

EVIDENCE FOR THE EXISTENCE OF GONADOTROPIN RECEPTORS
IN THE NUCLEI ISOLATED FROM RAT OVARY

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Specific binding of radiolabeled human chorionic gonadotropin (hCG) to nuclei isolated from pseudopregnant rat ovaries was studied. Incubation of cultured luteal cells or isolated nuclei with fluorescein isothiocyanate conjugated hCG showed concentration of fluorescence in the nuclear region. Isolated nuclei exhibited saturable high affinity binding of radiolabeled hCG with an apparent K_d of 3.42×10^{-10} M. The binding was inhibited by increasing concentrations of unlabeled hCG. Under dissociating conditions, the bound hCG was dissociated from the nuclei. However, unlike the plasma membranes, the hCG bound to nuclei was not degraded before dissociation. Radiolabeled hCG bound to the nuclei could also be dissociated by brief exposure to $MgCl_2$ or acidic incubation medium. The bound hCG was not extractable with 4M KCl or 2% Triton X-100. The available evidence suggest that nuclear receptors are distinct from plasma membrane receptors for hCG.

Receptor-mediated internalization of hCG in testicular tumor cell line (1) and luteal cells of pseudopregnant rat (2) and ewe (3) have been reported. Rao et al. (4) showed the presence of specific binding sites for hCG in the intracellular organelles prepared from bovine corpora lutea. Similar intracellular distribution of receptors of other peptide hormones such as insulin (5-7) and growth hormone (6) have also been demonstrated. In this communication, we report the existence of discrete hCG receptors in the nuclei isolated from rat ovary employing a fluorescent conjugate of hCG and iodinated hCG as probes.

MATERIALS AND METHODS

Animals: 21 day old female rats (Sprague Dawley strain) were injected with 50 IU of pregnant mare serum gonadotropin (PMSG) followed 56 h later (day 0) by 25 IU of hCG (8) to obtain highly luteinized ovaries. Rats were killed on day 4 and ovaries collected.

Preparation of Luteal Cells for Fluorescence Microscopy: Ovaries were collected from pseudopregnant rats and luteal cells were dispersed using collagenase and pancreatin and cultured in McCoy's 5A Medium (modified)

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(Grand Island Biological Co., NY), supplemented with 10% horse serum and 2.5% fetal bovine serum, amino acids and antibiotics in tissue culture chamber slides and maintained at 37°C in a humid atmosphere of 5% CO₂ and 95% air. Cell attachment occurred during overnight incubation. For microscopic observations the culture medium was removed by aspiration, the cells washed with fresh medium, and then 1 ml of medium containing 1 µg of FITC-hCG was added and incubation continued at 37°C. After incubation, the medium was removed and the cells were washed and observed under a Carl-Zeiss fluorescence microscope..

Isolation of Purified Nuclei from Rat Luteal Cells: Nuclei were isolated from luteinized rat ovaries by the procedure described by Blobel and Potter for liver tissue (9). Freshly collected ovaries were homogenized in 5 volumes of cold sucrose buffer containing 0.34 M sucrose, 15 mM magnesium acetate and 0.25 mM spermine (pH 7.4) in a glass homogenizer fitted with teflon pestle. The homogenate was mixed with 2.3 M sucrose (1:3) and centrifuged at 24,000 rpm for 40 min in a Beckman SW 25.1 rotor. The supernatant was discarded, the pellet was resuspended in the original volume of sucrose buffer by gentle rehomogenization and then mixed 1:3 with 2.3 M sucrose. This was layered over 5 ml of 2.15 M sucrose cushion in Ultracentrifuge tubes and centrifuged for 40 min at 24,000 rpm. The resulting pellet was resuspended in TMS buffer (0.01 M Tris pH 7.9, 5 mM MgCl₂, 5 mM Sucrose, 5 mM Dithiothreitol) and used for subsequent studies.

