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# Solubilization and Pharmacological Characterization of a Glucocorticoid Membrane Receptor from an Amphibian Brain

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**Physiological functions of steroid hormones involve activation of intracellular receptors as well as poorly understood membrane receptors. We report the pharmacological characterization of a solubilized corticosterone receptor from neuronal membranes. This receptor previously was shown to localize with plasma membrane subcellular fractions and to be involved in the modulation of courtship behaviors in the roughskin newt (*Taricha granulosa*). We describe procedures with non-ionic detergents that solubilize the receptor and maintain high affinity [$^3$H]corticosterone binding. The pharmacology of the solubilized corticosterone receptor resembles that of the membrane receptor with high affinity for [$^3$H]corticosterone and an identical rank-order potency for other steroid ligands (corticosterone>cortisol>aldosterone>dexamethasone). Unlike binding in membrane preparations, [$^3$H]corticosterone binding to the solubilized receptor is insensitive to negative modulation by guanyl nucleotides and only modestly sensitive to the presence of $Mg^{2+}$. We also identified two ligands that exhibit high affinity binding to the solubilized receptor and have the potential to be used in an affinity purification scheme. They are corticosterone-3-carboxymethyloxime (CORT-3-CMO), which may be covalently attached to a Sepharose resin, and a derivitized azide form of CORT-3-CMO which can be covalently coupled to the solubilized receptor itself. The stability of the solubilized [$^3$H]corticosterone receptor in the detergent system will facilitate further purification and molecular characterization.** © **1998 Elsevier Science Ltd. All rights reserved.**

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## INTRODUCTION

Steroid hormones use intracellular receptors that bind DNA in a sequence specific manner to modulate transcriptional activity of target genes. Evidence for rapid steroid actions through plasma membrane receptors has accumulated in recent years (review by Wehling [1]) and comes from pharmacological, electrophysiological and behavioral experiments.

Progestins [2, 3], glucocorticoids [4–6], estrogens [7], mineralocorticoids [8, 9] and $1,25(OH)_2D_3$ [10] have all been shown to bind specifically to cellular membrane sites. Rapid influences on electrolyte movement across cellular membranes are also induced by progesterone [11], glucocorticoids [12, 13], estradiol [14, 15], aldosterone [8], $1,25(OH)_2D_3$ [16] and testosterone [17]. Steroid hormones can also effect rapid behavioral changes, such as the rapid inhibition of mating behavior in male newts [4, 18].

Some steroid membrane receptors may use G-proteins in the signal transduction machinery. Cortisol-induced inhibition of $Ca^{2+}$ currents in guinea pig is diminished by pertussis toxin treatment or the presence of GDP$\beta$S [12]. Corticosterone binding in newt brains is inhibited by guanyl nucleotide analogs in a concentration dependent manner [19]. Aldosterone effects on HML cells involve 1,4,5-inositol trisphosphate and are sensitive to pertussis toxin, but not cholera toxin [8]. Estradiol inhibition of $Ca^{2+}$ currents

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in rat neostriatal neurons is reversed by GTPγS [15]. Testosterone stimulation of $Ca^{2+}$ currents and induction of $IP_3$ and DAG in rat osteoblasts is blocked by pertussis toxin [17]. All of these observations are both consistent with and characteristic of G-protein coupled signal transduction mechanisms.

A receptor for corticosterone found in newt (*Taricha granulosa*) neuronal membranes has been identified and characterized [4]. This protein also possesses characteristics of a G-protein coupled receptor [19]. A single intraperitoneal injection of corticosterone inhibits courtship behavior with a latency of less than 8 min [4] and inhibits stress-induced locomotion in a similar time frame [20]. The affinities of an array of steroids for this [$^3$H]corticosterone binding site are correlated with their respective potencies in newt behavioral assays [4]. The receptor is enriched in plasma membrane fractions prepared by sucrose density centrifugation and does not bind dexamethasone and other ligands which have high affinity for intracellular glucocorticoid receptors [4]. These data suggest that there is a membrane corticosterone receptor which is distinct from the intracellular corticosterone receptor.

Detergent solubilization followed by affinity purification of membrane receptors has proven useful in advancing molecular and biochemical understanding of various receptor systems. We have extended the work with the newt corticosterone membrane receptor by solubilizing the receptor in neuronal membranes using non-ionic detergents. We have characterized the solubilized receptor and identified ligands that can be used during affinity purification.

