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Progesterone Binding to Plasma Membrane and Cytosol Receptors in the Amphibian Oocyte

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We have found a single class of progesterone binding sites at the amphibian oocyte plasma membrane, whereas two progesterone receptor forms, similar to those in chick and human, are present in the cytosol. In this study both plasma membranes and 105,000 × g cytosol from *Rana pipiens* oocytes were photoaffinity labeled with the synthetic progestin [^{3}H]R5020. SDS-polyacrylamide gel electrophoresis of the photolabeled proteins in the oocyte cytosol indicate that the two forms have molecular weights essentially identical to that found for human breast tissue and chick oviduct, i.e., 80 and 110 kDa, and that the forms were present in approximately equimolar ratios. In contrast, the plasma membrane form is present as a single 110 kDa species and accounts for at least 50% of the total 110 kDa species. The presence of large amounts of the 110 kDa protein in both membrane and cytosol suggests that the plasma membrane receptor may not be unique, and that the 110 kDa form may function both in membrane and cytosol and/or that part of the cytosolic 110 kDa form represents progesterone receptor in the process of being transported to or from the plasma membrane.

There is growing recognition of the non-genomic effects of steroid hormones at the plasma membrane, in contrast to their intracellular role as regulators of transcription (reviewed in 1, 2). Progesterone initiation of meiosis in prophase-arrested amphibian oocytes was one of the first well-defined examples of a steroid effect on the plasma membrane, since it could be shown that exogenous, but not injected, progesterone induced meiosis and that many of the progesterone-induced changes associated with meiosis occurred in enucleated oocytes (reviewed in 3).

Studies in this laboratory have demonstrated that binding of [^{3}H]progesterone to the isolated *Rana* oocyte plasma-vitelline membrane is specific, temperature-dependent and has a slow off-rate (4). Structure-function analysis indicates that the most active physiological inducers of meiotic maturation are the Δ^4-3,20-diones (e.g. progesterone) (5). The estimated number of plasma membrane receptors is consistent with the quantity of progesterone binding necessary for induction of meiosis (4).

Rana oocytes have, in addition to progestin-binding receptors on their surface, two progestin (R5020) binding components in the cytosol (6). These cytosol components have binding coefficients and sephedex gel column elution characteristics similar to the A and B forms reported for chick oviduct (7). A report (8) that R5020 binding component in *Xenopus* plasma membranes has a molecular weight similar to the B component isolated from chick oviduct (110 kDa), led us to re-examined photoaffinity labeling of both the membrane and cytosolic forms using prophase-arrested *Rana pipiens* oocytes. R5020 binding to cytosol prepared from intact and enucleated oocytes, as well as to isolated plasma membranes, permits comparison of receptor forms in three compartments: plasma membrane, cytosol and nucleus. The present study indicates that the *Rana* oocyte cytosol and nucleus contain two forms with molecular weights similar to that in chick and human tissue (110 and 80 kDa) and that the oocyte plasma membrane contains a single progestin-binding form that co-migrates with the 110 kDa cytosolic form.

MATERIALS AND METHODS

Materials. Fully grown *Rana pipiens* oocytes, arrested in first meiotic prophase, were stripped of their follicular envelopes and freed from thecal cells by a modification (9) of Masui's method (10). These oocytes are herein referred to as "denuded". Oocytes were maintained in modified Ringer's solution (9) at room temperature and used within 1-2 h after isolation. Denuded oocytes were manually enucleated with fine-tipped jeweler's forceps as described previously (9) and allowed to reseal in Ringer's solution for 1 h. During enucleation the nuclei remain intact and can be removed from the medium. [^{3}H]R5020 (dimethyl-19-norpregna-4,9-diene-3,20-dione, 17, 21-[17-methyl-^{3}H]; sp. act. 85 Ci/mmol) and unlabeled R5020 were obtained from New England Nuclear, Boston, MA. Progesterone, 17β-estradiol and trypsin were obtained from Sigma Chemical Co., St. Louis, MO. Precast 8 and 10% Tris/Glycine

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slab gels and molecular weight markers were obtained from NOVEX, San Diego CA.

