Guanyl nucleotides modulate binding to steroid receptors in neuronal membranes
(corticosteroid receptors/G proteins/stress/sexual behavior)

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ABSTRACT The recently characterized corticosteroid receptor on amphibian neuronal membranes appears to mediate rapid, stress-induced changes in male reproductive behaviors. Because the transduction mechanisms associated with this receptor are unknown, we performed radioligand binding studies to determine whether this steroid receptor is negatively modulated by guanyl nucleotides. The binding of [3H]corticosterone to neuronal membranes was inhibited by nonhydrolyzable guanyl nucleotides in both equilibrium saturation binding and titration studies. The addition of guanyl nucleotide plus unlabeled corticosterone induced a rapid phase of [3H]corticosterone dissociation from membranes that was not induced by addition of unlabeled ligand alone. Furthermore, the equilibrium binding of [3H]corticosterone and the sensitivity of the receptor to modulation by guanyl nucleotides were both enhanced by Mg2+. These results are consistent with the formation of a ternary complex of steroid, receptor, and guanine nucleotide-binding protein that is subject to regulation by guanyl nucleotides. Therefore, rapid signal transduction through corticosteroid receptors on neuronal membranes appears to be mediated by guanine nucleotide-binding proteins.

Steroid hormones modulate behavior and neuroendocrine function through regulation of neuronal protein synthesis, an action mediated by intracellular steroid receptors (1, 2). However, not all effects of steroid hormones on brain function involve these genomic mechanisms (3). Evidence for nongenomically mediated actions of steroids includes changes in neuronal excitability or neurosecretion that occur within seconds of steroid administration, or that occur when de novo protein synthesis is inhibited or access to intracellular receptors is blocked, or that occur in brain regions lacking intracellular steroid receptors (4–8). These actions of steroids that occur independently of classical intracellular receptors may have important roles in the regulation of behavior. For example, progesterone induces reproductive behavior in female rats, in part through modulation of hypothalamic oxytocin receptors; this rapid steroid effect is independent of de novo protein synthesis (9).

The binding sites for steroids on the membranes of neurons (10, 11) as well as other cells (12–17) might mediate some rapid cellular responses to steroids. However, little is known about the transduction mechanisms associated with these recognition sites. Only a few general types of transduction mechanisms account for the multitude of cellular responses initiated by cell-surface receptors: a receptor may be an integral part of a ligand-gated ion channel, a transmembrane-regulated enzyme, or a transmembrane protein that couples to guanine nucleotide-binding regulatory proteins (G proteins) (18). It is now well established that some steroids can directly modulate the functioning of a ligand-gated ion channel, the γ-aminobutyric acid type A (GABA_A) receptor (19, 20). It appears that steroids may utilize alternative mechanisms as well. We recently characterized in neuronal membranes from an amphibian brain (21) a high-affinity corticosteroid receptor that appears to be physiologically relevant in mediating the stress-induced suppression of male reproductive behavior (22–24). This receptor is not associated with the GABA_A receptor chloride channel complex (21). Therefore, we have conducted studies to determine whether the transduction mechanism used by this steroid receptor involves G proteins.

Members of the superfamily of G protein-coupled transmembrane receptors exhibit common structural and regulatory motifs (25). One characteristic of these G protein-coupled receptors is that binding of hormone or neurotransmitter to the receptor is subject to heterotropic negative modulation by guanine nucleotides (26, 27). Therefore, to address this question with respect to the membrane-bound corticosteroid receptor, we performed a series of radioligand binding studies to determine whether [3H]corticosterone (CORT) binding is sensitive to regulation by guanyl nucleotides. The data support the conclusion that signal transduction through this corticosteroid receptor in neuronal membranes is mediated by G proteins.

METHODS

Animals. Adult male rough-skinned newts (Taricha granulosa) were collected locally (Benton Co., OR). Animals (mean weight, 20 g) were maintained in the laboratory in large tanks under a lighting and temperature regimen that approximated natural conditions. Animals were maintained and sacrificed in accordance with federal and institutional guidelines.