## MATERIALS

Male *T. granulosa* were collected locally and used immediately or stored in tanks containing de-chlorinated water for up to three weeks. Stored animals were feed *tubifix* worms on a three day cycle and were handled in compliance with accepted animal use guidelines.

[$^3$H]corticosterone (71.07 Ci/mmol) was purchased from NEN/Dupont (Boston, MA). Corticosterone, aldosterone, dexamethasone, digitonin, sodium cholate, bacitracin, trypsin inhibitor, dioxane and Sephadex were from Sigma (St. Louis, MO). Corticosterone-3-carboxymethyloxime (CORT-3-CMO) was from Steraloids (Wilton, NH). PMSF and leupeptin were from Boehringer Manheim (Indianapolis, IN). Sucrose was from Mallinkrodt (Paris, KY) HEPES from Research Organics (Cleveland, OH) and EDTA from JT Baker (Phillipsburg, NJ). *N*-(2-aminoethyl)-4-azido-2-nitroaniline was purchased from Molecular Probes (Eugene, OR).

## METHODS

### *Preparation of neuronal membranes*

Synaptosomes were prepared from newt brains by the method of Whittaker [21] with modifications by Orchinik [22]. Briefly, newt brains were homogenized in 40 vol. (per original weight) 0.32 M sucrose, 5 mM HEPES, pH 7.45 using a Teflon on glass tissue homogenizer. Homogenate was centrifuged at $1{,}000 \times g$ for 10 min at 4°C using a Beckman J2-21 centrifuge. Pellet (P1) was discarded and supernatant centrifuged at $16{,}000 \times g$ for 40 min at 4°C. Pellet (P2) was quick frozen and held at −70°C for at least 30 min then resuspended in 150 vol. (per original brain weight) 25 mM HEPES, 10 mM EDTA, pH 7.45 and held at 4°C for 2–3 h to allow the dissociation of any endogenous ligand. Suspension was centrifuged at $40{,}000 \times g$ for 15 min at 4°C. Pellet was resuspended in 25 mM HEPES, pH 7.45 for final wash and centrifuged again at $40{,}000 \times g$ for 15 min at 4°C. A protein analysis by the method of Lowry *et al.* [23] was usually performed prior to the final spin. Pellets were stored at −70°C for at least 30 min or until use. All above steps included a protease inhibitor cocktail consisting of PMSF (0.1 mM), trypsin inhibitor (100 μg/ml), leupeptin (0.7 μg/ml) and bacitracin (100 μg/ml).

### *Solubilization of membrane receptor*

Receptor was solubilized by the method of Peterson and Schimerlik [24] with some modification. Pellets were resuspended to a protein concentration of 5 mg/ml in 25 mM HEPES, pH 7.45. To this suspension was added 0.25 vol. of 4% digitonin, 0.8% sodium cholate, 25 mM HEPES, pH 7.45. Suspension was held at room temperature for 20 min with occasional gentle mixing. Suspension was ultracentrifuged at $>100{,}000 \times g$ using a Beckman L8-70M ultracentrifuge for 1 h at 2°C. Pellet was discarded and membrane extract held at 0°C until use. All steps included protease inhibitor cocktail as described above.

### *Synthesis of photoaffinity ligand*

Corticosterone-3-carboxymethyloxime was dissolved in 30% dioxane to a concentration of 10 mM. *N*-(2-aminoethyl)-4-azido-2-nitroaniline was dissolved in 30% dioxane to a concentration of 20 mM. The two reagents were mixed together in equal volume and then mixed with 0.1 volumes of 1 M aqueous 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). The reaction was incubated overnight at 4°C. The reaction mixture was extracted 3 times with equal volumes of ethyl acetate, aqueous phases were pooled and concentrated to dryness in a rotary speed-vac. Sample was resuspended in 60% acetonitrile/water and product purified from reactants over a $C_{18}$ column with an isocratic gradient at 60% acetonitrile,

collecting 0.5 ml fractions. Fractions were dried as above and resuspended in 50 $\mu$l 60% acetonitrile for analysis by $C_{18}$ thin layer chromatography. Fractions containing pure product were pooled and identity was verified by mass spectral analysis.