Preparation of plasma-vitelline membranes and [^{3}H]R5020 binding. Plasma-vitelline membranes were isolated in 0.24 M sucrose plus Ca^{2+} as previously described (4). Membranes from 10 oocytes were pooled and transferred with 20 μl of 0.24 M sucrose to a 1.5 ml Eppendorf microfuge tube containing 80 μl of medium with a final concentration of 0.24 M sucrose, 20 mM Tris-HCl (pH 7.5), 0.75 mM $CaCl_2$, 10 mM Mg^{2+} acetate and 2 nM [^{3}H]R5020 at 0-4°C. Membranes were incubated in the dark for 4 h at 0-4°C, collected by gentle centrifugation, rinsed 3× with ice-cold unlabeled sucrose-tris medium and photolysed for the times indicated. Photolysed membranes were then analyzed by SDS-polyacryalamide gel electrophoresis (SDS-PAGE, see below). Intact denuded oocytes were incubated in ice-cold Ringer's solution containing 2 nM [^{3}H]R5020 for 2 h in the dark, rinsed 3 times with ice-cold unlabeled Ringers solution, and photolysed from above in 35 mm diameter plastic Petri dishes with the pigmented hemispheres of the oocytes facing up. The plasma-vitelline membranes from photolysed oocytes were isolated as described above and analyzed by SDS-PAGE.

Preparation of cytosol and [^{3}H]R5020 labeling. 50 denuded nucleated or enucleated oocytes were homogenized in 2.5 ml sucrose-TEG buffer 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 105,000 × 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 in the dark with varying concentrations of [^{3}H]R5020 at 0-4°C in the presence or absence of 100-fold excess of R5020, progesterone or estradiol. Bound and free [^{3}H]R5020 were separated by LH-20 gel filtration at 0-4°C. Sephadex LH-20 (Pharmacia Fine Chemicals, Piscataway, NJ) was preswollen overnight in sucrose-TEG buffer at 0-4°C and 6 × 0.5 cm minicolumns prepared. Aliquots (150 μl) of cytosol from nucleated or enucleated oocytes were washed into LH-20 columns with 50 μl sucrose-TEG buffer. After 30 min samples were eluted with buffer and 12 fractions (250 μl) collected and counted using a Beckman LS 7500 Liquid Scintillation System. Counts were corrected for quenching using an external standard.

Photoaffinity labeling. Plasma membrane preparations labeled with [^{3}H]R5020 were resuspended in 100 μl sucrose-TEG buffer in 1.5 ml microcentrifuge tubes and irradiated from above at 254 nm in a 4-bulb Rayonet photoreactor (Model RMR-600) for 2-10 min at 4°C. A filter prevented the transmission of light of $\lambda < 300$ nm. Variations from this procedure are noted in the figure legends. The first 6.0 ml eluted from LH-20 columns were pooled and photolysed as described for plasma membrane preparations. The pooled material contained the bulk of the [^{3}H]R5020-cytosol binding proteins.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. [^{3}H]-R5020 labeled and photolysed plasma-vitelline membrane and cytosol samples were treated with an equal volume of 10% ice-cold trichloroacetic acid, placed in an ice bath for 15 min, and centrifuged. The precipitate was washed 3× with fresh 5% TCA, 5× with diethyl ether and resuspended in sample buffer (10% glycerol, 5% β-mercaptoethanol, 3% SDS, 70 mM Tris, pH 6.8), boiled for 2 min and electrophoresed by the method of Laemmli (11) on 8% Tris/Glycine slab gels. Gels were sliced into 2 mm segments, and the protein eluted overnight by incubation in NCS tissue solubilizer (Amersham Corp., Boston, MA). Radioactivity in each slice was measured as described above and corrected for quenching using an external standard.

RESULTS

Comparison of [^{3}H]R5020 Binding to Cytosol Prepared from Enucleated and Nucleated Oocytes

105,000g cytosol was prepared from both intact and manually enucleated prophase *Rana* oocytes. Comparison of progestin-binding with and without nuclei indicates the distribution of receptor between the two compartments. Receptor associated with the plasma-vitelline membrane comes down with the particulate fraction with minimal contamination of the cytosol. The 105,000g cytosol was incubated with the non-metabolizable progestin [^{3}H]R5020 at 0-4°C. Bound and free R5020 were separated by chromatography on LH-20 columns. The flow-through contained protein-bound R5020 while free R5020 remained on the column. Cytosol prepared from nucleated and enucleated oocytes did not differ significantly in protein content as measured by the Lowry method. LH-20 analysis of the medium in which oocytes were enucleated did not indicate measurable [^{3}H]R5020 binding. As shown in Fig. 1, a comparison of [^{3}H]R5020 elution in blank and cytosol-containing samples indicates that the bulk of the bound [^{3}H]R5020 eluted in the first 6.0 ml and that unbound [^{3}H]R5020 accounted for less than 5% of the total [^{3}H]-R5020 eluted from the LH-20 columns in the void volume. Elution of bound [^{3}H]R5020 as a function of incubation time indicated that maximal [^{3}H]R5020 binding to cytosol components occurred by 4 h at 0-4°C. Therefore, a 4 h incubation in the dark at ice-bath temperatures was used in the cytosol [^{3}H]R5020-binding experiments outlined in these studies.

