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Partial purification and biochemical characterization of a membrane glucocorticoid receptor from an amphibian brain

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Abstract

A membrane receptor for corticosterone (mGR) in the brain of the roughskin newt (*Taricha granulosa*) has been previously identified. This manuscript reports the evaluation of several chromatographic resins for enrichment of the newt mGR solubilized from neuronal membranes. A protein with an apparent molecular weight of 63 kDa was purified to near homogeneity following sequential purification using ammonium sulfate fractionation, wheat germ agglutinin (WGA)-agarose chromatography, hydroxylapatite chromatography, and an immobilized ligand affinity resin (Corticosterone-Sepharose). Other studies employed a novel protein differential display strategy and a photoaffinity labeling strategy to visualize candidate receptor proteins following SDS–PAGE. Both of these techniques also identified a 63 kDa protein, agreeing with the estimation of molecular weight from the purification data. Furthermore, the use of 2D SDS–PAGE following the photolabeling procedure showed the candidate 63 kDa protein to have a p*I* of approximately 5.0. Taken together these data suggest that the newt mGR is an acidic glycoprotein with an apparent molecular weight of 63 kDa. Because these characteristics of newt mGR are inconsistent with the characteristics of intracellular glucocorticoid receptors, these two receptor proteins are apparently distinct.

1. Introduction

Many mechanisms of steroid hormone action remain elusive. The classical model for steroid action relies on the interaction of the steroid with an intracellular receptor that modulates transcriptional activity of target genes. This signaling route for steroids is well-characterized and accounts for many of the known effects of steroid hormones. However, some responses to steroids fail to fit the classical genomic model and, instead, appear to utilize mechanisms involving membrane receptors and second messengers. Several recent reviews focus on steroid membrane receptors and non-genomic actions of steroids [1–8].

Evidence for the existence of membrane steroid receptors comes from several types of observations. First, some effects of steroid hormones are too rapid to be explained by the classical model. For example, electrophysiological studies find that membrane conductance can be modified within seconds of exposure to estradiol [9], progestins [10] and corticosteroids [11]. Second, the pharmacological specificity of rapid responses to steroids does not always match the specificity of steroid binding to known intracellular steroid receptors. The progesterone-induced acrosome reaction in sperm [12,13], androgenic modulation of second messenger systems in osteoblasts [14], and corticosteroid-induced inhibition of reproductive behaviors [15] are induced by different sets of steroids than those that bind intracellular progesterone, androgen, and glucocorticoid receptors, respectively. Third, steroids can induce rapid responses even when prevented from entering the cell by being conjugated to BSA. Progesterone-BSA can modulate hypothalamic neuronal

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activity [16] and behavioral responses [17], and estradiol-BSA can potentiate kainate-induced currents in CA1 neurons [18]. Fourth, some steroids can bind to and modulate the actions of known membrane receptors. Progesterone apparently binds to the oxytocin receptor and, thereby, inhibits oxytocin binding and oxytocin induced Ca^{2+} currents and inositol triphosphate (IP_3) turnover [19]. Progesterone metabolites also directly modulate the $GABA_A$ receptor/chloride channel [20].

The present study focused on a membrane glucocorticoid receptor (mGR) found in the brain of an amphibian, the roughskin newt (*Taricha granulosa*). Ligand-binding studies demonstrated that this receptor is localized in neuronal membranes, and binds corticosterone (CORT) with high affinity (sub-nanomolar K_D) and a specificity that distinguishes it from classical intracellular glucorticoid receptors [15]. This receptor has characteristics of a G-protein coupled receptor in that [^{3}H]CORT specific binding is enhanced by Mg^{2+} and inhibited by non-hydrolyzable guanyl nucleotides [21]. Evidence that this mGR regulates brain function and behavior in newts comes from studies showing that the rank-order potency of specific steroids to inhibit [^{3}H]CORT specific binding to neuronal membranes (CORT > cortisol > aldosterone > RU 28,362 > dexamethasone) matches the rank-order potency of these steroids to rapidly inhibit (within 20 min) male reproductive behavior [15]. Similarly, CORT administration, but not dexamethasone, can rapidly inhibit stress-induced increases in locomotor activity [22] and suppress the neuronal activity in medullary neurons in newts [23,24].

Other studies indicate that a mGR that is similar to the newt mGR may exist in other vertebrates. Ligand-binding studies using membrane preparations from rat brains [25,26], rat liver tissue [27], and mouse pituitary glands [28] find binding sites with high specificity for corticosteroids. Neurophysiological studies also reveal that corticosteroid administration causes rapid changes in Ca^{2+} currents in guinea pig hippocampal CA1 neurons and that this rapid response appears to be mediated by a G-protein coupled receptor because it is sensitive to pertussis toxin (PTX) and GDPβS [29]. Similarly, corticosteroid administration induces rapid changes in neuronal activity in rats [11,30] and cats [31]. Physiological studies in fish show that cortisol administration can rapidly inhibit prolactin secretion and reduce cAMP activity in lactotrophs [32]. Behavioral studies report rapid effects of CORT administration on locomotor activity in birds [33] and rats [34].

Detergent solubilization and chromatographic purification strategies have helped to identify several membrane steroid receptors and binding proteins. The membrane receptor for 1-alpha,25-dihydroxyvitamin D3, which is associated with rapid transport of Ca^{2+} across intestinal basal-lateral membranes [35], has been solubilized and partially purified from chick intestinal epithelial membranes [36]. The membrane-associated cortisol binding protein in rat liver has been solubilized and purified [27]. The progesterone binding protein in porcine liver membranes has been solubilized, purified, and partially sequenced [37]. When this putative membrane receptor for progesterone was subsequently cloned, it was found to be a single transmembrane protein of 194 amino acids with low sequence similarity to known proteins [38], to be localized to endomembranes [39], and have a pharmacological signature that is reminiscent of the sigma receptor [40].

Other studies have investigated steroid membrane receptors and binding proteins using photoaffinity labels. Photoactive progestins have labeled proteins in plasma membranes of *Xenopus* oocytes [41], mouse cerebellum [42] and porcine liver [43]. Photoactive aldosterone derivatives have labeled a protein in the plasma membrane of human mononuclear leukocytes [44]. The photoactive glucocorticoid, dexamethasone mesylate, has been used to label membrane proteins from lymphocytes [45]. This approach with photoactive steroids can provide biochemical information about steroid binding proteins and be used in conjunction with purification strategies.