*[$^3$H]corticosterone binding to solubilized receptor*

All binding assays contained 80 $\mu$l membrane extract, 0.1% ethanol (as ligand vehicle), 25 mM HEPES, pH 7.45 in a final volume of 200 $\mu$l. [$^3$H]corticosterone was present at 0.5 nM and $MgCl_2$ at 10 mM unless indicated otherwise. Binding reactions were held for 5–8 h (unless indicated otherwise) at 30°C after which protein bound ligand was separated from free ligand by gravity filtration over G-50 Sephadex at 4°C, using binding assay buffer as the mobile phase. A protease inhibitor cocktail as described above was present throughout the binding assay. All experiments were analyzed using GraphPad Prism software (GraphPad, San Diego, CA).

## RESULTS

*[$^3$H]corticosterone association and dissociation kinetics*

Kinetic experiments were performed to determine that [$^3$H]corticosterone binding was reversible and to establish parameters for subsequent equilibrium experiments (Fig. 1). The kinetic data were indicative of a simple bimolecular reaction with a monophasic association and dissociation. The half-time for equilibrium association at a 0.5 nM radioligand concentration was 45 min with a $k_{obs}$ of $0.015 \pm 0.002$ min$^{-1}$. Dissociation had a half-time of 60 min with a $k_{-1}$ of $0.012 \pm 0.001$ min$^{-1}$.

*Equilibrium saturation analysis*

Saturation experiments were performed for an estimation of the affinity of [$^3$H]corticosterone for the solubilized receptor and to determine the receptor specific activity (Fig. 2). These data showed saturable, high affinity [$^3$H]corticosterone binding to a homogenous population of receptors with a $K_d$ of $0.29 \pm 0.02$ nM; in good agreement with previous reports for the membrane bound form [4]. The $B_{max}$ value in this system was approximately 50 fmol/mg protein; similar to values found with the membrane bound receptor, suggesting no significant enrichment of specific activity. A Scatchard–Rosenthal plot of the data (Fig. 2, inset) was linear and therefore consistent with the labeling of a homogenous population of sites. The signal to noise ratio is unchanged by the solubilization procedure with approximately 80% specific binding at the $K_d$.

*Equilibrium competition analysis*

To further evaluate the pharmacological signature of the solubilized [$^3$H]corticosterone receptor, competition experiments were performed (Fig. 3). The rank-order potency of the tested compounds is identical to previous reports [22] for the membrane receptor with the following relative affinities: corticosterone>cortisol>aldosterone≫dexamethasone. This provides strong evidence that the receptor solubilized in this study is indeed the membrane corticosterone receptor identified previously.

In addition to these steroid ligands, two useful derivatives of corticosterone were evaluated for binding activity at the corticosterone receptor (structures shown in Fig. 3b). The first, corticosterone-3-carbox-

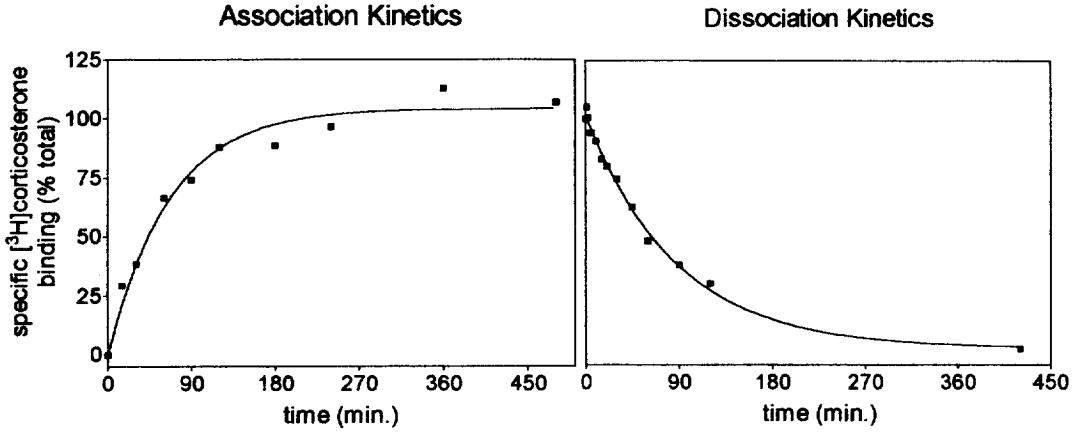


**Fig. 1. Kinetics of [$^3$H]corticosterone association and dissociation. Binding conditions were as described in Section 3 with reactions terminated at times indicated. The fits for association and dissociation were generated using a single phase exponential association and a single phase exponential decay, respectively, with GraphPad Prism software. Half-time of association at a 0.5 nM radioligand concentration was 45 min with a $k_{obs}$ of $0.015 \pm 0.002$ min$^{-1}$. Dissociation half-time was 60 min with a $k_{-1}$ of $0.012 \pm 0.001$ min$^{-1}$. Data is representative of two independent experiments with duplicate time points.**

