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CHARACTERIZATION OF A PROGESTIN-BINDING MACROMOLECULE IN THE AMPHIBIAN OOCYTE CYTOSOL

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SUMMARY

A [^{3}H]-progestin-binding macromolecule has been isolated from R. pipiens oocyte cytosol and characterized using binding assays, gel filtration, DEAE-cellulose chromatography and ultracentrifugal techniques. Macromolecules present in the prophase oocyte cytosol have a high affinity and specificity for the synthetic progestin R5020 and the intact oocyte will concentrate both R5020 and progesterone 20-40 fold from the medium. This may be the first published case of a cytosol steroid binding macromolecule in a cell system in which the steroid appears to act at an extranuclear level.

INTRODUCTION

The action of progesterone on the amphibian oocyte is considered to be unique in that it acts at an extra-nuclear level. Progesterone initiates the resumption of meiosis (1,2) and the oocyte completes one and one-half meiotic divisions before becoming arrested at second meiotic metaphase. An increase in protein synthesis apparently required for the successful completion of meiosis (1,3) coincides with nuclear breakdown during the first meiotic division and occurs even when the oocyte has been enucleated (4). This finding together with a lack of inhibitory effect of either exogenous (3,5,6) or injected (7,8) actinomycin D on the sequence of maturational events has been taken as evidence against obligatory RNA synthesis. This is in contrast to other steroid target cells (9) where the physiologically active steroid first binds to a cytosol receptor and then moves into the nucleus to induce the synthesis of a new mRNA and then that of protein.

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Progesterone is believed to exert its primary action at the oocyte surface, since exogenous but not injected (4) progesterone initiates the resumption of the meiotic divisions. The concept of a membrane receptor is supported by autoradiographic studies that indicates steroid to be localized mainly at the level of the cell membrane and underlying cytoplasm (8). Ozon and Belle (10) and Brachet et al. (8) have reported specific binding of progesterone to cortical melainin containing granules and to a 15-18S membrane component, respectively. Ozon and Belle have mentioned (10) a failure to detect specific binding to a cytosol fraction using techniques such as equilibrium dialysis, Sephadex chromatography, sucrose gradient or charcoal adsorption.

Interpretation of "progesterone binding" in amphibian oocytes is complicated by the finding that progesterone is rapidly metabolized by ovarian cytosol at 0-4° C (11) and by the fact that previous studies used preparations containing theca and/or follicle cells in addition to oocytes. By using cytosol prepared from denuded Rana pipiens oocytes and R5020, a non-metabolizable highly potent progestin (12), we report the presence of a high affinity, saturable, progestin binding macromolecule. This macromolecular complex has been characterized in some detail using gel filtration, DEAE chromatography, and sucrose gradient centrifugation.

MATERIALS AND METHODS

Preparation of Oocytes

Sexually mature Rana pipiens females were obtained from the Lake Champlain region of the United States and denuded prophase arrested oocytes isolated as described elsewhere (13). [^{3}H]R5020 (Dimethyl-19-norpregna-4,9-Diene-3,20-Dione, 17, 21-[17-Methyl-^{3}H]; specific act. 86 Ci/mmole) was obtained from New England Nuclear (Boston, Mass.) and purity checked on Silica gel G using benzene:ethyl acetate (60:40, v/v) as the mobile phase.

Preparation of Cytosol and Steroid Binding Assays

100 denuded oocytes were homogenized in 5.0 ml medium containing 0.25 M sucrose, 0.05 M Tris-HCl, 1.5 mM EDTA, 10 mM monothioglycerol, pH 7.4 at 0-4° C and centrifuged at 240,000 x g for 30 min. After discarding the upper fatty layer, the supernatant, or cytosol, was carefully removed and adjusted to about 5 mg protein/ml. Cytosol was incubated with varying concentrations of [^{3}H]R5020 at 0-4° C and specific binding determined using both the Charcoal-dextran (14) and the DEAE cellulose filter

assays (15) in the presence or absence of 1000-fold excess of non-labeled progesterone. Maximum binding occurred after about 4 h. Equilibrium dissociation constants were obtained from Scatchard analyses (16) of binding data.