This paper describes the effectiveness of several chromatographic resins for enriching the mGR from newt brains, a biochemical characterization of the mGR, and the development of novel strategies for visualizing proteins labeled with photoactive steroids. These studies used newt brains for the mGR because [^{3}H]CORT has sub-nanomolar affinity for the newt mGR with approximately 85% specific binding at its K_D [15,46]. In contrast, [^{3}H]CORT binding studies in rat brain show a lower affinity of approximately 100 nM with 70% specific binding [25,26]. Furthermore, previous studies have provided detailed information about the pharmacology and physiology of the newt mGR and have validated procedures for maintaining high-affinity binding after solubilization of this receptor [46].

2. Materials and methods

All buffers, salts, detergents, DEAE-Sepharose, WGA-agarose and Donkey anti-Sheep IgG monoclonal antibody were purchased from Sigma (St. Louis, MO). CM-Sepharose and EAH-Sepharose were from Pharmacia Biotech (Piscataway, NJ). Macro-Prep Ceramic hydroxylapatite resin and glass chromatography columns with flow adapters were from Bio-Rad (Hercules, CA). Disposable 2.5 ml plastic chromatography columns were from IsoLabs (Akron, OH). Silver staining kit was from Novex (San Diego, CA).

Anti-CORT antibody was from Cortex Biochemical (San Leandro, CA). Enhanced Chemiluminescence Super-Signal Substrate was from Pierce (Rockford, IL). [^{3}H]Corticosterone (79 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Roughskin newts (*Taricha granulosa*) were collected from local ponds and maintained in tanks containing continuously flowing de-chlorinated water until used. Newts were fed tubifix worms on a 3–5 day cycle. Animals were treated in compliance with IAUAC standards and guidelines as approved by the Animal Use and Care Committee at Oregon State University.

All procedures discussed were repeated several times and shown to be reproducible. Chromatographs given in the figures come from individual experiments but are representative of results obtained across experiments.

2.1. Stock solutions

10 × detergent	4% digitonin, 0.8% sodium cholate, 25 mM HEPES, pH 7.45
Buffer A	25 mM HEPES, pH 7.45, 100 μM PMSF.
Buffer B	Buffer A with 0.8× detergent solution
Buffer C	Buffer B with 10 mM $MgCl_2$

2.2. Preparation of neuronal membranes and solubilization of membrane proteins

P2 membrane pellets were prepared by the method of Whittaker [47] with some modification as described previously [46]. Membrane proteins were solubilized by the method of Peterson and Schimerlik [48] with the modifications described previously [46].

2.3. Ammonium sulfate fractionation

Ammonium sulfate was added directly to membrane extract to achieve concentrations given in the results section. Each precipitation step proceeded for 15 min on ice followed by a 5 min centrifugation at 15,000 × *g* at 4°C. Supernatants were transferred to fresh centrifuge tubes for the next precipitation step and pellets were resuspended in buffer C for use in radioligand binding assays, total protein quantification or further purification.

2.4. Preparation of chromatography columns

Chromatographic resins were poured as slurries into disposable 2.5 ml plastic chromatography columns for hydroxylapatite and ion exchange resins, or glass columns with adjustable flow adapters for wheat-germ agglutinin (WGA)-agarose and CORT-Sepharose resins. All columns were equilibrated with 10 volumes of buffer B prior to loading. For hydroxylapatite resin, the dry ceramic resin was resuspended as a 10% slurry in 0.1 M potassium phosphate, pH 6.8 prior to equilibration with buffer B. Membrane extracts were diluted 2.5-fold with buffer A to achieve buffer B conditions prior to loading. All columns were loaded and run under gravity, with flow rates slowed to ~0.5 ml/min with an adjustable stopcock if necessary. Fractions of 0.5 ml were collected for binding assays and A_{280} readings.

2.5. Synthesis of CORT-Sepharose

EAH-Sepharose was washed with 80 ml of 0.5 M NaCl per 1 ml settled resin on a sintered glass filter followed by at least 10 volumes of distilled deionized water (ddH_2O). Resin was drained and resuspended as a 50% slurry with 20 mM corticosterone-3-carboxymethyloxime (CORT-3-CMO) in 30% dioxane, pH 4.5. One-tenth volume of 1 M *N*-ethyl-*N*″-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was added to catalyze the condensation reaction. Coupling was allowed to proceed overnight at 4°C on a gentle-rocking shaker. Resin was washed with 3 × 10 volumes of 30% dioxane to remove excess ligand followed by alternate washes with 0.5 M NaCl, pH 9 and 0.5 M NaCl, pH 4. Finally, the resin was washed with ddH_2O and drained prior to loading into glass chromatography columns.

2.6. Radioligand binding assays

[^{3}H]CORT binding assays were performed as previously described for membrane fractions [15] and solubilized fractions [46].

2.7. SDS–PAGE

For one-dimensional (1D) gels proteins were separated on SDS–PAGE gels as described by Laemmli [49] using a BioRad Mini Protean II electrophoresis system. For two-dimensional (2D) gels, samples were resuspended in 125 μl IPG strip rehydration buffer (8 M urea, 0.3% DTT, 1% triton X-100, 1% CHAPS, 2% ampholytes (3–10 l, Pharmacia)) and used to rehydrate 7 cm, 3–10 l immobiline dry strips (Pharmacia) overnight. Proteins were focused using the Pharmacia Multiphor II system using a linear gradient program as follows: Step 1, 200 V, 2 mA, 7 W, for 1 min; Step 2, 3500 V, 2 mA, 7 W, for 90 min; Step 3, 3500 V, 2 mA, 7 W, for 125 min. Focused IPG strips were incubated 10 min in equilibration buffer (0.1 M Tris–HCl,

pH 6.8, 6 M urea, 30% glycerol, 1% SDS) with 1% DTT followed by a 10 min incubation in equilibration buffer with 4.5% iodoacetamide and 0.1% bromophenol blue. Equilibrated strips were laid across a 12% SDS–PAGE gel, sealed in with 0.5% agarose in stacking gel buffer [49] and run as 1D gels. Gels were either stained using a Novex Silver Express silver staining kit or transferred to nitrocellulose for western blot analysis.

2.8. Western blotting

SDS–PAGE gels were transferred to nitrocellulose using the Bio-Rad Mini-Protean II transfer cell in transfer buffer (25 mM Tris–base, 192 mM glycine, pH 8.03, 0.01% SDS, 20% methanol) at room temperature at 50 V for 45 min for 1D gels (0.75 mm) or 90 min for 2D gels (1.5 mm). Blots were blocked with 5% BSA, in TBS-T (0.1 M Tris–HCl, pH 7.2, 0.2 M NaCl, 0.1% tween-20) at 10°C overnight. Blots were then incubated with 1:1000 polyclonal sheep anti-CORT 1° antibody for 1 h in 5% BSA in TBS-T at room temperature followed by 3×15 min washes with TBS-T. Blots were then incubated with monoclonal donkey anti-sheep IgG 2° antibody in TBS-T for 1 h at room temperature followed by 7×10 min washes in TBS-T. Blots were developed using ECL SuperSignal Substrate and visualized by autoflourograph detection using Fuji RX X-ray film.

