

Rapid Corticosteroid-Dependent Regulation of Mineralocorticoid Receptor Protein Expression in Rat Brain

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Corticosteroid hormones regulate many aspects of neural function via mineralocorticoid receptors (MR) and glucocorticoid receptors (GR). Although GR expression is negatively regulated by endogenous corticosteroids, the autologous regulation of MR expression has been less well studied, partly due to limitations of receptor binding assays that cannot measure the ligand-activated form of MR. Using MR-reactive antibodies and Western blot, we examined relative MR protein expression in rat brain and its potential autoregulation by corticosteroids. We found that MR protein expression is autoregulated in a negative fashion by adrenal steroids. Compared with GR, we see a more rapid regulation of MR, such that there is a substantial increase in MR protein within 12 h after adrenalectomy, whereas GR levels show very little increase until more than 24 h after adrenalectomy. Also, in contrast to GR, which has been found to be regulated by both MR and GR, adrenalectomy-induced increase in MR was prevented by treatment with the MR selective agonist, aldosterone, but not the GR selective agonist, RU28362. Interestingly, acute treatment of adrenalectomized rats with corticosterone

produced a significant decrease in whole-cell MR protein within 45 min, suggesting ligand-induced rapid degradation of MR. Chronic high levels of corticosterone also produced a significant decrease in MR protein levels below adrenal-intact rat levels. These results have important implications for previous studies that estimated the proportion of MR that are occupied *in vivo* by various circulating levels of corticosterone. Those studies compared available MR binding levels in adrenal-intact rats with 24-h adrenalectomized rats, with the assumption that there were no differences between the various conditions in total receptor expression. Those studies concluded that MR is nearly fully occupied by even the lowest circulating corticosterone levels. Given the 2- to 3-fold increase in MR protein that we have observed within 24 h after adrenalectomy, it is likely that those studies significantly overestimated the proportion of MR that were occupied by low basal corticosterone levels. These results support the prospect that MR as well as GR can participate in the transduction of phasic corticosteroid signals. (*Endocrinology* 143: 4184–4195, 2002)

CORTICOSTEROIDS HAVE A WIDE range of regulatory effects on brain function. Besides the classically described negative feedback effects of corticosteroids on hypothalamic-pituitary-adrenal (HPA) axis action (1), these effects include regulation of the levels of a number of neurotransmitters and their receptors, and regulation of signal transduction pathways that impinge on neuronal excitability, growth, and survival (2, 3). Some of these regulatory effects depend on tonic permissive or proactive effects of circulating corticosteroids, whereas others depend on the dynamic actions of stress-induced phasic increases in corticosteroid secretion (4, 5).

In many cases, corticosteroid effects are mediated by two closely related intracellular receptors, mineralocorticoid receptors (MR) and glucocorticoid receptors (GR). These receptors function as hormone-activated transcription factors and are members of a large family of structurally related nuclear hormone receptor proteins (6). Although hormone-activated MR and GR are believed to bind to the same DNA hormone response elements, they each can have unique protein-protein interactions. These interactions may be independent of DNA binding or subsequent to DNA binding, and consequently these two receptor types can transduce distinct effects in various corticosteroid target cells (7). The

two receptors also differ in their distribution within the brain. Although GR has a widespread distribution, with a notable enrichment in the hippocampus and paraventricular nucleus of the hypothalamus, MR is predominantly found in the hippocampus and is virtually absent from the paraventricular nucleus (8, 9).

An additional distinction between MR and GR is that MR has a 4- to 10-fold higher affinity than GR for the endogenous corticosteroids, corticosterone, or cortisol (10, 11). Consequently, the proportional occupancy of the two receptors is likely to differ across the range of circulating corticosteroid levels, with MR being occupied by hormone to a greater extent than GR. Researchers have estimated *in vivo* occupancy of these two receptors by various levels of corticosteroids, and indeed find that this is the case (10, 12, 13). These estimates have relied on the fact that only the unactivated form of the receptor can be measured in an *in vitro* radioligand exchange assay (14, 15). Thus, the amount of MR or GR receptor binding present in a tissue sample is believed to reflect the amount of unoccupied (available) receptors present. Using this strategy, researchers have noted that, during very low basal levels of corticosterone secretion in the rat, there is very little available MR receptor binding, even in MR-abundant hippocampal tissue (10, 12, 13). On the other hand, a substantial amount of GR receptor binding is available during these low circulating hormone levels. As hormone levels increase due to stress or circadian drive, the

Abbreviations: GR, Glucocorticoid receptor; HPA, hypothalamic-pituitary-adrenal; MR, mineralocorticoid receptor.

