight markers, BSA and carbonic anhydrase (CA). In Lanes B and C, five prominent membrane proteins were labeled by [^{125}I]-INA following treatment of plasma membranes with EITC-hCG and EITC-oLH, respectively.

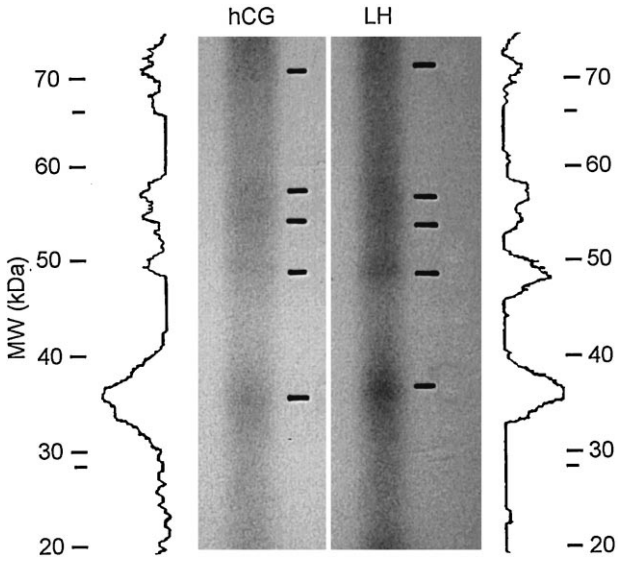

Fig. 3. Autoradiographs of the SDS-gel show the membrane proteins subjected to [^{125}I]-INA labeling. Optical density scans corrected for low spacial frequency background absorbance are shown at the sides of each gel. Five peptides with molecular weights of 71, 57, 55, 49, and 36 kDa were detected following labeling of plasma membrane preparations with EITC-hCG (left lane) and EITC-oLH (right lane).

We then employed a fluorescence energy transfer method to determine whether multiple copies of the LH receptor were present in the receptor complex. This photobleaching fluorescence energy transfer method is based on the reduced rate of irreversible photobleaching of donor fluorophores when acceptor fluorophores are present [13]. Slower rates of fluorescence decay for cells labeled with donor and acceptor (D + A) fluorophores than for cells labeled with fluorescence donor only (D) are indicative of energy transfer from fluorescence donor to acceptor and occur only when the donor and acceptor are separated by distances less than R_0 which is characteristic of the specific donor/acceptor pair [14]. Energy transfer was quantitated as % energy transfer efficiency.

For these experiments we used a fluorescence microscope photometer based on an inverted-configuration Zeiss Axiomat microscope equipped with a fluorescence vertical illuminator, photometer module, thermoelectrically controlled thermal stage and scanning stage. Fluorescence isolated by the photometer module was conducted by 3-mm glass fiberoptic bundles to a Hamamatsu R943 photomultiplier tube

mounted in a thermoelectrically-cooled housing. Fluorescence excitation was provided by a Coherent Radiation Innova 100 argon ion laser operating under light control at 488 nm. The intensity of the laser radiation focused on the cell was 15–20 mW and this quantity was held constant between measurements on membranes labeled with fluorescein isothiocyanate (FITC)-derivatized LH or hCG only or on membranes labeled with FITC-derivatized hormone plus tetramethylrhodamine isothiocyanate (TRITC)-derivatized hormone. The $1/e^2$ Gaussian spot diameter was 29 μm. Fluorescence intensities were measured via photon counting using a Princeton Applied Research 1182 amplifier/discriminator and expressed as counts per second (cps). Donor fluorescence from FITC was isolated with a standard fluorescein filter set together with a short pass fluorescein-selective filter to remove red tetramethylrhodamine fluorescence. This combination was highly effective in rejecting TRITC fluorescence. In individual experiments membranes were identified and centered in the microscope field and, at time zero, an electronically controlled shutter was opened to allow laser radiation to impinge on the sample. Simultaneously, a computer program was activated to record the output of the photomultiplier measuring membrane fluorescence. Data were collected at 0.01 s intervals for 10 s. Typically, about 20 sites in each sample were photobleached in this manner. The data traces were analyzed to give the energy transfer efficiency as has been described in detail previously [13].