2.9. Photoaffinity labeling studies

Azido-CORT was synthesized as described previously [46]. Membrane extract was diluted 2.5-fold with buffer/0.2 M NaCl and samples were incubated with either 10 μM CORT or vehicle (0.1% ethanol) for 1 h at room temperature. Azido-CORT was then added to sample to a concentration of 2 μM ($10\times$ K_i) and incubated at room temperature for 2 h. Samples were incubated on ice for 10 min then exposed to 254 nm UV irradiation using a Spectroline hand held UV lamp (Spectronics Corporation, Westbury, New York) for 5 min with gentle shaking on ice. Receptor was enriched by WGA-agarose chromatography and analyzed by western blot.

3. Results

3.1. Ammonium sulfate fractionation

To determine if the newt mGR could be enriched by ammonium sulfate fractionation, solubilized newt neuronal membrane proteins were fractionated by precipitation with increasing concentrations of ammonium sulfate. Pellets from each precipitation step were resuspended for quantification of total protein by absorbance at 280 nm (A_{280}) and quantification of receptor binding activity by [^{3}H]CORT binding assays (Fig. 1A). The greatest amount of receptor binding activity occurred in the 80–100% saturated ammonium sulfate fractions. Whereas the majority of total protein precipitated at earlier points. Since the receptor precipitated toward the extreme end of total protein precipitation (Fig. 1B), a second experiment was performed with expanded resolution in the receptor positive range. A single cut of 70–95% saturated ammonium sulfate produced the optimal enrichment and yield of the receptor binding activity, and therefore, this cut was utilized for future experiments.

3.2. Ion exchange chromatography

A weak anion exchange resin, DEAE-Sepharose, and a strong cation exchange resin, CM-Sepharose, were used to evaluate the effectiveness of ion exchange chromatography for enriching the newt mGR. The flow-through and wash fractions of the anion exchange resin contained no [^{3}H]CORT binding activity. Whereas these fractions from the cation exchange resin contained 95% of the loaded [^{3}H]CORT binding activity (data not shown). These findings suggest that mGR is an anionic protein at physiological pH. However, recovery of receptor from the DEAE-Sepharose resin was poor ($<20\%$) and inconsistent from run to run. Furthermore, no enrichment of receptor over total protein was observed. Thus ion-exchange chromatography was not utilized in mGR purification studies.

3.3. Hydroxylapatite chromatography

To evaluate the effectiveness of hydroxylapatite resin for enriching the mGR, solubilized membrane proteins were loaded onto a hydroxylapatite column and then step eluted with low potassium phosphate, followed by high potassium phosphate (Fig. 2). The majority of receptor binding activity eluted at the high salt concentration; whereas, greater than 50% of total protein eluted at the low potassium phosphate. At least 0.5 M potassium phosphate was required for efficient ($>50\%$) recovery of receptor activity. Although the interactions of proteins with hydroxylapatite are not completely understood, they are predominately ionic. The observation that the receptor activity elutes at higher salt concentrations than the bulk protein suggests that the receptor is a highly charged protein.

3.4. Lectin affinity chromatography

Lectins bind carbohydrate moieties on glycoproteins and have been used to purify many membrane pro-

teins. The present study evaluated the lectin affinity resin, WGA-agarose for enriching mGR. Solubilized membrane proteins were loaded onto WGA-agarose resin and then eluted with n-acetylglucosamine (NAG) (Fig. 3). Because NAG had significant negative effects on [^{3}H]CORT binding assays, the eluent had to be desalted over a G-50 Sephadex column prior to assaying for receptor binding activity. Approximately 90% of total protein remained unbound to the WGA-agarose resin, and was collected in the flow-through and wash fractions, as compared to a loss of about 15% of the receptor binding activity in these fractions. About 40% of receptor binding activity occurred in the eluent fractions with only about 4% of total protein. Recovery of the receptor protein was probably underestimated, because the G-50 desalting step resulted in protein loss. These data not only show that WGA-agarose can be used to enrich the newt mGR, but also indicate that the mGR is a glycoprotein.