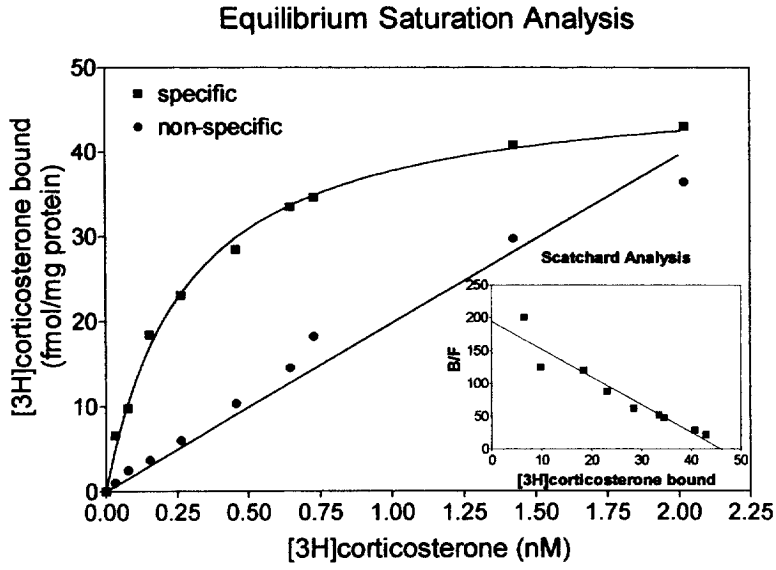


**Fig. 2. Equilibrium saturation analysis. [$^3$H]corticosterone concentration was varied over a 40-fold range as indicated under binding conditions described in Section 3. Specific binding (■) was calculated by subtracting non-specific binding (•), as defined in the presence of 10 $\mu$M corticosterone, from total binding (not shown). The fit was generated assuming single site binding with GraphPad Prism software. A $K_d$ of 0.29 $\pm$ 0.02 nM and a $B_{max}$ of 50 fmol/mg protein were calculated from the data. Inset is a Scatchard–Rosenthal transformation of the saturation binding data. The line is a linear regression with $r^2$=0.96 and gives a $B_{max}$ estimate of 46 fmol/mg protein and an approximate $K_d$ of 0.24 nM. Data is representative of two independent experiments with total binding determined from triplicate points and non-specific binding determined from duplicates in each experiment.**

ymethyloxime (CORT-3-CMO) has a free carboxyl group that can be coupled to the free amine of a solid support for the production of an affinity resin. The second, a photoactive derivative of CORT-3-CMO, CORT-azide, can be used as a photoaffinity label. Binding studies with this azide derivative were performed in the dark to minimize photoactivation during the incubation step. Corticosterone, cortisol, aldosterone, CORT-3-CMO and CORT-azide completely inhibited specific [$^3$H]corticosterone binding with $K_i$ values of 0.24 nM (95% CI, 0.19 to 0.30 nM), 10.5 nM (95% CI, 7.5 to 14.5 nM), 211 nM (95% CI, 175 to 255 nM), 2.6 nM (95% CI, 1.8 to 3.8 nM) and 234 nM (95% CI, 135 to 402 nM) respectively. Dexamethasone only inhibited 15% of specific binding at a concentration of 10 $\mu$M. The low affinity of dexamethasone suggests that the intracellular corticosterone receptor does not contribute to the [$^3$H]corticosterone specific binding in the solubilized preparation.

*Lack of modulation of [$^3$H]corticosterone binding by guanyl nucleotides*

The effects of guanyl nucleotides were examined because previous reports [19] showed significant negative modulation of [$^3$H]corticosterone binding in the presence of these compounds (Fig. 4). We found no negative effect of these nucleotides on [$^3$H]corticosterone binding in the detergent solubilized system. A modest increase in [$^3$H]corticosterone binding was observed in the presence of 100 $\mu$M GDP.