DEAE Cellulose Chromatography

Cytosol was incubated with 50 nM [^{3}H]R5020 at 0-4° C for 4 h and was applied to a 1 x 20 cm microgranular DEAE-cellulose (Whatman DE-52) column equilibrated with a buffer containing 10 mM thioglycerol, 1.5 mM EDTA, and 50 mM Tris-HCl, pH 7.5. Elution was carried out with 100 ml of a 0-0.5 M KCl gradient in the same buffer and 2.0 ml fractions collected and analyzed for radioactivity, conductivity, and absorbance at 280 nM.

Agarose gel filtration

Cytosol was incubated with 50 nM [^{3}H]R5020 at 0-4° C for 4 h and was applied to a 1 x 20 cm Agarose bead column (Bio-gel A-0.5m, Bio-Rad, Rockville Center, N.Y.) equilibrated with a buffer containing 10 mM thioglycerol, 1.5 mM EDTA, 50 mM Tris-HCl, pH 7.5 (low salt) or the same buffer containing 0.3 M KCl (high salt). The column was eluted with either low or high salt buffer (flow rate 8-10 ml/h) and 2.0 ml aliquots collected and analyzed for radioactivity.

RESULTS

R5020 Binding to Oocyte Cytosol

The specific binding of [^{3}H]R5020 to the oocyte cytosol was measured using both the charcoal-dextran and DE-52 filter paper assays. A typical Scatchard plot of [^{3}H]R5020 binding to oocyte cytosol is shown in Figure 1. By both techniques the binding was specific and saturable. The mean K_D value at 0-4° C obtained from determinations on cytosol preparations from three frogs was $2.8 \pm 0.4 \times 10^{-8}$. Extraction of the cytosol radioactivity after incubation periods of up to 12 h at 0-4° C, followed by chromatography on silica gel G indicated no detectable (< 1%) conversion or metabolism of [^{3}H]R5020.

When oocyte cytosol was incubated with [^{3}H]R5020 for 4 h at 0-4° C and applied to an Agarose gel column, the radioactivity peak was detected in the exclusion volume of the gel (Figure 2). The apparent molecular weight was greater than 5×10^5. No other peak was detected in either low salt or 0.3 M KCl elution buffer. Essentially identical results were obtained with cytosol preparations from three frogs.

Figure 3 shows that when an oocyte cytosol-[^{3}H]R5020 preparation was applied to a DEAE-cellulose column and subsequently eluted with a KCl gradient, two peaks of radioactivity were resolved eluting at 0.08 M and