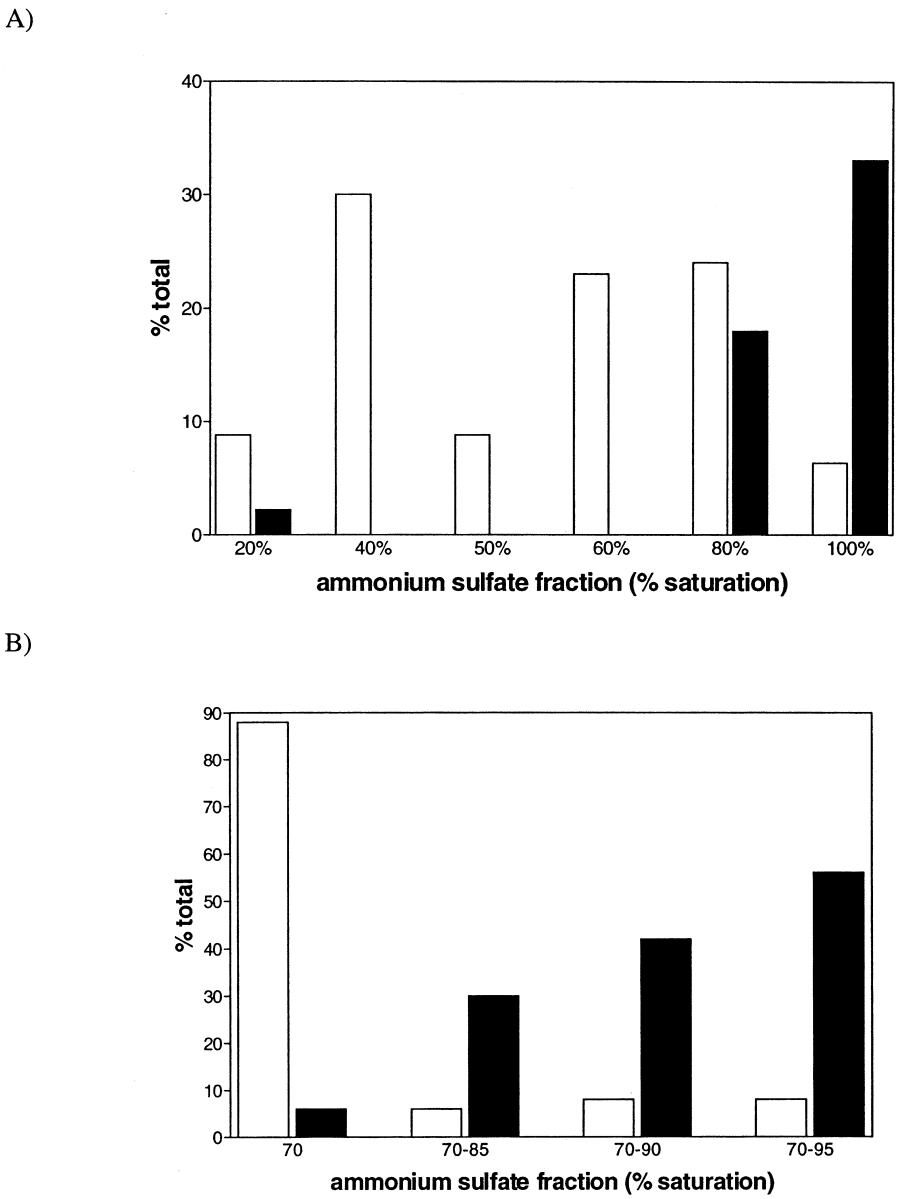


Fig. 1. Ammonium sulfate fractionation of solubilized neuronal membrane proteins. Proteins were precipitated with increasing concentrations of ammonium sulfate followed by removal of precipitate by centrifugation and resuspension of pellets in buffer C. Total protein (open bars) was quantified by absorbance at 280 nm and receptor binding activity (filled bars) was quantified by [^{3}H]CORT binding assays. Values are reported as percent total in reference to total protein or total specific binding activity in the pre-precipitate sample. (A) Initial fractionation over a broad range of ammonium sulfate concentrations. (B) Expanded fractionation through ammonium sulfate concentrations positive for precipitation of mGR binding activity.

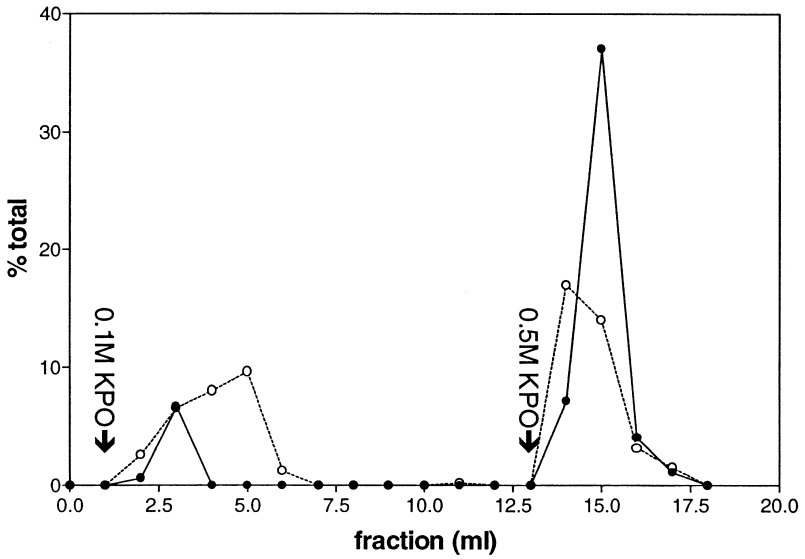


Fig. 2. Hydroxylapatite chromatography. 0.4 ml membrane extract diluted with 0.6 ml buffer A (1 ml total) was loaded onto a 0.5 ml hydroxylapatite column equilibrated with buffer B/20 mM potassium phosphate, pH 7.45. Proteins were step eluted with potassium phosphate as shown on the graph. Total protein (○) was quantified by absorbance at 280 nm and receptor binding activity (●) with [^{3}H]CORT binding assays.

3.5. CORT-Sepharose chromatography

Corticosterone-Sepharose was evaluated for efficacy in a purification scheme, initially by determining whether specific binding of [^{3}H]CORT was depleted by incubating mGR with the affinity resin. Solubilized membrane proteins were loaded onto either CORT-Sepharose or blank Sepharose (after blocking free amines). As shown in Fig. 4, the flow-through fractions for both resins contain similar amounts of total protein (approximately 70%, compared to pre-load controls). Similiarly, flow-through fractions from blank Sepharose contained 70% of the [^{3}H]CORT specific binding activity. Flow-through fractions from CORT-Sepharose only retained 10% of receptor binding activity. These data indicate that the CORT-Sepharose affinity resin selectively depletes mGR from solubilized membrane preparations.

Two strategies were employed to displace mGR binding to the CORT-Sepharose resin by [1] treating

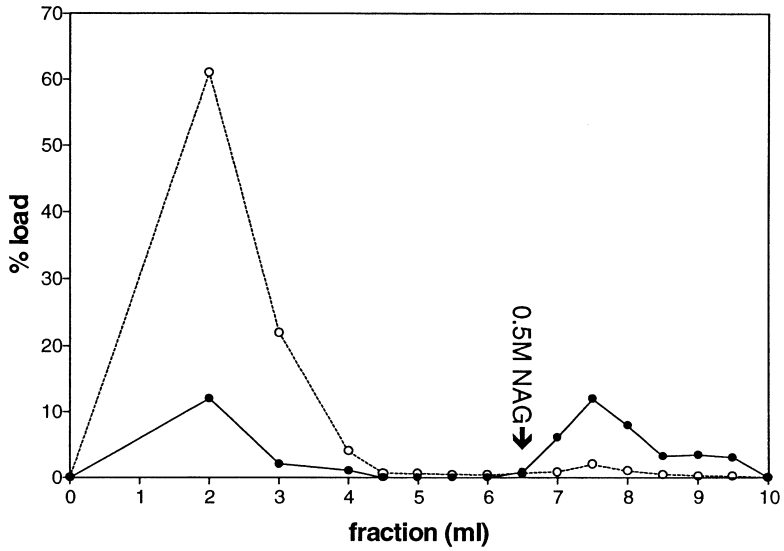


Fig. 3. WGA-agarose chromatography. Two ml of solubilized membrane proteins in buffer B/0.2 M NaCl were loaded onto 1ml of wheat germ agglutinin agarose resin and allowed to mix as a slurry overnight at 4°C in a sealed glass column in an end-over-end manner. The column was then drained and washed with 10 volumes buffer B/0.2 M NaCl. Proteins were eluted by resuspending the drained resin in 0.2 M NAG/buffer B/0.2 M NaCl. The resultant slurry was then incubated one hour prior to draining the eluent fraction from the resin. Total protein (○) was quantified by absorbance at 280 nm and receptor binding activity (●) by [^{3}H]CORT binding assays after desalting over a G-50 Sephadex column.