Preparation of FITC Conjugated hCG: Conjugation of hCG with Fluorescein isothiocyanate (FITC) was carried out as described by Nairn (10) with slight modifications. HCG (1 mg; CR-121; 13,450 IU/mg) was dissolved in 0.27 ml of PBS (pH 7.4). To this was added 5000 cpm of [¹²⁵I]hCG in 25 µl of PBS and 54 µl of 0.2 M Na₂HPO₄, followed by the dropwise addition of FITC (in 0.1 M Na₂HPO₄ to give a final concentration of 0.4 mg/ml). The pH of the mixture was adjusted to 9.3 with 0.1 M Na₂HPO₄ and then 50 µl of 0.145 M NaCl was added. The reaction mixture was stirred for 2 h at 4°C in the dark and then loaded on a Sephadex G 25 column (1 x 20 cm) equilibrated with PBS. The column was eluted with PBS and 0.5 ml fractions collected. The radioactivity in each fraction was counted to detect hCG and the optical density at 492 nm was read on a spectrophotometer to detect the FITC. Fractions 14-18 contained FITC-conjugated hCG which was used for subsequent labeling studies.

Binding of [¹²⁵I]hCG in Isolated Nuclei: Isolated nuclei (100 µl) containing about 10-20 µg DNA were incubated with [¹²⁵I]hCG (40 cpm/pg) in a total volume of 400 µl buffer (containing Tris 5 mM, Sucrose 125 mM, Calcium chloride 0.5 mM, Sodium chloride 75 mM and BSA 0.5%, pH 7.4) at 37°C with constant shaking. After the incubation, 2 ml of incubation buffer was added, the tubes were centrifuged at 10,000 rpm, the supernatant removed, the pellet resuspended in 2 ml of incubation buffer and centrifuged again. The pellet was counted for radioactivity in a scintillation counter. Nonspecific binding was determined by parallel incubation in the presence of 1000 fold excess of unlabeled hCG.

RESULTS

To obtain direct visual evidence for hCG internalization, we have employed the FITC-hCG conjugate as a probe. When the cells were incubated with FITC-hCG for 10 min, bright fluorescent patches or clusters were seen along the periphery of the cells (Fig. 1a). On prolonged incubation for 3 h, the