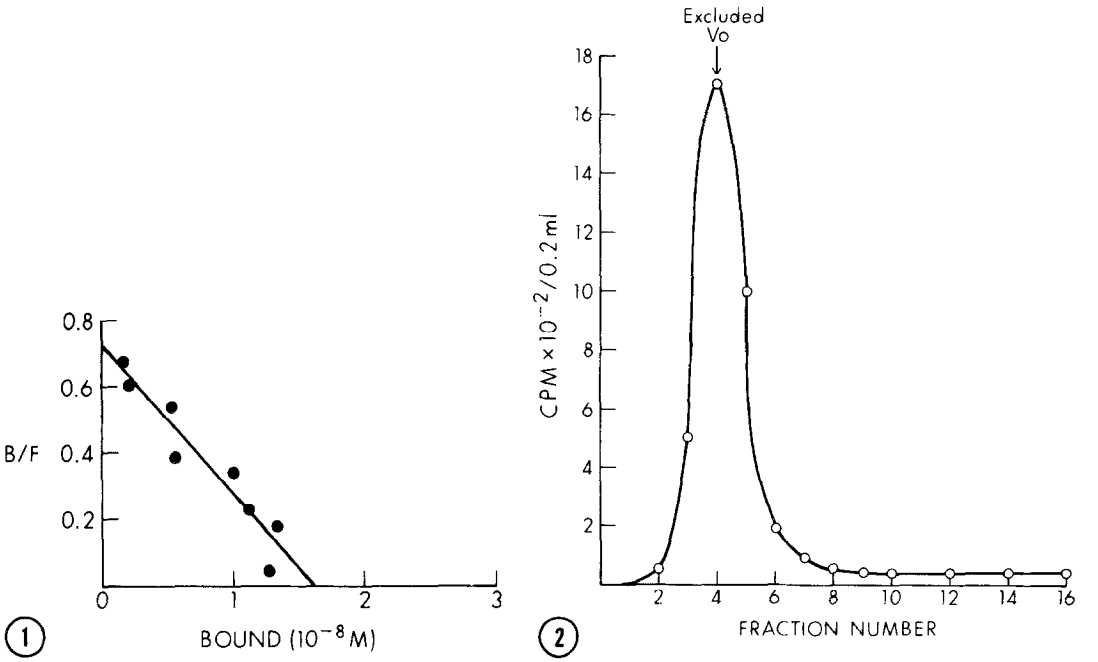

Figure 1. Scatchard plot of [^{3}H]R5020 binding to Rana pipiens oocyte cytosol. 0.2 ml of cytosol was incubated at 0-4° for 4 h in the presence of varying amounts of [^{3}H]R5020 ± 1000 fold excess unlabeled progesterone.

Figure 2. Gel filtration on Agarose A-0.5 m of 1.0 ml [^{3}H]R5020-oocyte cytosol incubation mixture.

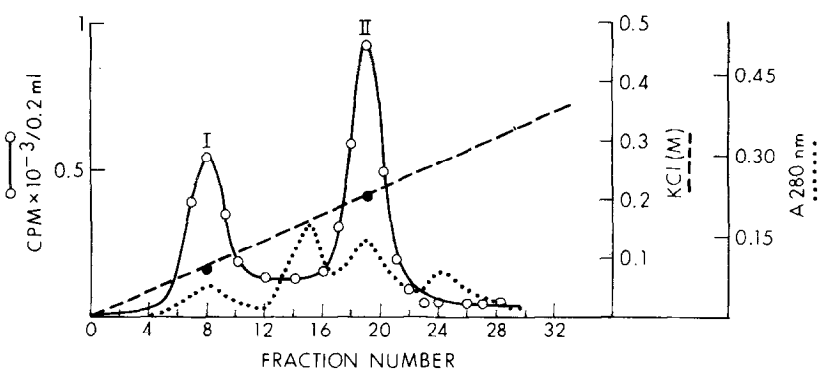

Figure 3. Exchange chromatography on DEAE-cellulose of [^{3}H]R5020-cytosol incubation mixture (1.0 ml).

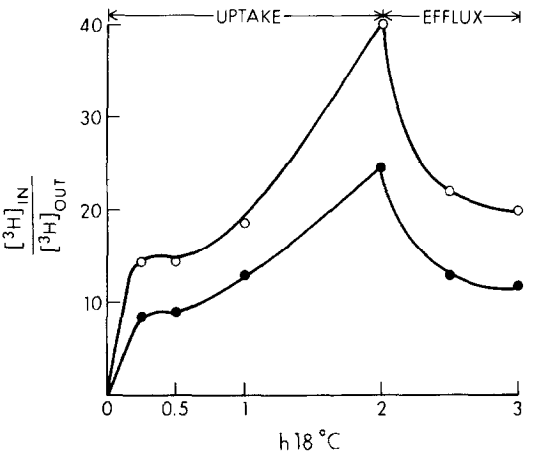

Figure 4. Uptake of [^{3}H]R5020 (0.15 μM) by denuded R. pipiens oocytes in progesterone-free Ringer's solution (●-●) and after 1 h pretreatment in Ringer's solution containing 15 μM progesterone (● - ●). After a 2 h uptake, oocytes were quickly rinsed and transferred to R5020 and progesterone-free Ringer's solution and efflux measured. Ten oocytes were taken at each point indicated, rinsed 3X, and counted. Uptake and efflux were at 18° C.

0.2 M KCl, respectively. About 35% of the total counts were recovered in the peak eluting at 0.08 M KCl and 55-60% eluted with 0.2 M KCl.

Analysis by sucrose density centrifugation of the oocyte cytosol-[^{3}H] R5020 preparation in low and high (0.3 M KCl) salt gradients failed to reveal any detectable peak of radioactivity (data not shown). The [^{3}H]R5020-macro-molecular complex was found to be sedimented to the bottom of the gradient indicating that the binding macromolecule tends to aggregate during centrifugation. As with Agarose gel chromatography (Figure 2), both DEAE cellulose chromatography (Figure 3) and sucrose density centrifugation gave very similar patterns for cytosol preparations from oocytes of three frogs.