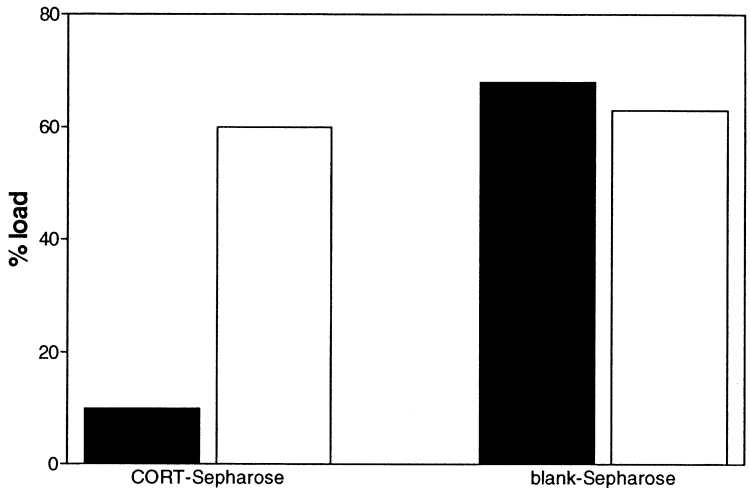


Fig. 4. CORT-Sepharose affinity chromatography. 0.5 ml of CORT-Sepharose and 0.5 ml of blank (blocked) Sepharose were loaded with 1 ml of solubilized membrane proteins in buffer B/0.5 M NaCl and allowed to mix overnight at 4°C in an end-over-end manner in sealed glass columns. Columns were drained and the unbound fraction assayed for total protein (open bars) concentration by absorbance at 280 nm and for receptor binding activity (filled bars) using [^{3}H]CORT binding assays.

the columns with excess free CORT or [2] stripping the column with a high salt/low pH shock (1 M NaCl, pH 4.0). Control experiments found that both strategies interfered with the [^{3}H]CORT binding assays. Gel-filtration, microconcentration/dilution, or dialysis were used as methods to restore [^{3}H]CORT binding assay conditions after treating solubilized fractions with either excess CORT or a high salt/low pH shock. However, no [^{3}H]CORT binding activity could be recovered in these control experiments. Therefore, it was impossible to estimate receptor protein in eluents from the CORT-Sepharose affinity resin because the elution process eliminated receptor binding activity.

3.6. Partial purification of mGR with sequential chromatographies

The chromatographic methods that provided reasonable enrichment of the receptor were performed sequentially to achieve partial purification of the receptor protein. Because of the limited amounts of starting material, binding assays and A_{280} readings were limited to pooled flow-through and eluent fractions and the data are reported in tabular format (Table 1). The mGR was initially enriched by ammonium sulfate fractionation followed by sequential chromatographic purification over WGA-agarose, hydroxylapatite and CORT-Sepharose. Eluents from each step were assayed for total protein and receptor binding activity. Also, an aliquot of each eluent was analyzed by SDS–PAGE (Fig. 5). Receptor enrichment could be estimated through the first three purification steps, culminating in approximately 50-fold enrichment in the hydroxylapatite resin eluent. Because radioligand binding assays were not feasible with the CORT-Sepharose column eluent fraction (see above), the final enrichment could not be calculated. Silver-stained SDS–PAGE gels (Fig. 5), however, revealed the purification of a protein to near homogeneity in the CORT-Sepharose affinity column eluent. This protein has an observed molecular weight of approximately 63 kDa.

3.7. Protein differential display with CORT-Sepharose

Because of the inability to recover binding activity in the CORT-Sepharose column eluent, an alternative

Table 1
Progress of receptor purification. Fractions were taken from each point in the purification strategy as listed in the table and subjected to quantification of total protein by A_{280} and quantification of receptor activity by [^{3}H]CORT binding assays

Fraction	Specific activity (fmol/mg protein)	Yield (% total)	Fold enrichment
1. Membranes	76	100	1
2. Detergent extract	66	64	0.87
3. Ammonium sulfate pcpt.	210	31	2.8
4. WGA-agarose eluent	370[a]	13[a]	4.9[a]
5. Hydroxylapatite eluent	3760	10	50

[a] Receptor enrichment in WGA-agarose eluent is probably underestimated because NAG interferes with [^{3}H]CORT specific binding to mGR.

strategy was developed to visualize receptor protein on a SDS–PAGE gel. For this, two CORT-Sepharose affinity columns were run in parallel. The first column (column 1) was loaded with solubilized membrane proteins, containing the receptor that could bind to the affinity resin. The second column (column 2) was loaded with membrane proteins pre-incubated with CORT, which was done to occupy the receptor's binding site and block specific interactions between mGR and affinity resin (Fig. 6B). Thus, mGR protein should be present in the eluent from column 1 and absent in the eluent from column 2. SDS–PAGE analysis of these eluents is shown in Fig. 6A. A protein with an apparent molecular weight of 63 kDa is seen in the eluent from column 1 and absent from that of column 2. These data are consistent with the identification of a 63 kDa protein by the purification scheme described by Fig. 5.

3.8. Photoaffinity labeling of mGR

Previous work described the synthesis of a novel azido-CORT ligand that had nanomolar affinity for

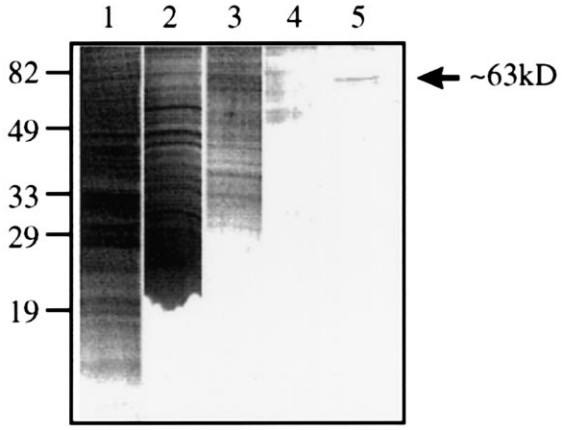
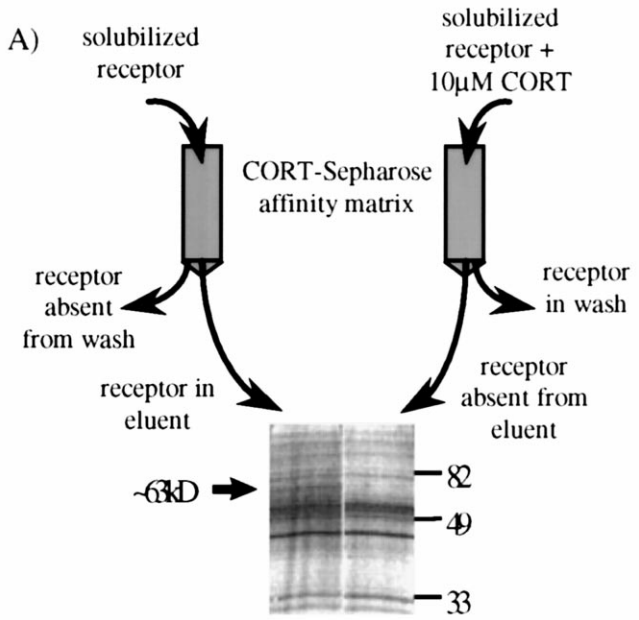