Membrane Preparation. Brains were rapidly removed and homogenized in 40 vol (original wt/vol) of ice-cold 0.3 M sucrose containing 5 mM Hepes (pH 7.45). The whole brain homogenate was centrifuged at 1000 g (15 min), and the resulting supernatant was centrifuged at 30,000 g (40 min; 4°C). The P2 pellet was frozen and thawed and then resuspended in 150 vol (original wt/vol) of cold buffer [25 mM Hepes/10 mM EDTA free acid/bacitracin (60 µg/ml), pH 7.45] for 2–3 hr at 4°C to dissociate endogenous ligands and remove endogenous cations. The suspension was centrifuged at 30,000 × g (30 min). The resulting pellet was washed in 150 vol of 25 mM Hepes buffer (pH 7.45) and centrifuged again at 30,000 × g for 30 min. The final pellet was resuspended in ~900 µl of assay buffer/brain to a protein concentration of 250–350 µg/ml. Assay buffer contained 25 mM Hepes and 10 mM MgCl_2 (unless specified otherwise) (pH 7.45). Protein concentration was determined by the Bradford method using

Abbreviations: GABA, γ-aminobutyric acid; G protein, guanine nucleotide-binding regulatory protein; CORT, corticosterone; GTP[yS] guanosine 5'-[γ-thio]triphosphate.

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The addition of neuronal membranes. 0.75 analysis of CORT to or 5.0 nM were to experiments, a using filters a 30°C (21). The addition of MgCl₂ enhanced [3H]CORT binding with EC₅₀ = 0.51 ± 0.17 mM. The hyperbolic fit shown was derived by least-squares regression analysis with GRAPH PAD-IN PLOT (San Diego).

Pierce Coomassie protein assay reagent (Rockford, IL) and bovine serum albumin standard.

Radioligand Binding Assays. The binding of radiolabeled CORT to crude synaptic membranes was initiated by the addition of 100 μl of [3H]CORT (final concentration, 0.75 nM) to 100 μl of the membrane preparation and 100 μl of inhibiting compound or buffer. For equilibrium saturation binding experiments, a range of [3H]CORT concentrations from 0.05 to 5.0 nM were used. The assays were incubated for 2 hr at 30°C (21) unless specified otherwise, and the reactions were terminated by rapid filtration and a 9-ml rinse with cold buffer [25 mM Tris base (pH 7.45) over Whatman GF-C filters, using a Brandel harvester (M-24R)]. Radioactivity bound to the filters was quantified by standard liquid scintillation spectroscopy. Nonspecific binding was defined as that occurring in the presence of 1 or 10 μM unlabeled CORT. At the concentration of [3H]CORT used, specific binding was typically 80% of total binding. The data from saturation and competition experiments were analyzed by nonlinear regression analysis using LUNDON analysis software (Lundon Software, Cleveland) and EBDA (Elsevier-Biosoft, Cambridge, U.K.), respectively.

Kinetic experiments were performed to study the guanine nucleotide-induced dissociation of [3H]CORT from membrane receptors. [3H]CORT (0.75 nM) was allowed to equilibrate with Taricha brain membranes for 2 hr as described above. Dissociation was initiated by addition of 25 μl of 25 μM unlabeled CORT or CORT plus 1 mM guanosine 5′-y-thio)triphosphate (GTP[yS]). Estimates of the kinetic parameters were obtained by using a nonlinear least-squares curve-fitting program KINETIC (Elsevier-Biosoft).

All binding experiments were performed in triplicate and were repeated at least twice with similar results.

Materials. [3H]CORT ([1,2,6,7-3H(N)]corticosterone; specific activity, 85–88 Ci/mmol; 1 Ci = 37 GBq) was purchased from New England Nuclear. Unlabeled CORT and guanine nucleotides were obtained from Sigma.

RESULTS

The specific binding of [3H]CORT to Taricha neuronal membranes was enhanced in a concentration-dependent manner by the addition of MgCl₂ (Fig. 1). The EC₅₀ of Mg₂⁺ for stimulation of [3H]CORT binding was 0.51 ± 0.17 mM. This enhancement of binding appeared to be cation specific; NaCl and CaCl₂ did not appreciably alter [3H]CORT specific binding (data not shown). In subsequent assays, 10 mM MgCl₂ was included in the assay buffer. Equilibrium saturation experiments (Fig. 2) indicated that [3H]CORT bound to a single population of high-affinity recognition sites with a KD of 0.14 ± 0.01 nM and a Bmax of 183 ± 4 fmol per mg of protein. Specific binding of [3H]CORT was reduced by GTP[yS], a nonhydrolyzable analog of GTP (Fig. 2). In the presence of 100 μM GTP[yS], the saturation isotherm was

![Fig. 1](image1.png)

**Fig. 1.** Effect of MgCl₂ on specific binding of [3H]CORT to neuronal membranes. The membrane preparation was incubated with 0.75 nM [3H]CORT and increasing concentrations of MgCl₂. The addition of MgCl₂ enhanced [3H]CORT binding with EC₅₀ = 0.51 ± 0.17 mM. The hyperbolic fit shown was derived by least-squares regression analysis with GRAPH PAD-IN PLOT (San Diego).