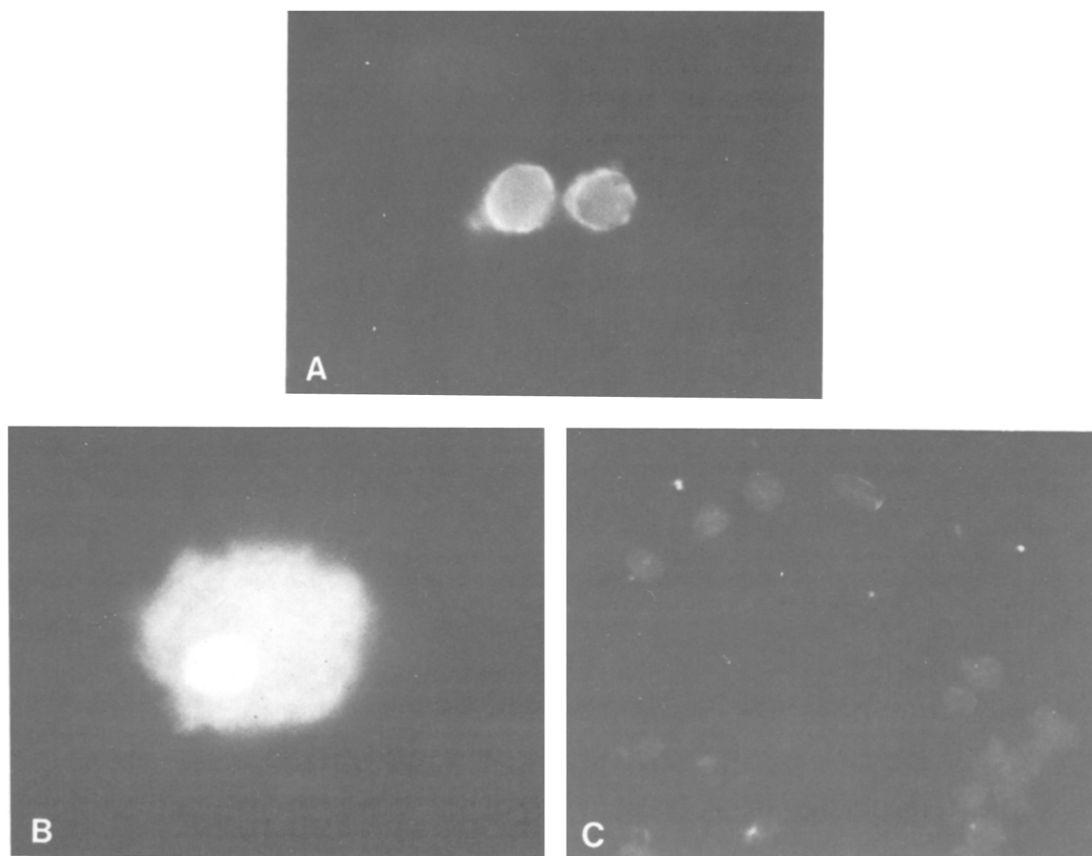


Fig. 1: Fluorescent photomicrographs of rat luteal cells incubated with FITC-hCG.

Cultured rat luteal cells were incubated with 1 ml of McCoy's medium containing 1 μ g of FITC-hCG at 37°. After incubation, the medium was removed, the cells washed and observed under a fluorescence microscope. (a) Cells viewed after 10 min incubation. (b) Cells viewed after 3 h incubation. (c) Isolated nuclei incubated with FITC-hCG for 10 min. Original magnification, (a) and (c) x336, (b) x866.

fluorescence was seen inside the cell as well with heavy accumulation on the nucleus making it distinctly visible (Fig. 1b). To confirm whether hCG in fact binds to the nucleus, freshly isolated nuclei from corpora lutea were incubated with FITC-hCG for 10 min. The nuclei were pelleted by centrifugation, washed, and observed under the microscope. A significant uptake of the fluorescence by the nuclei was observed (Fig. 1c).

To examine the nuclear binding of hCG, freshly isolated nuclei from pseudopregnant rat corpora lutea were used. When the nuclei were incubated

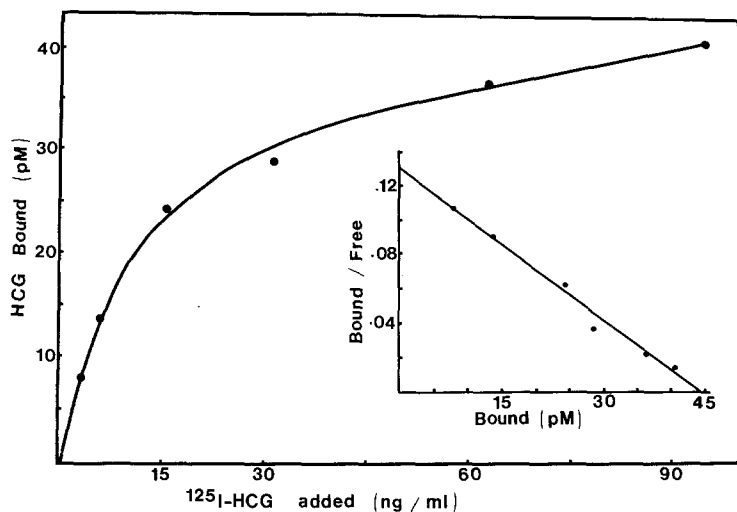


Fig. 2: Binding of [^{125}I]hCG to nuclei isolated from rat luteal tissue. The isolated nuclei were incubated with increasing concentrations of [^{125}I]hCG for 3 h at 37°. The nuclei were pelleted by centrifugation and counted for radioactivity. Inset: Scatchard plot of the same data.

for 3 h with increasing concentrations of [^{125}I]hCG, saturable binding of [^{125}I]hCG by the nuclei was observed (Fig 2). Scatchard analysis (11) of the data showed a K_d of 3.42×10^{-10} M. When the nuclei were incubated with 500,000 cpm of [^{125}I]hCG in the presence of increasing doses of unlabeled hCG, the binding of [^{125}I]hCG decreased in a concentration-dependent manner