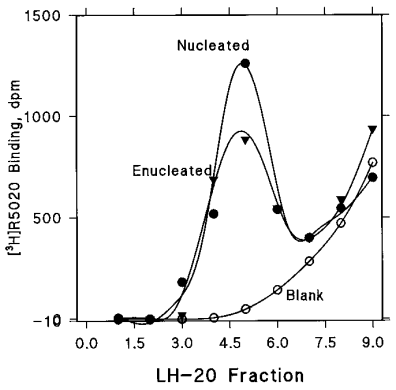

FIG. 1. Elution of [^{3}H]R5020-cytosol complex from nucleated or enucleated *Rana pipiens* oocytes on LH-20 columns. 105,000 × g cytosol was prepared from prophase *Rana* oocytes with intact nuclei (●—●) or from manually enucleated oocytes (▲—▲). Cytosol preparations were incubated with [^{3}H]R5020 for 2 h at 0–4°C prior to LH-20 elution. Blank (○—○) indicates [^{3}H]R5020 elution without added cytosol.

Comparison of [^{3}H]R5020 binding to cytosol from enucleated and intact oocytes found that enucleated oocytes contained 66±3% of the total [^{3}H]R5020 binding capacity (Figure 1). Proton relaxation times of oocyte water indicate that the oocyte nucleus contains about 25% of the total oocyte water (12). Comparison of cytosol receptors in the intact oocyte (Table 1) with those of enucleated oocytes (Fig. 1) indicates that the soluble R5020 receptors are about 60% more concentrated in the nucleoplasm than in the cytoplasm. [^{3}H]-

TABLE 1

Recovery of Photolabeled [^{3}H]R5020 Peaks from Plasma Membrane and Cytosol Preparations Eluted from Sodium Dodecyl Sulfate Polyacrylamide Gels

Preparation	Peak A (dpm/oocyte)[a]	Peak B (dpm/oocyte)[a]
Plasma membranes	—	842 ± 31
105,000 × g Cytosol	698 ± 25	465 ± 33

[a] Mean ± SD (N = 3).

R5020 binding to either nucleated and enucleated cytosol was largely abolished by 100-fold excess of unlabeled R5020 or progesterone, but not by 17β-estradiol.

A Comparison of [^{3}H]R5020-Crosslinked Receptors in the Oocyte Plasma Membrane and Cytosol

Photoaffinity labeling with [^{3}H]R5020 was used to compare the A and B forms of the oocyte cytosol receptor with the species recovered in the plasma membrane. The progesterone or R5020 binding moiety in the *Rana* oocyte plasma membrane is not readily solubilized by detergents but can be released by SDS treatment. The [^{3}H]R5020-labeled plasma membranes and the pooled eluent from the first 6 ml of [^{3}H]R5020-cytosol flow-through from the LH-20 columns (Figure 1) were photolysed as described in methods, and then fractionated on 8% SDS-polyacrylamide gels. As shown in Figure 2, 1-D gel electrophoresis of the photolabeled cytosol proteins indicates that the two major cytosolic forms have molecular weights similar to that found for human breast tissue (13)and chick oviduct (7), i.e. about 80 and 110 kDa, and that the forms were present in approximately equimolar ratios. In contrast, the plasma membrane form exists as a single ~110 kDa form. [^{3}H]R5020 binding in the membrane is also abolished by pretreatment with 100-fold excess progesterone or unlabeled R5020, but not by estradiol. Incubation of isolated plasma membranes with 10 μunits/ml of trypsin (pH 7.8) for 10 min at 20°C prior to the photoaffinity labeling step eliminated the ~110 kDa [^{3}H]R5020 binding component (data not shown).

Alternatively, intact oocytes were incubated in Ringers solution containing [^{3}H]R5020 for 1 h at ice-bath temperatures, rinsed with cold Ringers solution, photolysed for 1 min and the membranes isolated and subjected to SDS-PAGE. A similar single peak migrating in the 110 kDa region was seen, indicating that it is possible to photolabel the progestin receptor on the surface of the intact oocyte (data not shown). Cytosol prepared from the same oocytes demonstrated no significant [^{3}H]R5020 labeling, indicating that the uv radiation did not penetrate the melanin-pigmented cortex. Exposure to uv radiation was minimized to prevent cell damage; oocytes so exposed were capable of responding to progesterone and began meiosis as indicated by completion of nuclear breakdown. The cortical granules containing melanin pigment may protect oocytes both from uv damage as well as prevent significant photolysis of [^{3}H]R5020 binding components in the cytosol.