*Modulation of [$^3$H]corticosterone binding by $Mg^{2+}$*

To further assess the solubilized receptor with regards to characteristics typical of G-protein coupled receptors, we evaluated the effect of the divalent cation $Mg^{2+}$ on [$^3$H]corticosterone binding (Fig. 5). $Mg^{2+}$ typically stabilizes the receptor high affinity state of G-protein coupled receptors by promoting G-protein–receptor interaction. The presence of $Mg^{2+}$ in the detergent extract nominally increased binding by approximately 15% at a concentration of 3 mM. This is in contrast to a 50% increase observed with the membrane receptor [19] and is consistent with the loss of guanyl nucleotide sensitivity.

## DISCUSSION

We have described the pharmacological characterization of a corticosterone membrane receptor solubilized from newt neuronal membranes. This is the first example of solubilization of a steroid membrane receptor from neuronal tissue. A cortisol-binding protein has been purified from rat hepatic plasma membranes [25], a progesterone-binding site has been purified from porcine liver membranes [26] and cloned from porcine vascular smooth muscle cells [27], and a 1,25(OH)$_2$D$_3$-binding protein has been solubilized and purified from basal lateral mem-

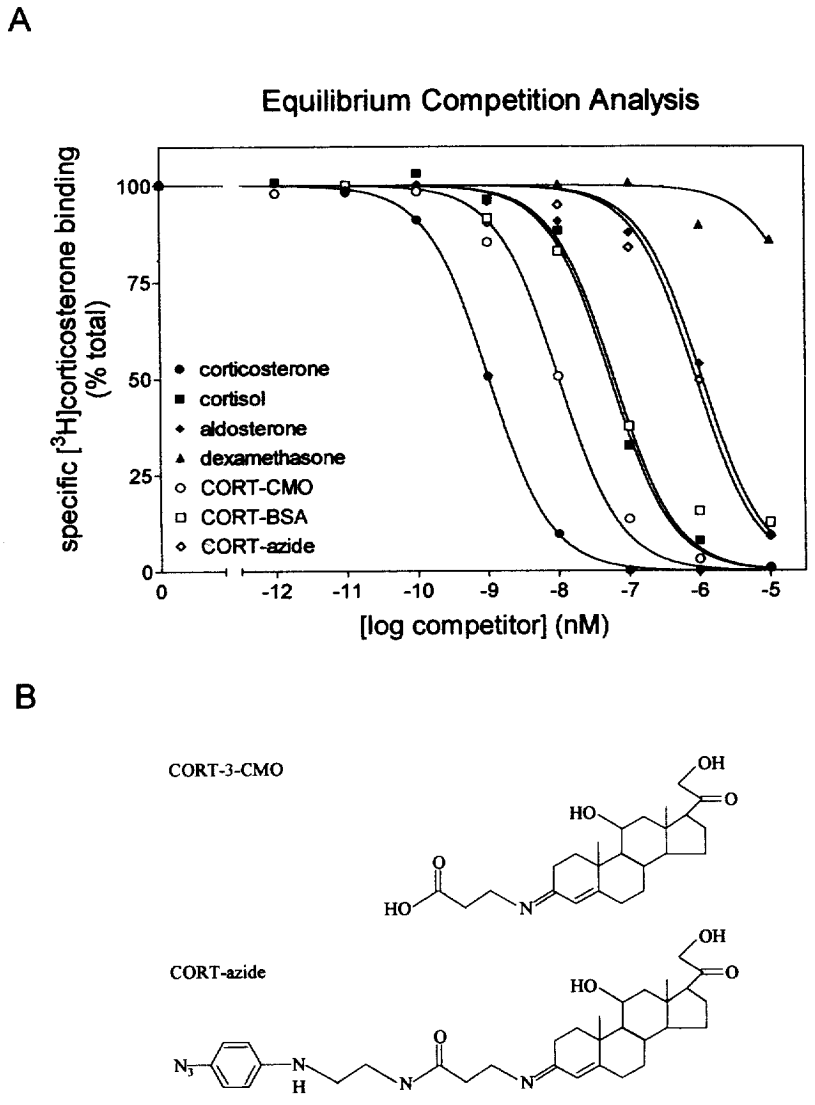


**Fig. 3. Equilibrium competition analysis. 0.5 nM [$^3$H]corticosterone binding was inhibited by increasing concentrations of various steroid hormones. Corticosterone (•), cortisol (■), aldosterone (◆), CORT-CMO (○) and CORT-azide (◇) completely inhibited specific [$^3$H]corticosterone binding with $K_i$ values of 0.24 nM (95% CI, 0.19 to 0.30 nM), 10.5 nM (95% CI, 7.5 to 14.5 nM), 211 nM (95% CI, 175 to 255 nM), 2.6 nM (95% CI, 1.8 to 3.8 nM) and 234 nM (95% CI, 135 to 402 nM), respectively. Dexamethasone (▲), at 10 μM, only inhibited 15% of control binding. Data were analyzed assuming a single site competition model. Top values were constrained for all data sets, to the value observed in the absence of any competitor. Data was obtained from pooling two independent experiments.**

branes of chick intestinal epithelium [10]. Solubilization of the newt corticosterone membrane receptor is an important step towards the purification and identification of this protein.