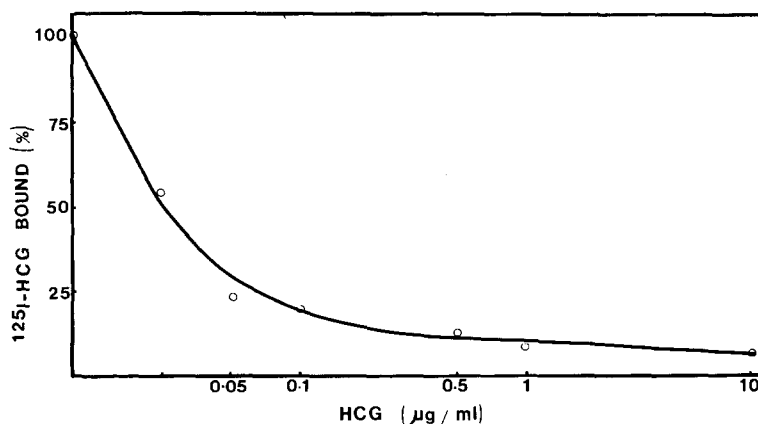


Fig. 3: Binding of [^{125}I]hCG to nuclei isolated from rat luteal tissue. The isolated nuclei were incubated with 500,000 cpm of [^{125}I]hCG in the presence of various concentrations of unlabeled hCG for 3 h at 37°. The radioactivity associated with the nuclear pellet was counted after removal of unbound hormone.

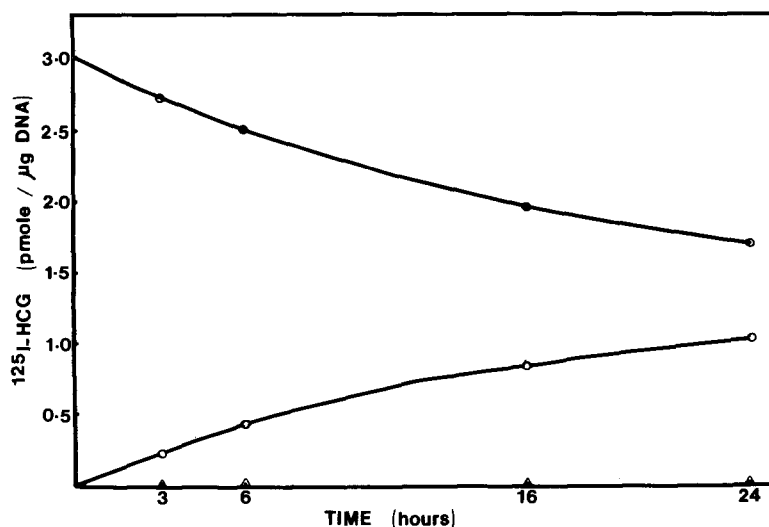


Fig. 4: Dissociation and degradation of [^{125}I]hCG bound to the nuclei. Isolated nuclei were incubated with [^{125}I]hCG (5,000,000 cpm/ml) for 3 h at 37°. The unbound hCG was removed by washing 3 times and the washed nuclei were suspended in 1 ml fresh buffer. At the indicated intervals, 100 μl aliquots in duplicate were drawn, centrifuged, and the pellet and supernatant counted for radioactivity. To the supernatant was added 10% cold trichloroacetic acid and, after 30 min, centrifuged. To the supernatant 10 μl KI (40%) and 40 μl H_2O_2 were added and the free iodine was extracted with chloroform. The aqueous phase was counted in a gamma counter to determine degradation.

(Fig. 3). The dissociation kinetics of [^{125}I]hCG bound to the nuclear binding sites was different from that of hCG bound to the plasma membrane receptors. As shown in Fig. 4, under dissociating conditions, approximately 45% of the bound hCG was dissociated from the nuclei by 24 h. In sharp contrast with the plasma membrane receptor system (2), the dissociated hCG from nuclei did not show evidence for degradation (Fig. 4). Exposure of nuclear receptor bound hCG to MgCl_2 resulted in dissociation of the hormone. This chaotropic agent also caused dissociation of hCG bound to plasma membranes (Fig. 5), although the extent of dissociation of the plasma membrane bound hCG was higher than the nuclear bound hCG. At lower pH, the dissociation of the hormone bound to the nuclei proceeded similar to the plasma membrane bound hCG (Fig. 5). KCl had no effect on the dissociation of either type (Fig. 6). The nuclear receptors were, however, not extractable with Triton-X-100 in contrast with plasma membrane receptors.