![Fig. 2](image2.png)

**Fig. 2.** Equilibrium saturation binding of [3H]CORT to neuronal membranes in the presence or absence of guanyl nucleotide. Neuronal membranes were incubated with increasing concentrations of [3H]CORT. Nonspecific binding (c) was defined as that occurring in the presence of 1 or 10 μM unlabeled CORT. Specific binding in the absence of guanyl nucleotide (c) was described by KD = 0.14 ± 0.01 nM and Bmax = 183 ± 4 fmol per mg of protein (Hill coefficient, 1.08). In the presence of 100 μM GTP[yS] (○), the saturation isotherm was described by KD = 0.21 ± 0.02 nM and Bmax = 140 ± 3 fmol per mg of protein (Hill coefficient, 1.12). In both cases, the data were best fit by a one-site model using LUNDON I software and LIGAND software (Elsevier-Biosoft, Cambridge, U.K.). (Inset) Linear Scatchard–Rosenthal replot of saturation data. B/F, bound/free.
FIG. 3. Guanyl nucleotides inhibit specific binding of [3H]CORT in Taricha brain membranes. Membranes were incubated with 0.75 nM [3H]CORT and increasing concentrations of the nucleotides shown. The titration curves were replicated three times with similar results. IC_{50} estimates ± SE were obtained for GTP[yS] (43.2 ± 11 μM) and p(NH)ppG (104 ± 65 μM) using EDBA software. The inhibition of [3H]CORT binding by GTP (data not shown) varied between experiments, with maximal inhibition ranging from 20% to 35%.

best fit by a one-site model yielding estimates of K_d = 0.21 ± 0.02 nM and B_{max} = 140 ± 3 fmol per mg of protein; the increase in K_d was not significant (T = 3.13; P = 0.089).

In titration experiments (Fig. 3), the nonhydrolyzable guanyl nucleotides GTP[yS] and 5'-guanyl imidodiphosphate [p(NH)ppG] inhibited up to 88% of the specific binding of [3H]CORT in a concentration-dependent manner; IC_{50} = 43.2 ± 11 μM and 104 ± 65 μM, respectively. The inhibition of [3H]CORT binding by GDP[yS] was temperature sensitive; GTP[yS] was less potent at 15°C (IC_{50} = 234 μM) than at 30°C. The rank order potency of nucleotides to inhibit [3H]CORT binding was GTP[yS] > p(NH)ppG >> GDP ≥ GMP >> ATP. The potency of GDP (data not shown) was variable between experiments but was generally similar to GDP. The limited potency of the endogenous nucleotide is presumably due to the high level of GTPase activity in neuronal membranes (26, 28, 29).

As shown in Fig. 4, the ability of guanine nucleotides to inhibit [3H]CORT binding was enhanced by MgCl_2. The efficacies of GTP[yS] and p(NH)ppG as modulators of [3H]CORT binding were markedly decreased when MgCl_2 was excluded from the assay buffer.

To determine the effects of guanyl nucleotides on dissociation kinetics, we compared the dissociation of [3H]CORT initiated by unlabeled CORT alone with that initiated by GTP[yS] plus CORT (Fig. 5). Dissociation of [3H]CORT initiated by CORT alone was monophasic, yielding a dissociation rate constant (k_{-1}) of 0.014 ± 0.006 min^{-1}. In contrast, dissociation of [3H]CORT initiated by the simultaneous addition of CORT plus GTP[yS] was biphasic (Fig. 5). The initial rapid phase of dissociation was described by k_{-1} = 0.515 ± 0.134 min^{-1}, in which 22% of [3H]CORT dissociated. The subsequent slow dissociation was described by a rate constant (k_{-1} = 0.015 ± 0.004 min^{-1}) similar to the k_{-1} obtained by the addition of CORT alone. The addition of GTP[yS] alone induced a rapid dissociation of ≈20% of [3H]CORT binding within 2 min (data not shown).

Considering that coupling of membrane receptors to G proteins may be dependent on the oxidation state of cysteine residues (30, 31), we performed GTP[yS] titration experiments in the presence or absence of 100 μM dithiothreitol, a disulfide bond reducing agent. The equilibrium binding of [3H]CORT was slightly decreased, but the potency of GTP[yS] to inhibit [3H]CORT binding was dramatically reduced when membranes were homogenized and assayed in buffers containing dithiothreitol (Fig. 6).

DISCUSSION

Our results provide evidence for a molecular mechanism for rapid modulation of brain function and behavior by steroid hormones. The data support the conclusion that signal transduction through the recently described corticosterone receptor in neuronal membranes is mediated by guanyl nucleotide-binding proteins. Of the >100 different G-protein-coupled receptors that have been identified (32), none have been steroid hormone receptors. However, the current findings that ligand binding to the CORT receptor was negatively modulated by guanyl nucleotides in equilibrium saturation, titration, and kinetic experiments provide strong evidence that this receptor is allosterically regulated by heterotrimeric G proteins. Given these findings, it appears that there are multiple receptor and transduction mechanisms that steroid hormones may use to modulate neuronal activity, including...
the regulation of transcription via soluble intracellular receptors, direct modulation of ligand-gated ion channels, and regulation of cellular effector mechanisms via G-protein-coupled receptors.