The values shown in the electrophoretic gel patterns in Figure 2 represent the actual dpm in the gel slices after correction for quenching and background. Table 1 compares the relative contribution of peaks A and B in membrane and cytosol calculated as dpm [^{3}H]R5020 bound per oocyte. Since the R5020 binding protein in the oocyte plasma membrane is not solubilized under the conditions used to prepare cytosol, minimal membrane binding protein is carried over into the cytosol fraction. As shown in Table 1, if it is assumed that photolabeling efficiency is about the same in cytosol and plasma membrane preparations, about 65% of the total R5020 binding component corresponding to the 110 kDa component is present in the plasma-vitelline membrane of the prophase-arrested oocyte. For comparison, the plasma membranes contain about 6 μg protein per oocyte, whereas the cytosol contains about 200 μg protein/oocyte, indicating that the ~110 kDa component is concentrated in the plasma membrane.

DISCUSSION

The progesterone cytosol receptor is unusual in that it consists of A and B forms in chick (7), frog (6) and human (13), although the rabbit may have a single form (14). We have used photoaffinity labeling with the non-metabolizable progesterone [^{3}H]R5020 to compare the A and B forms of the oocyte cytosol progesterone

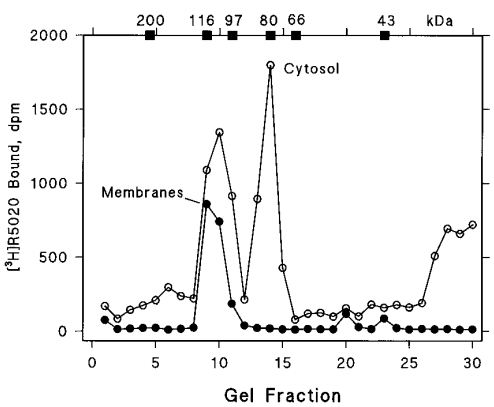

FIG. 2. SDS-Polyacrylamide gel electrophoresis (PAGE) of isolated plasma-vitelline membranes and 105,000 × g cytosol preparations *from Rana pipiens* oocytes following photoaffinity labeling with [^{3}H]R5020. Plasma-vitelline membranes and cytosol preparations were incubated with [^{3}H]R5020 for 2 h at 0–4°C prior to photolysis for 2 min at ice-bath temperatures. PAGE was carried out on 10% SDS Tris glycine gels (NOVEX). The upper abcissa indicates the position of marker proteins.

receptor with the progesterone-binding receptor recovered in the plasma membrane. 1-D gel electrophoresis of the photolabeled proteins indicates that the A and B forms have molecular weights very similar to that found for human breast tissue and chick oviduct, i.e. ~80 and ~110 kDa. In contrast, the plasma membrane form exists as a single ~110 kDa form and accounts for 60-70% of the total ~110 kDa component in the oocyte.

The presence of a ~110 kDa R5020-binding component in both the plasma membrane and cytosol suggests that those receptors may be similar or identical. Alternatively, the ~110 kDa form found in the oocyte cytosol may not be a cytosol/nuclear receptor, but may represent the plasma membrane receptor associated with the endosomal traffic between site of synthesis and/or degradation and the oocyte surface. Progesterone binding may initiate the translocation of regions of the plasma membrane to intracellular sites.

The region which differentiates the A form of the progesterone receptor from the B form in the chick cytosol receptor is a 128-amino acid sequence located at the N terminus of the B protein and is acidic in amino acid composition by virtue of a 25-residue glutamic acid track (15). The N terminus extension of the B form of the progesterone receptor could serve as a plasma membrane anchor. Since we can photoaffinity label the plasma membrane receptor in intact oocytes (above), and exogenous but not injected progesterone will initiate meiosis (reviewed in 3), the hydrophobic region within the steroid binding domain must be accessible to progesterone at the oocyte surface. Our structure-function studies have found that the spatial arrangement of substituents on the upper β surface of the steroid molecule is of critical importance in the induction of meiosis in *Rana pipiens* oocytes; the 3, 20-dione, 21-ol configuration being the most active (5). This must include an angular methyl group at carbon 19. The most potent inducers have an unsubstituted α surface, and any introduction of a polar group diminishes or abolishes activity. We have previously shown that progesterone binding to the plasma membrane receptor acts via G protein(s) to sequentially activate, within seconds, at least three lipid messenger systems: phospholipid N-methyltransferase (16,17), sphingomyelin synthase (17) and phosphatidylcholine-specific phospholipase D (18). This results in the sequential release of at least three different molecular species of 1,2-diacylglycerol that act as progesterone second messengers to trigger meiosis. Thus, if the A and B forms are structurally homologous except for the extension at the N terminus, these receptor forms are involved in markedly different signal processing in the cell.