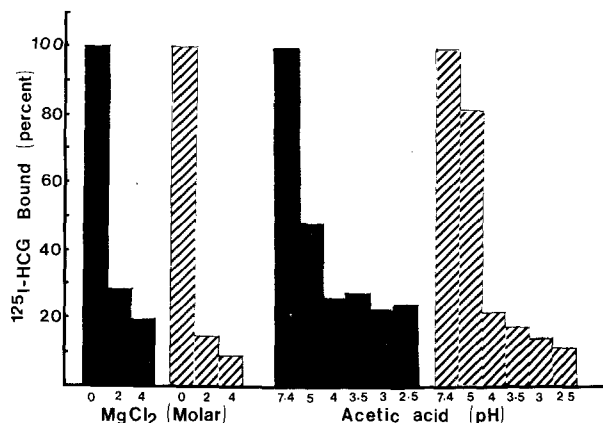


Fig. 5: Effect of Magnesium chloride and pH on the binding of [¹²⁵I]hCG to nuclei isolated from rat luteal tissue. Isolated nuclei were incubated with [¹²⁵I]hCG for 3 h at 37°. The unbound hormone was removed by washing and the nuclei suspended in MgCl₂ or 0.145 M NaCl and acetic acid (pH ranging from 2.5-7.5) for 10 min. The nuclei were pelleted by centrifugation and radioactivity determined. Parallel assays were conducted using rat ovarian plasma membranes solubilized in 1% Triton-X-100. [] Nuclei, [/] Plasma membrane.

which were readily solubilized in the presence of the detergent (Fig. 6).

DISCUSSION

The above data demonstrate the presence of high affinity saturable binding sites for hCG in the nuclei isolated from rat ovary. The hCG bound to

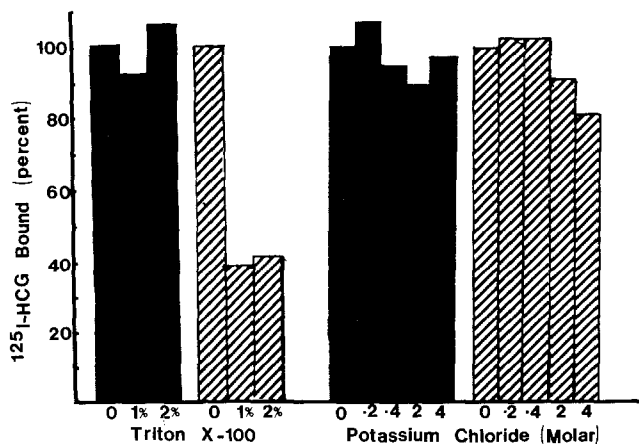


Fig. 6: Effect of Triton-X-100 and KCl on the binding of [¹²⁵I]hCG to nuclei isolated from rat luteal tissue. Isolated nuclei were incubated with [¹²⁵I]hCG for 3 h at 37°. The unbound hormone was removed by washing and the nuclei suspended in Triton-X-100 or KCl at the indicated concentrations. The incubation was continued at 37° for 1 h. The nuclei were pelleted and the radioactivity determined. [] Nuclei, [/] Plasma membrane.

plasma membrane receptors is dissociated by exposure to low pH (3, 12) or 2 M $MgCl_2$ (13, 14). These treatments also released hCG from the hCG-nuclear receptor complex. However, the nuclear receptor-bound hCG was not solubilized by Triton-X-100 whereas plasma membrane receptors were readily solubilized. This suggests that the nuclear binding is not due to contamination with plasma membrane receptors. Direct uptake of fluorescent hCG conjugate by the nuclei confirmed this observation. The hCG binding to the nuclei further differed from plasma membrane binding in that the dissociation of the former is not associated with degradation. We have reported earlier that more than 50% of the plasma membrane bound [^{125}I]hCG is degraded before dissociation from the binding sites (2).

It is known that treatment of intact nuclei with Triton-X-100 will remove much of the outer nuclear membrane (15). The observation that Triton-X-100 did not remove any of the bound hCG shows that the hormone is not bound to the nuclear membrane. This conclusion is confirmed by the fluorescence micrograph of isolated nuclei exposed to FITC-hCG (Fig. 1c) which showed distribution of the fluorescence throughout the nuclei. The exact location of the binding sites cannot be inferred from the present data but it suggests that the receptors are not localized exclusively on the nuclear membrane. Whether the nuclear and plasma membrane receptors for hCG/LH are structurally and immunologically identical is not known, nor is it understood whether the nuclear receptors for hCG/LH has any physiological role.

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