Since the discovery that GTP regulates the binding of guanylyl nucleotides, it has become clear that the negative modulation of agonist binding by guanyl nucleotides is a generalized phenomenon among G-protein-coupled receptors (34, 35). This regulation is believed to be due to a heterotropic interaction between nucleotide binding to G protein and agonist binding to receptor. In the absence of receptor-bound ligand, G proteins exist as heterotrimers with GDP bound. The binding of agonist to the receptor facilitates the formation of an agonist–receptor–G-protein complex and the dissociation of GDP. GTP is exchanged for GDP; this promotes dissociation of the G protein into α and βγ subunits, which may activate effector molecules, and dissociation of the receptor from the G-protein–receptor complex. When dissociated from G proteins, the free receptor displays a low affinity for agonists (26, 27). Dissociation of the ligand from the receptor, and hydrolysis of bound GTP, allows for continued activation of the cycle in response to hormone or transmitter.

The results from each of the experiments presented here are consistent with the formation of a receptor–G-protein complex that is subject to allosteric regulation by guanyl nucleotides. The binding of [3H]CORT to Taricha brain membranes was inhibited in a concentration-dependent manner by guanyl nucleotides. As in most G-protein-coupled receptor systems, the nonhydrolyzable nucleotides had greater efficacy in inhibiting ligand binding than the hydrolyzable compounds, and the adenosine nucleotide was without effect. Also, GTP[yS] induced a rapid phase of [3H]CORT dissociation from membrane receptors that was not induced by CORT alone, consistent with a nucleotide-induced shift in the affinity state of the CORT receptor. We also found that MgCl2 enhanced the specific binding of [3H]CORT, an effect not induced by CaCl2 or NaCl. In many receptor systems, Mg2+ facilitates formation of the high-affinity state of the receptor, presumably by binding to the G protein (26, 27). Therefore, the increase in binding elicited by Mg2+ likely reflects a conversion from the low- to the high-affinity state of the receptor for CORT. The EC50 value (0.51 mM) for this Mg2+ effect is similar to the Km of Mg2+ for hormone receptor-mediated activation of Gs (36).

The inhibition of [3H]CORT binding by GTP[yS] in saturation isotherms was described by an apparent decrease in the number of binding sites, rather than a decrease in affinity. The increase in Kd (from 140 to 210 μM) was modest, similar for, example, to the affinity shift induced in GABAA receptors by GTP (37). However, the rapid dissociation of [3H]CORT binding induced by GTP[yS] suggests a more pronounced affinity shift, at least in a subpopulation of CORT receptors (Fig. 5). In many systems, guanyl nucleotides induce a 50- to 100-fold decrease in receptor affinity, usually measured as a decreased ability of agonists to displace radiolabeled antagonist binding. No antagonists to the CORT receptor are currently available (21), and the highest concentration of [3H]CORT used was 5 nM, which effected a 97% occupancy of high-affinity recognition sites. Therefore, a 50- to 100-fold shift in affinity of a subpopulation of receptors would not be detectable under the assay conditions used but rather would be manifest as a reduction in the apparent Bmax. Similar decreases in apparent Bmax have been reported in saturation isotherms studying guanyl nucleotide regulation of 3H-labeled agonist binding to G-protein-coupled receptors, such as α- (38) and β-adrenergic (39) receptors.

Many of our observations are consistent with findings in the β-adrenergic receptor system, where the receptor–G-protein interactions have been extensively characterized (25). The efficacies of guanyl nucleotides to regulate agonist binding to EDTA-washed membranes were enhanced by MgCl2 in both the CORT (Fig. 4) and β-adrenergic (40) receptor systems. This phenomenon may be related to the ability of Mg2+ to promote formation of the ternary complex (agonist–receptor–G protein), which increases the guanyl nucleotide sensitivity of [3H]CORT binding. The modulation of [3H]CORT binding by guanine nucleotides was also temperature sensitive in a manner similar to the β-adrenergic system (40). In addition, treatment of the membranes with 100 μM diithiothreitol resulted in a slight reduction of the equilibrium binding of [3H]CORT but a much greater reduction in the efficacy of GTP[yS] as an inhibitor of [3H]CORT binding. Similarly, in the β-adrenergic system, the oxidation state of cysteine residues appears to be important not only for
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