Fig. 5. Partial purification of mGR with sequential chromatographies. 10 ml membrane extract (lane 1), approximately 2 mg/ml, was precipitated with ammonium sulfate and the pellet resulting from a 70% to 95% saturated ammonium sulfate cut was resuspended in 1 ml buffer B/0.2 M NaCl (lane 2). Suspension was loaded onto 1 ml WGA-agarose and incubated as a slurry overnight at 4°C. Resin was drained and washed with five ml buffer B/0.2 M NaCl then eluted with 2 × 1 ml 0.5 M NAG in buffer B/0.2 M NaCl (lane 3). WGA-agarose eluent was loaded onto a 0.5 ml hydroxylapatite column followed by washing with 5 ml 0.1 M potassium phosphate and elution with 2 ml 0.5 M potassium phosphate in buffer B (lane 4). Hydroxylapatite eluent was loaded onto 1 ml of CORT-Sepharose affinity resin and allowed to incubate overnight as a slurry at 4°C. Resin was washed with 5 ml buffer B/0.5 M NaCl then eluted with 2 ml 1 M NaCl, 0.2 M sodium acetate, pH 4.0 (lane 5). Each lane was loaded with approximately 2 ng of receptor based on radioligand binding assays. For affinity column eluent a loss of 50% of receptor from previous step was assumed to attempt equal loading. Proteins were visualized by silver staining following SDS–PAGE on a 12% acrylamide gel.



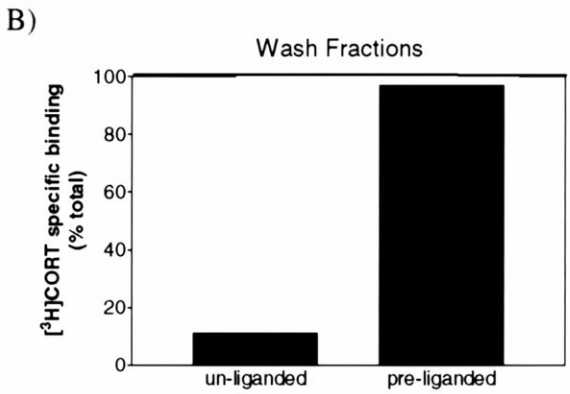


Fig. 6. mGR differential display. (A) Solubilized membrane proteins (2.5 ml) in buffer B/0.5 M NaCl were equilibrated for 1 h at room temperature with either 10 μM corticosterone or vehicle (0.1% ethanol) then loaded onto identical 1 ml Corticosterone-Sepharose affinity resins and allowed to mix overnight at 4°C in and end-over-end manner. Columns were drained and washed with 5 column volumes buffer B/0.5 M NaCl. Columns were then eluted with 0.2 M sodium acetate, pH 4.0, 1 M NaCl. Eluent was desalted by washing in a 5 K molecular weight cut-off centrifugal concentrator and finally concentrated to a volume of 10 μl for dilution into SDS–PAGE loading buffer for resolution of proteins by 12% gels. Proteins were visualized by silver staining. (B) Columns were loaded as in A except proteins were pre-liganded with either 50 nM [^{3}H]CORT (instead of 10 μM cold CORT) or vehicle prior to loading columns. [^{3}H]CORT was then added to the flow-through fraction of the unliganded sample so that total CPMs in both the pre-liganded and un-liganded flow through fractions were equal prior to determing [^{3}H]CORT binding activity in these fractions. [^{3}H]CORT binding activity is plotted normalized to total protein concentration and relative to sample loaded onto columns.