Uptake of R5020 by Denuded Oocytes

Figure 4 compares the uptake of [^{3}H]R5020 at 18° C by denuded oocytes without prior exposure to exogenous progesterone and by oocytes exposed to 15 μM progesterone for 1 h prior to R5020. As can be seen, untreated oocytes concentrated [^{3}H]R5020 40-fold from the medium by the end of 2 h.

Pretreatment with progesterone reduced R5020 uptake by about 40%. The results also suggest 2-step kinetics, a rapid uptake approaching saturation within 30 min, and a second non-saturable uptake that continues for at least 2 h. As is also shown in Figure 4, transfer of oocytes to R5020-free medium was followed by a rapid efflux of about 50% of the [^{3}H]R5020 over a 0.5 h period. Extraction of [^{3}H]R5020 from the incubation medium and from oocyte homogenates at the end of a 2 h uptake period, followed by chromatography, indicated no significant (< 1%) metabolism. Although not shown, denuded oocytes concentrate [^{3}H]-progesterone 20-30 fold over the same period. Uptake is non-saturable and independent of $[\text{Progesterone}]_o$.

DISCUSSION

The results presented here demonstrate for the first time the isolation and characterization of a progestin [^{3}H]R5020 binding macromolecule in the amphibian oocyte cytosol using binding assays, gel filtration, DEAE-cellulose chromatography and ultracentrifugal techniques. It should be noted that the denuded oocyte is a single cell essentially free of exogenous serum proteins and somatic cells (2). As reported here, macromolecules present in the oocyte cytosol have a high affinity and specificity for the synthetic progestin and the intact oocyte will concentrate both [^{3}H]R5020 and progesterone from the medium. Progesterone effectively competes with [^{3}H]R5020 uptake in the intact oocyte. A detailed analysis of the stereochemical requirements which determine steroid affinity to the cytosol macromolecule(s) will be presented elsewhere.

In low and high salt sucrose density gradients the labeled R5020-complexes tend to aggregate and are primarily recovered from the 105,000 x g pellet. The eluent from Agarose A-0.5 m contained the bound [^{3}H]R5020 in the exclusion volume and on DEAE-cellulose columns the [^{3}H]R5020-macromolecular complex resolved into two distinct peaks at 0.08 M and 0.2 M KCl. Schrader and O'Malley (17) using DEAE-cellulose columns with sequential elution by KCl, have resolved chick oviduct progesterone-receptor 6 S dimer complex

into components "A" and "B". Like progesterone receptor complex of chick oviduct (18), the [^{3}H]R5020-macromolecular complex from amphibian oocyte cytosol exhibits various states of aggregation or asymmetry depending on the technique of isolation and detection.

In vitro, progesterone is known to induce normal resumption of meiotic maturation in amphibians (4). The use of RNA and protein synthesis inhibitors (1,3,5-8) has revealed that the physiological response to progesterone depends upon protein synthesis but not RNA synthesis. This has suggested that the synthesis of specific proteins necessary for the initiation of the meiotic divisions might be induced by progesterone without intervening RNA synthesis. In this regard, it is noted that preliminary experiments in this laboratory indicate no significant binding of [^{3}H]R5020 macromolecular complex to isolated nuclei or to calf-thymus DNA-cellulose. At present, we have no biochemical data upon which to reconstruct the molecular events by which progesterone increases protein synthesis at the translational level. However, Molinari et al. (19) have recently suggested an interesting analogy between the binding of heparin to "native" estradiol receptor complex and the initiation factors of protein synthesis. The finding that both estradiol receptors and initiation factors interact with ribonucleoprotein particles, and that both "native" receptors and initiation factors have a tendency to behave as a multiprotein aggregate complex, suggest a common physiological function. Thus, the "native" cytosol receptor may exert a regulatory function in the initiation of protein synthesis in the extranuclear compartment. The possibility that the [^{3}H]R5020 binding macromolecule of the oocyte cytosol exerts an influence on protein synthesis at the translational level is currently being investigated.

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