available MR binding becomes undetectable and the GR binding levels decrease. Interestingly, 24 h after adrenalectomy there is a substantial amount of MR binding in the hippocampus, whereas GR-binding levels remain similar to those observed in the adrenal-intact rat with low circulating corticosterone levels (16). These observations have led to the conclusion that MR is predominantly occupied (upward of 90%) by endogenous hormone, even under the lowest levels of basal secretion, whereas GR only becomes occupied by hormone as corticosterone increases during times of stress or during the circadian rise in basal secretion (17, 18). It is important to recognize that these estimates rely on the assumption that neither MR or GR protein levels have up-regulated within 24 h after adrenalectomy. This differential pattern of MR and GR occupancy profiles has led to the well-reasoned hypothesis that MR primarily mediates the tonic permissive effects of corticosteroids, whereas GR mediates corticosteroid effects that have a phasic influence (4, 5).

One prediction derived from the above hypothesis has been that regulation of MR-dependent actions of corticosteroids will take place primarily at the level of regulation of MR protein expression (18). However, because the majority of MR is believed to be tonically occupied by endogenous corticosteroids, the prediction has been that MR expression is not controlled in an autologous manner by corticosteroids, as is the case for GR (19). In support of this prediction, corticosteroid receptor binding studies have led to the conclusion that MR does not up-regulate after adrenalectomy (16, 20, 21). Although some studies have found alterations of MR binding following various experimental manipulations (22–26), these studies have been limited by the inability of the receptor binding assay to measure total receptor levels in the presence of circulating hormone levels. Thus, these studies have required that animals be adrenalectomized before tissue collection. There is the possibility that the stress of the surgery as well as the complete clearance of endogenous corticosteroids impacts on MR protein expression.

As an alternative approach to studying MR protein expression in rat brain, we have used the Western blot approach. We describe here two MR-specific antibodies that appear to fully recognize both the unactivated as well as the activated form of the receptor. Using these antibodies, we have reexamined the extent to which MR protein expression in rat brain is autoregulated by corticosteroids.

Materials and Methods

Subjects

Subjects were male Sprague Dawley rats (Harlan Labs, Indianapolis, IN) ranging in weight from 250–350 g at the start of treatment. Rats were housed in wire mesh hanging cages (three per cage) and had *ad libitum* access to laboratory rat chow and water. They were maintained on a 12-h light, 12-h dark schedule (lights on at 0700 h) and were allowed at least 2 wk from their date of arrival to acclimate to the colony before use. All procedures were approved by the University of Colorado's Animal Care and Use Committee.

Surgery

Animals were fully anesthetized with an ip injection of ketamine (60 mg/kg) and xylazine (13 mg/kg). Surgery was performed under aseptic conditions. Animals were adrenalectomized bilaterally through two

dorsal-lateral incisions just below the rib cage. Sham-operated animals received identical anesthesia, laparotomy, and wound closure. Except for adrenal-intact subjects, 0.9% saline was provided as drinking water postsurgery. All surgery occurred between 0800 and 01300 h (within the first half of the animals' light period).

Sham surgery does not affect hippocampal MR protein levels

To validate that sham-adrenalectomized rats were the appropriate control group to use for experiments using adrenalectomy, it was important to establish that sham surgery had no effect on MR protein levels. It is possible that the stress from sham surgery resulted in an increase in HPA axis activity in the hours following surgery that resulted in down-regulation of MR that was not present in adrenalectomized rats due to their lack of sustained corticosterone secretion. Therefore, hippocampal MR protein levels, as assessed by whole-cell Western blot (see below), were compared between a no-surgery control group of animals ($n = 6$) and sham-surgery rats ($n = 6$). The sham surgery rats were part of a subsequent experiment (see Results: Brain region comparison of MR and GR protein up-regulation). No-surgery rats were killed in pairs at the same time as the sham-surgery rats (1, 3, or 5 d after surgery). There was not a significant difference in mean (\pm SEM) hippocampal MR protein levels between the no-surgery (OD = 0.12 \pm .005) and sham-surgery groups (OD = 0.12 \pm .007).

Steroid administration

For acute corticosterone administration, rats were given ip injection with a dose of 2.5 mg/kg. Corticosterone was dissolved in vehicle (44% propylene glycol, 16% absolute ethanol, and 40% PBS vol/vol) for a final concentration of 2.5 mg/ml. For long-term (5 d) administration of corticosterone, corticosterone pellets weighing approximately 100 mg each were implanted sc in the back region of the animal (21). Minisomotic pumps (Alzet osmotic pump 2001; Alza Corp., Palo Alto, CA) that delivered 1 μ l/h of solution were used for long-term (5 d) administration of aldosterone (Sigma-Aldrich, St. Louis, MO) and RU28362 (gift of the former pharmaceutical company, Roussel-Uclaf, France). Aldosterone (10 μ g/ μ l) and RU28362 (10 μ g/ μ l) were dissolved in propylene glycol, and either compound alone or in combination were loaded into minisomotic pumps. Pumps were surgically implanted sc in the back region.