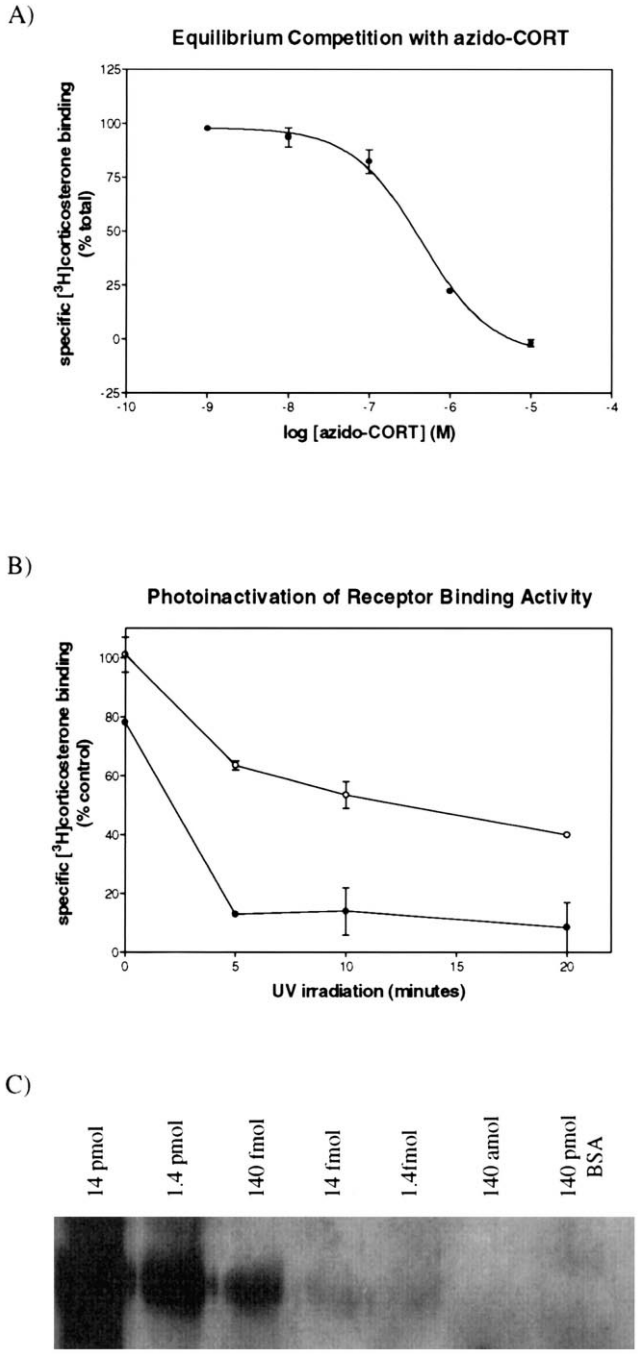


Fig. 7. Preliminary studies of azido-CORT photolabeling strategy. (A) Azido-CORT was synthesized as described previously (46) then used in an equilibrium competition assay. P2 membrane preparations were used with approximately 100 μg total protein per assay tube. All tubes received [^{3}H]CORT to a final concentration of 0.8 nM and azido-CORT to concentrations indicated on graph. Binding assays were done in 300 μl 25 mM HEPES, pH 7.45, 10 mM $MgCl_2$, 100 μM PMSF and allowed to incubate 4–6 h at room temperature in total darkness. Assays were terminated by rapid filtration over GF/C glass fiber filters equilibrated in cold assay buffer. A K_i of 163 nM was calculated from this batch of azido-CORT in reasonable agreement with previous batches. (B) P2 membrane preparations of approximately 1 mg protein each were incubated with either azido-CORT (●) or vehicle (○) (four preparations for each condition). Membranes were chilled by shaking in an ice bath for 10 min then irradiated for the time shown on graph. Membranes were pelleted by centrifugation at 40,000 × g and washed three times in 25 mM HEPES, pH 7.45 by resuspension and re-centrifugation. Final pellets were subjected to [^{3}H]CORT binding assays as above. (C) CORT-BSA was subjected to western blot analysis as described in methods. Lanes were loaded with amounts given in the figure expressed as mols CORT. Detection of CORT was between 14 and 140 fmol. BSA was run as a control to ensure the antibody was not detecting epitopes on BSA itself. Ten-fold more BSA than the highest level of BSA-CORT showed no signal.

the newt mGR [46]. This ligand was used in a photolabeling strategy as an independent method to identify mGR on a SDS–PAGE gel. Our photolabeling strategy was unique because it does not use a radiolabeled ligand to detect photolabeled proteins. Instead a western blot methodology was used with an antibody against corticosterone to visualize proteins that had been labeled with azido-CORT. As shown in Fig. 7, azido-CORT binds the membrane corticosterone receptor with a K_i of approximately 200 nM (Fig. 7A) and

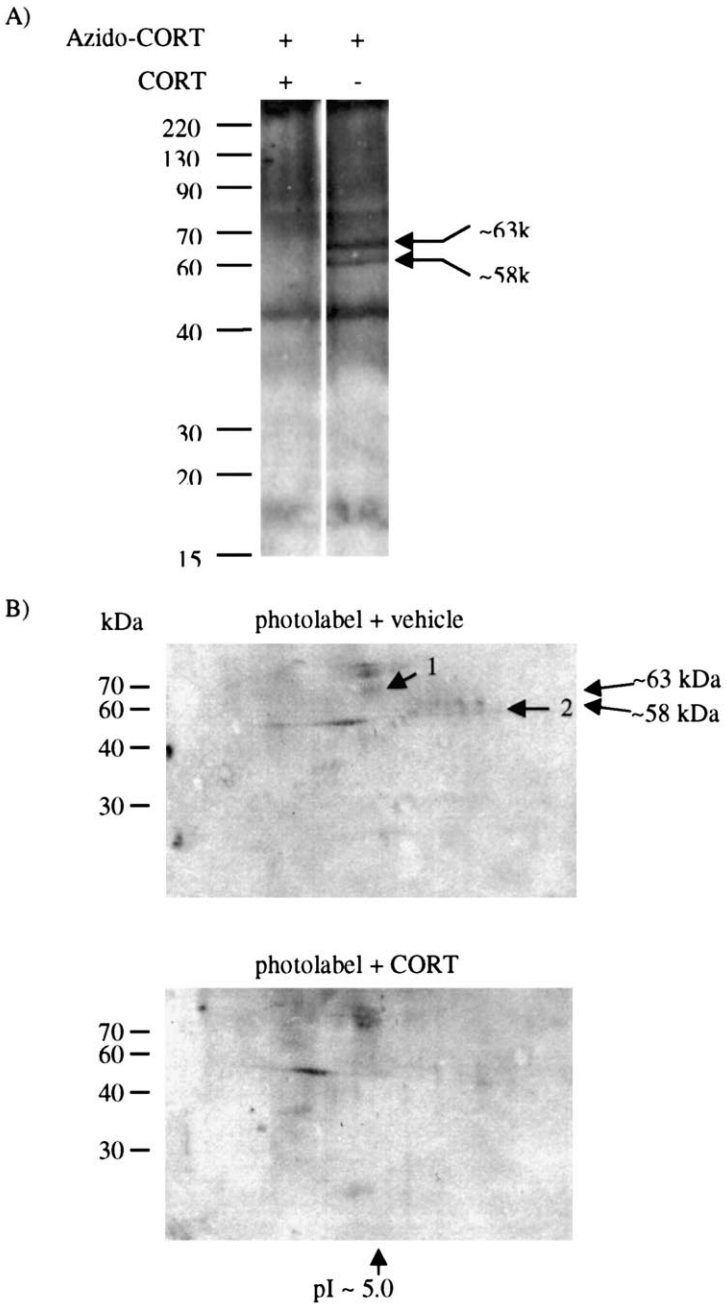


Fig. 8. Detection of mGR by photoaffinity labeling. (A) One ml of membrane extract was diluted to 2.5 ml with buffer A and incubated with either 10 μM CORT or vehicle (0.1% ethanol) for 1 h at room temperature. Proteins were then labeled with 2 μM azido-CORT (10× it's K_i) for 2 h at room temperature. Reactions were chilled 10 min in an ice bath prior to UV irradiation at 254 nm for 5 min while shaking on ice. Reactions were loaded directly on to 1 ml WGA-agarose resin and receptor enriched as described in the WGA-agarose chromatography section. Eluents were desalted by centrifugal concentrators, diluting eluent 2 × 20-fold with 25 mM HEPES, pH 7.45 prior to precipitation of total proteins with 80% acetone at −20°C overnight. Proteins were pelleted at 2000 × g and resuspended in SDS–PAGE loading buffer for electrophoresis and western blot analysis as described in methods. (B) Samples were prepared as in A except acetone pellets were resuspended in IPG rehydration buffer and run out on 2D gels as described in Section 2.

cross-links the receptor with greater than 50% efficiency (Fig. 7B). Also, a dilution series of CORT-3-CMO-BSA analyzed by the western blot procedure using the anti-CORT primary antibody (Fig. 7C) shows successful detection of labeled proteins with femtomolar sensitivity. The covalent linkage of CORT to BSA is identical to the linkage of CORT to the azido moiety in the azido-CORT molecule [46]. Thus, the CORT epitopes available on the CORT-3-CMO-BSA should be identical to the CORT epitopes available on the azido-CORT. This western blot procedure proved sensitive enough to detect approximately 50 fmol of CORT. BSA alone was used as a negative control to verify that the signal was not obtained from any BSA epitopes.