Progesterone and/or R5020 binding to *Rana pipiens* and/or *Xenopus* oocyte plasma membrane preparations have been studied in several laboratories (4,8,9,19). The binding constants (K_d), binding capacities and estimated molecular weights of progesterone or R5020 binding components are compared in Table 2. The number of binding sites per μm^2 membrane surface area has been corrected for the membrane capacitance of the prophase-arrested *Rana pipiens* oocyte (20). Capacitance measurements indicate that the surface area is about 16 times that estimated for the oocyte as a sphere, and is in good agreement with a factor of 10-12 times determined from electron micrographs (4). The number of progesterone binding sites indicated for *Xenopus* oocytes have been based on the surface area of a sphere and should also be divided by 10-16. This would make the sites/μm^2 reported by Sadler and Maller (8) comparable to our value for *Rana* oocytes. Sadler and Maller (8) and Blondeau and Baulieu (20) have found photoaffinity labeling of a 110 and 15-30 kDa peptides, respectively, from *Xenopus* plasma membrane preparations. We describe a single ~110 kDa binding protein in *Rana* plasma membranes, similar to that found by Sadler and Maller in *Xenopus*. The report of 30 and 15 kDa R5020 binding components in *Xenopus* plasma membrane preparations by Blondeau and Baulieu (20) might be due to proteolysis in their preparation. Blondeau and Baulieu used differential centrifugation to prepare a cell fraction enriched with plasma membranes but do not mention the addition of protease inhibitors.

Progestin binding studies with both *Rana* and *Xenopus* oocyte plasma membranes demonstrate affinity

TABLE 2

Comparison of [^{3}H]Progesterone and/or [^{3}H]R5020 Binding to Amphibian Oocyte Plasma Membrane Receptors as Reported by Various Laboratories

Species	Progestin	K_d	Sites/μm^2	M.W. kDa	Oocyte fraction used
Rana pipiens	Progesterone	5 × 10-7	4000[a]	~110	Plasma-vitelline membrane
Xenopus	R5020	1 × 10-6	65000	~110	Plasma-vitelline membrane with follicle cells
Xenopus	R5020	1 × 10-6	n.d.	~30	10000 × g fraction
		1 × 10-5		~15	
Xenopus	Progesterone	10-9	181	n.d.	10000 × g fraction

[a] Corrected for membrane capacitance (see ref. 21).

constants in the μM range. The difference between the K_d for progesterone (10^{-7} M) binding to *Rana* membranes and R5020 (10^{-6} M)binding to *Xenopus* membranes is consistent with the affinity differences between the natural and synthetic progestins. More recently, Liu and Patino have reported the K_d for progesterone binding to a fraction containing plasma membrane fragments prepared from whole *Xenopus* ovaries to be about 10^{-9} M (19). However, the estimated number of progesterone binding sites per μm^2 of *Xenopus* plasma membrane surface is at least an order of magnitude less than that necessary for induction of meiosis. Since progesterone is readily metabolized by reductases in oocyte preparations, even at ice-bath temperatures (22), the apparent low progesterone binding values in *Xenopus* oocyte membrane preparations may be due to endogenous enzymatic activity.

Progesterone binding sites have recently been identified on the surface of human sperm and appear to potentiate Ca^{2+} influx, which is necessary for the acrosome reaction (e.g. 23, 24). Progesterone receptor(s) have also been reported in synaptic plasma membranes from brain (25,26), in membranes from mouse cerebellum (28) and have been photoaffinity labeled in rat liver plasma membranes (27). Steroid effects at the neuronal cell surface include the modulation of GABA-induced Cl^- flux by the natural metabolites of progesterone and deoxycorticosterone and the rapid release of dopamine and acetylcholine by progesterone (1). The present work indicates a possible relationship between steroid receptors for both genomic and non-genomic effects. Studies are now in progress to isolate and compare the 110 kDa forms in the oocyte plasma membrane and in the cytosol and to describe the attachment of the B form to the plasma membrane.

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