Tissue collection for immunoblot and receptor binding assays

Animals were killed by decapitation. Rapid dissection of the brain to obtain hippocampal formation (hippocampus), parietal cortex (cortex), and general diencephalic region (hypothalamus) was carried out on frosted glass kept cold on crushed ice. Dissected tissue samples were immediately frozen using dry ice and stored at -80 C.

Western blotting

Tissue was processed in such a manner as to extract MR and GR from the whole-cell (cytoplasm + nucleus; Ref. 14). Frozen tissue was homogenized in buffer (0.5–1.0 ml/100 mg tissue; 50 mM Tris; 6 mM MgCl₂; 10% sucrose; 1 mM phenylmethylsulfonyl fluoride; 1 mM leupeptin; 1 μ g/ml of pepstatin A; 1 μ g/ml antipain; 1 μ g/ml aprotinin; 1 μ g/ml of soybean trypsin inhibitor in distilled water, pH 7.2; and 0.5% sodium dodecyl sulfate). Samples, 100 μ g/25 μ l (unless noted otherwise), were mixed with 10 μ l 3.5 \times Laemmli's sample buffer and were denatured by boiling for 3 min. Twenty to 30 μ l of this sample were loaded onto each lane depending on lane size. Samples and prestained molecular weight markers (Bio-Rad Laboratories, Inc., Hercules, CA) were electrophoresed on 8% Tris-glycine polyacrylamide gels and then were electrophoretically transferred onto Immobilon-P (Millipore Corp., Marlborough, MA) membranes. The membranes were blocked for 30 min at room temperature with 10% Carnation dried milk in Tris-buffered saline with Tween-20 [TBST; Tris (20 mM), NaCl (137 mM), 0.05% Tween-20, pH 7.6]. Membranes were then washed with TBST buffer and incubated overnight at 4 C with one of two polyclonal anti-MR antibodies, MR 214 a rabbit polyclonal antibody directed against a rat MR cDNA derived peptide (bases 1275–1868); diluted 1:50,000 in Tris buffer; gift from Dr.

Stanley Watson, University of Michigan, Ann Arbor, MI) or MCRN-17 (a goat polyclonal antibody directed against a human/rat MR aminoterminal-derived peptide; diluted to 0.05 $\mu\text{g}/\text{ml}$ in Tris buffer; Santa Cruz Biotechnology, Santa Cruz, CA), or anti-GR antibody, BuGR2 (a mouse monoclonal antibody recognizing a sequence of rat GR near the hinge region (27); Affinity BioReagents, Inc., Golden, CO; 0.025 $\mu\text{g}/\text{ml}$ Tris buffer). After washing with TBST, membranes were incubated with horseradish peroxidase-linked antibody (antirabbit and antimouse antibody, Amersham Pharmacia Biotech, Arlington Heights, IL; diluted 1:3,000 in TBST; or anti-goat antibody, Sigma; diluted 1:15,000 in TBST) for 1 h at room temperature. Proteins were detected by incubating membranes with horseradish peroxidase chemiluminescent substrates (enhanced chemiluminescence reagent, Amersham Pharmacia Biotech) and exposure to autoradiography film (enhanced chemiluminescence hyperfilm, Amersham Pharmacia Biotech). Both MR-reactive antibodies, MR214 and MCRN-17, yielded a prominent band in brain tissue at approximately 110 kDa, which corresponds to the approximate molecular weight of rat MR (28). For both antibodies, this band was not present on lanes loaded with thymus tissue (data not shown), a tissue that expresses very little if any MR (29, 30), or when incubating the blot with primary antibody and the antigenic-specific peptide (see Fig. 1, for example, using MCRN-17 \pm blocking peptide). We have previously demonstrated using rat hippocampal tissue that the GR-reactive antibody BuGR2 produces a prominent immunoreactive band at approximately 97 kDa, which corresponds to the approximate molecular mass of rat GR (14).

Quantification of Western blot films

Measurement of OD of MR and GR-immunoreactive bands were determined using a computerized densitometry system (NIH Image). Measurements for total OD were taken from the darkest region from the band being quantified. Background OD was measured in a space (*i.e.* where no discernible protein band was present) immediately above or below that band within the same lane. Total OD was then subtracted from background OD, and this value was used for all analyses and figures containing Western blot data. Equivalent total sample protein (detergent-compatible assay; Bio-Rad Laboratories, Inc.) was loaded onto each lane, and samples from each treatment group were counter-balanced across lanes within a particular gel. On most occasions, samples were run on duplicate or triplicate gels and the mean OD for a particular sample used for subsequent analyses. All samples for a particular experiment were run on the same gel, or set of gels run at the same time, under the same conditions. Because exposure times, transfer efficiency, and other factors may vary between runs, specific immunoreactive OD values (relative MR or GR protein levels) were only directly compared within a set of gels run at the same time.