From these data, a calculation indicates that 1 mg of total starting protein is required to successfully detect mGR by this methodology. This calculation is derived from a receptor specific activity of 100 fmol/mg protein in P 2 membrane preparations [15], a photolabel cross-linking efficiency of 50%, and a detection limit of 50 fmol CORT. High non-specific labeling in initial experiments, however, indicated that a preliminary purification step was required in order to visualize any specifically labeled proteins. WGA-agarose was chosen for this because it showed the highest level of enrichment of receptor protein compared to the other purification methods. Also, since WGA-agarose binds carbohydrate moieties of glycoproteins, it was assumed that photolabeling of the receptor's binding site would not interfere with this purification technique. Azido-CORT labeled mGR was thus visualized by western blot methodology following enrichment by WGA-agarose chromatography (Fig. 8A). This showed two bands of approximately 63 and 58 kDa in which label was specifically displaced by excess CORT. To further evaluate the proteins identified in the photolabeling strategy, the labeling experiments were repeatedly followed by 2D gel electrophoresis (Fig. 8B) and two spots with apparent molecular weights of 63 and 58 kDa were identified, as seen on the 1D gel. The 63 kDa spot had an approximate p*I* of 5.0 and a multi-spot pattern characteristic of a glycoprotein. This observation is consistent with the characteristics of the mGR predicted by the chromatographic data, namely an acidic glycoprotein with an apparent molecular weight of approximately 63 kDa.

4. Discussion

The current study evaluated and applied methods for purifying and characterizing mGR from newt neuronal membranes. Ammonium sulfate fractionation followed by WGA-agarose and hydroxylapatite chromatographies provided significant enrichment of the newt mGR. Purification of a 63 kDa protein to near homogeneity was completed with the use of a CORT-Sepharose affinity resin. Furthermore, using photoaffinity labeling that was visualized by western blots following 1D or 2D SDS–PAGE gels, differential display identified a protein with a molecular weight of 63 kDa, consistent with the purification experiments. These studies have successfully identified steps with efficacy in the purification of the newt neuronal mGR that can be used in future experiments for a large-scale purification of this protein to gain primary amino acid sequence information. The calculations of receptor specific activity reported in the current study are most likely underestimated due to the inability to accurately measure protein concentrations by A_{280} at the low levels of protein present in these experiments. This hypothesis is supported by the observation that total protein visualized by silver staining on the SDS–PAGE gels is much less intense than expected for the protein loads.

In addition to identifying purification techniques, these studies provided insights into the biochemical characteristics of newt mGR. Ion exchange and hydroxylapatite chromatographies suggest that mGR is an anionic protein with a high charge. WGA-agarose chromatography provided evidence that mGR is a glycoprotein. Three independent strategies (purification by sequential chromatographies, differential display using CORT-Sepharose affinity chromatography, and photolabeling with western blot detection) all identified a protein with an apparent molecular weight of 63 kDa. Two-dimensional SDS–PAGE gels following photolabeling revealed that this protein has a p*I* of approximately 5.0, confirming that it is anionic. Taken together, these data suggest that the newt neuronal mGR is an acidic glycoprotein with an apparent molecular weight of 63 kDa.

Recent evidence indicates that, at least in some cases, steroid membrane receptors might be classical intracellular steroid receptors (or isoforms of) that become associated with the plasma membrane and functionally link to second messenger systems. Antibodies specific for the intracellular glucocorticoid receptor (iGR) label membrane proteins in lymphocytes [45,50–52]. Anti-estrogen receptor (ER) antibodies have been found to specifically label proteins in rat pituitary tumor cell membranes [53,54], hypothalamic dendrites [55] and plasma membranes of CHO cells transfected with ER cDNA [53]. Furthermore, the transfection of ER cDNA into CHO cells produced rapid inositol tris-phosphate turnover and adenyl cyclase activity in response to estradiol [53].

The current study provides evidence that the newt neuronal mGR and iGR are distinct proteins. The iGR is known to migrate on SDS–PAGE at around 96 kDa (consistent with its predicted molecular weight)

and is not known or expected to be glycosylated, whereas the mGR appears to be a glycoprotein with an apparent molecular weight of 63 kDa. These biochemical properties considered together with the pharmacological, behavioral and neurophysiological properties of mGR, discussed in Section 1, strongly suggest that the newt mGR and iGR are indeed distinct receptor proteins. However, the structure of mGR and its relationship, if any, to iGR can only be elucidated by complete molecular characterization.

Our photolabeling studies identified two proteins, including a 58 kDa protein that is unlikely to be the mGR. If this 58 kDa protein were the mGR, then in the photolabeling studies it would be expected to interact specifically with the azido-CORT label and exhibit an enhanced signal compared to total protein stains. The signal for the 58 kDa was not enhanced, as it was for the 63 kDa protein. Furthermore, only the 63 kDa protein was identified by the purification and differential display strategies. One explanation for specific labeling of the 58 kDa protein might be that it closely associates with mGR and may therefore be labeled, because of its juxtaposition to the mGR binding site.

Previous reports on the purification of other steroid membrane receptors have interesting parallels with the current study. The purification of a cortisol binding protein from rat hepatic membranes revealed two proteins of 52 and 57 kDa when analyzed by SDS–PAGE [27]. High resolution size-exclusion chromatography suggested that these two proteins may heterodimerize to form the functional receptor. Using an azido-progesterone photolabel, another study found two proteins in porcine liver microsomes with apparent molecular weights of 60–65 kDa and 90–100 kDa [43]. Native gel analysis suggests that these progesterone-labeled proteins may be components of larger heteromeric complex [43]. These published observations and the current data set (namely that azido-CORT labeled two proteins) indicate that some membrane steroid receptor may require heterodimerization to achieve functional binding activity. That characteristic could explain why expression cloning strategies have failed to identify steroid membrane receptors.

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