The corticosterone receptor from newt neuronal membrane has previously been characterized pharmacologically [22] and physiologically [28–30] and shown to be highly enriched in plasma membrane fractions using sucrose density gradient centrifugation [4]. There is a strong correlation between the pharmacology of the membrane receptor ($IC_{50}$'s for corticosterone binding) and the potencies of various corticosteroids to inhibit courtship behaviors ($EC_{50}$'s in behavioral assays) [4]. The localization of corticosterone binding sites in the plasma membrane, the lack of affinity for dexamethasone and other intracellular glucocorticoid receptor ligands, and the rapid behavioral and physiological effects of corticosterone, all provide strong evidence for a plasma membrane corticosterone receptor with physiological significance.

Evidence for involvement of G-protein coupled signalling pathways exists for many steroid membrane actions. Previous studies with membrane preparations from newt brain found significant negative modulation of [$^3$H]corticosterone binding by guanyl nucleotides [19]. There is also evidence that the inhibition of calcium currents in guinea pig hippocampal CA1 neurons by cortisol involves a G-protein coupled mechanism [12]. The loss of guanyl nucleotide sensi-

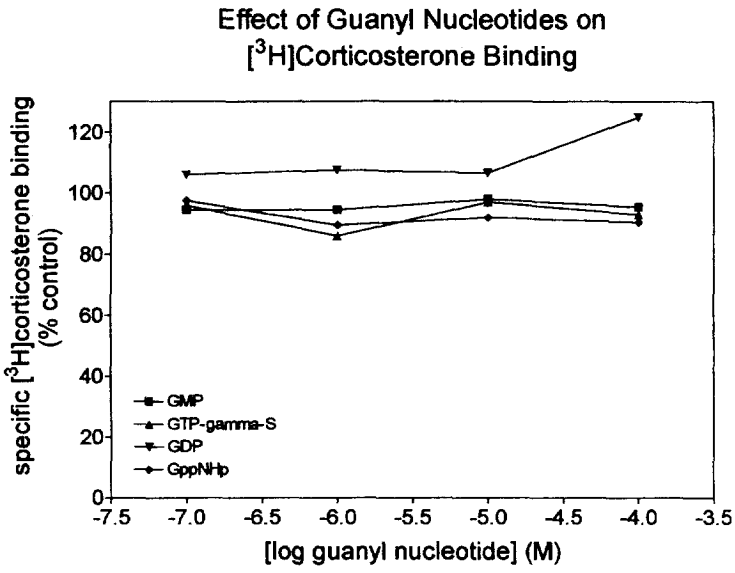


**Fig. 4. Lack of modulation of [$^3$H]corticosterone binding by guanyl nucleotides. 0.5 nM [$^3$H]corticosterone binding was measured in the presence of increasing concentrations of various guanyl nucleotides as indicated. With concentrations up to 100 $\mu$M no negative effect of guanyl nucleotides on [$^3$H]corticosterone binding was observed. $Mg^{2+}$ was present at 1 mM for all guanyl nucleotide assays. Data is representative of three independent experiments for GTP$\gamma$S and Gpp(NH)p and two experiments for GDP and GMP.**

tivity in a detergent solubilized system, as reported here, has precedent. Several receptors known to be members of the G-protein coupled receptor superfamily have previously been detergent solubilized with disparate characteristics regarding maintenance of guanyl nucleotide sensitivity after solubilization. $A_1$ [31] and $A_{2a}$ [32] adenosine receptors, muscarinic acetylcholine receptors [24, 33] and a variety of dopamine receptors [34–36] have all been successfully co-solubilized with G-proteins; while GLP-1 receptors [37], angiotensin II receptors [38] and CRF receptors [39] have lost detectable G-protein association in a solubilization procedure.