As shown in Fig. 4, there was no significant difference between the initial rate of fluorescein photobleaching in the presence or absence of TRITC-derivatized LH (left panel) or TRITC-hCG (right panel). This suggests that the receptors are physically separated from one another by a distance greater than about 56 Å, the R_0 value for this donor/acceptor pair [15]. From these results it appears that LH receptors on bovine corpora lutea are contained within large molecular weight structures comprised of a number of membrane proteins in addition to individual copies of the receptor itself. [^{125}I]-INA derivatization of a family of membrane proteins following binding of EITC-LH and EITC-hCG to the LH receptor is consistent with our previous time-resolved phosphorescence anisotropy studies of the LH receptor on bovine corpora lutea membranes [5] in which the bovine LH receptor appeared to exist within a large complex that was rotationally

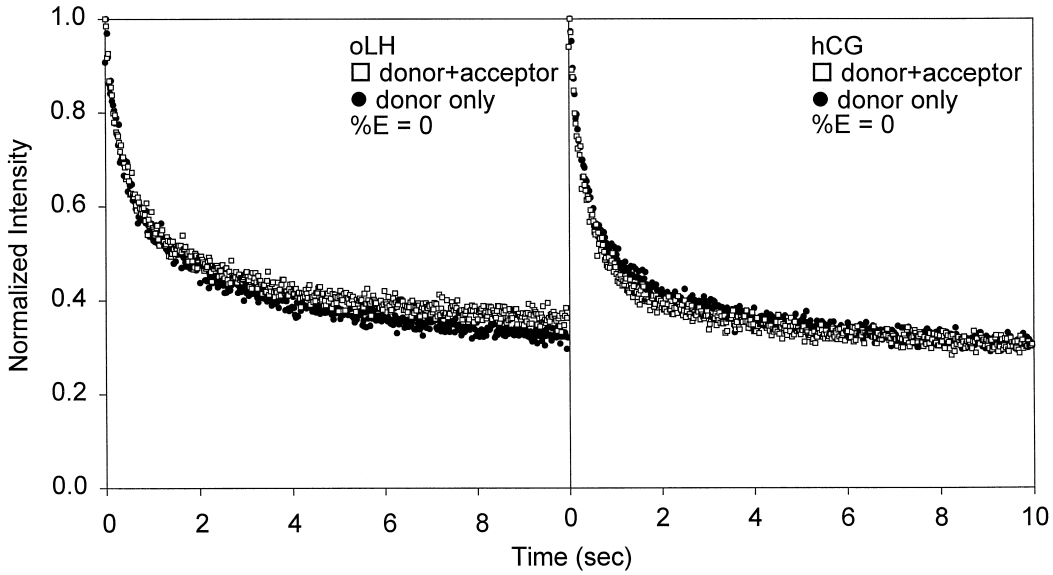

Fig. 4. Fluorescence energy transfer between LH- (left panel) and hCG-occupied (right panel) LH receptors on bovine plasma membranes. There was no significant difference in fluorescein fluorescence decay between membrane labeled with fluorescence donor alone (●) and fluorescence donor and acceptor (□). For both LH- and hCG-occupied receptors, energy transfer efficiency was not significantly different from zero.

immobile on the 1000 μs time scale of our experiments. This is the case for a number of receptors including epidermal growth factor receptor [16,17] which is present in aggregated structures following hormone binding. These structures can contain other non-receptor proteins. The T cell and B cell receptors are both coprecipitated with a number of proteins which comprise part of a large receptor-containing complex [18,19]. Similarly, Ortega et al. [20] have shown that, on rat basophilic leukemia cells, there is recruitment of non-receptor proteins into structures containing the Type I Fcε receptor.

Although [^{125}I]-INA derivatizes proteins near the LH receptor, we do not yet know the identity of these proteins or their function. However, one can propose several possible functions for these proteins including involvement in signal transduction or in maintaining the conformation of LH receptor in the lipid bilayer. It is not likely that these proteins are randomly present near the receptor since they were reproducibly identified in repeated experiments and since similar proteins were identified in samples treated with oLH and hCG. However, as was the case for experiments described by Raviv et al. [6,7] and Meiklejohn et al. [8], the efficiency of [^{125}I]-labeling of membrane proteins was low. Thus, we cannot determine whether these INA-derivatized proteins represent the entire complement of membrane proteins near the LH receptor.

Nonetheless, the absence of fluorescence energy transfer between LH receptors suggests that LH receptors are greater than about 100 Å apart on these membrane preparations. This is in contrast to other studies in which we have measured positive values for fluorescence energy transfer between LH receptors on ovine luteal cells [21]. LH receptors on intact ovine luteal cells and on plasma membrane preparations exhibit slow rotational lateral diffusion following binding of hCG [5]. Evidence from other laboratories also suggests that small clumps of LH receptors are present on the cell membrane. Scanning confocal microscopy studies of rat ovarian cells reveals fluorescently-labeled LH receptors that are non-uniformly distributed over individual cells [22]. Electron microscopic studies show that the receptors are concentrated on microvilli in rat luteal tissue [23] and that, following hormone binding, there is a rapid aggregation of receptors into larger clusters [24]. From electron microscopy studies, it appears that the size of receptor-hormone aggregates increases on cells exposed to LH concentrations at or greater than those which result in maximum progesterone secretion [25]. In summary, photoproximity labeling of proteins near the LH receptor, together with fluorescence energy transfer measurements of inter-receptor distance, has the potential to reveal protein interactions between receptors and other non-receptor proteins involved in signal transduction and enhances our understanding of membrane protein organization following hormone binding.

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