G-protein coupled receptors can exist in a state of either high or low affinity for their respective ligands. The high affinity state is promoted by interaction with G-proteins and the low affinity state achieved by uncoupling from G-proteins. The maintenance of high affinity [$^3$H]corticosterone binding in the absence of guanyl nucleotide sensitivity in the detergent system is thus paradoxical but can be explained by two alternative hypotheses. (1) Receptor–G-pro-

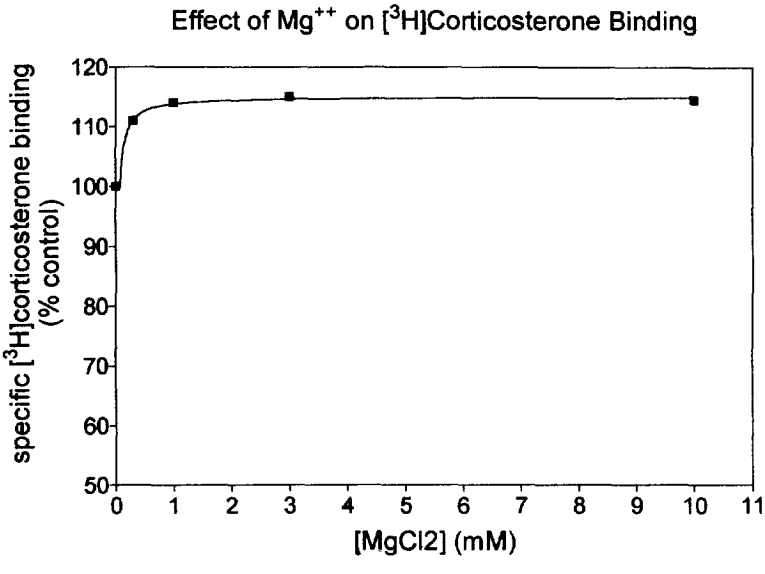


**Fig. 5. Effect of $Mg^{2+}$ on [$^3$H]corticosterone binding. $Mg^{2+}$ was added to binding assay tubes as $MgCl_2$ to final concentrations indicated. A 15% increase in specific [$^3$H]corticosterone binding was observed with an $EC_{50}$ of approximately 10 $\mu$M and a maximal effect occurring by 3 mM. Fit was generated using GraphPad Prism single site binding. Data is representative of two independent experiments.**

tein interaction is lost in the detergent system but the receptor high affinity state is favored by the detergent environment. (2) Receptor–G-protein interaction is maintained in the detergent system but GTP–GDP exchange is inhibited by the detergent environment. The latter hypothesis is consistent with the present data showing a nominal increase in binding in the presence of either $Mg^{2+}$ or GDP. Either excess $Mg^{2+}$ or GDP could drive the association of a small population of uncoupled receptors towards G-protein coupling. This would increase the number of receptors in the high affinity state and thus modestly increase observed specific [$^3$H]corticosterone binding. Further studies are needed to determine whether either hypothesis is tenable.

Steroid derivatives have been successfully used to photo-label and partially purify steroid binding proteins [40, 41]. We have shown that the newt corticosterone membrane receptor can retain high affinity binding activity as a solubilized protein and may thus be purified by exploiting this property. The receptor is stable with no significant loss of binding activity over a three week period when stored at 0°C. Furthermore, we have identified ligands, CORT-3-CMO, and CORT-azide which can be used in an affinity purification scheme.

Evidence for membrane steroid receptors has been accumulating for nearly two decades and, although the molecular identity of steroid membrane receptors is still unresolved, these data have physiological relevance. Steroid hormones play an integral part in both homeostatic and behavioral processes and are of significant clinical value. An understanding of the broad scope of steroid signalling mechanisms may help design improved therapeutic agents, perhaps enabling drug targeting to specific pathways of steroid hormone action. Cloning and expression of membrane steroid receptor proteins will provide an opportunity for detailed systematic research into the molecular nature and physiological significance of steroid membrane receptors.

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