Receptor binding assay

Both cytosolic MR and GR available radioligand binding were simultaneously measured according to previously described methods (12). Briefly, frozen tissue was homogenized in binding homogenization buffer (0.5 ml/100 mg tissue; 10 mM Tris; 1 mM EDTA; 20 mM molybdc acid; 5 mM dithiothreitol; and 10% glycerin in double-distilled water, pH

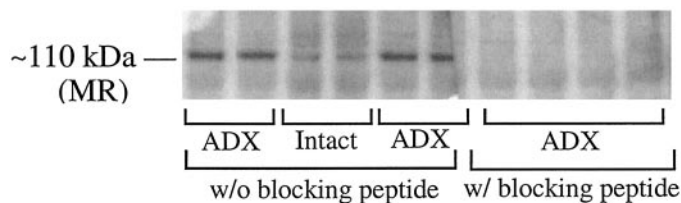


FIG. 1. Demonstration of MR-specific immunoreactivity on Western blot. Hippocampal whole-cell extracts from 5-d adrenalectomized rats (ADX) or adrenal-intact rats (Intact) were subjected to SDS-PAGE and electrophoretically transferred to membrane. The membrane was cut in half. One half was incubated with MCRN-17 antibody and the other half incubated with a mixture of MCRN-17 antibody and the peptide fragment of MR (0.05 $\mu\text{g}/\text{ml}$) used to generate MCRN-17 antibody.

7.4) using a motor-driven pestle and centrifuged at $105,000 \times g$ for 30 min at 4 C. This procedure is likely to extract soluble corticosteroid receptors from both the cytoplasmic and nuclear compartments but appears to exclude activated receptors that are tightly bound to chromatin (14). Three aliquots from each sample were incubated for approximately 20 h at 4 C in the presence of 15 nM [6,7- ^3H]dexamethasone (NEN Life Science Products, Boston, MA) and either 1) 0.5 μM RU 28362, 2) 10 μM dexamethasone, or 3) equivolume binding homogenization buffer. Dexamethasone has been found to bind MR with high affinity *in vitro*, and similar treatment effects on MR receptor binding level have been observed using either ^3H -dexamethasone or ^3H -aldosterone as radioligand (12). Absolute MR binding levels, however, may be about 30% lower when ^3H -dexamethasone is used as radioligand rather than ^3H -corticosterone (17). Incubation solutions were filtered through columns containing 1.25 ml of LH-20 Sephadex (Amersham Pharmacia Biotech, Piscataway, NJ) at 4 C. The eluent containing bound steroid was collected in scintillation vials, mixed with scintillation cocktail (Ultima Gold, Downers Grove, IL), and tritium radioactivity was counted on a scintillation counter (Packard Series 1600). MR binding was derived by subtracting radioligand binding in the presence of the GR specific ligand RU 28362 (MR binding + nonspecific binding) from binding in the presence of nonlabeled dexamethasone (nonspecific binding). GR binding was determined by subtracting radioligand binding in the presence of homogenization buffer (total binding) from binding in the presence of RU 28362. Specific binding is presented as femtomoles per milligram of cytosolic protein and comprised greater than 85% of total binding. Protein content was determined by the method of Bradford, with use of BSA as protein standard.

Immunohistochemistry

Animals were deeply anesthetized with ketamine (80 mg/kg) and xylazine (17 mg/kg), after which 0.1 ml heparin was injected into the left ventricle and allowed to circulate. Subjects were then exsanguinated by transcardiac perfusion of 0.01 M PBS + 0.1% heparin (pH 7.4; 50 ml in 1 min) and fixed by perfusion of heparinized 4% paraformaldehyde in 0.01 M phosphate buffer (pH 7.4; 500 ml in 10 min). Brains were postfixed in 4% paraformaldehyde for 48 h and were then sectioned (50 μm) on a Vibratome. For the detection of MR, floating sections were incubated overnight (4 C) in the anti-MR antibody MCRN-17 (Santa Cruz Biotechnology) at a 1:500 dilution in 0.01 M PBS (pH 7.5) plus 0.3% Triton X-100, and 0.5% BSA. The following day, sections were rinsed in PBS and incubated for 1 h in biotinylated secondary antibody at a 1:500 dilution (Vector Laboratories, Burlingame, CA). Immunoreactivity was detected with the Vectastain ABC method (Vector Laboratories), using diaminobenzidine (0.5 mg/ml Tris) and 0.005% nickel ammonium sulfate as chromagen. Brain sections were then mounted on slides, dried, delipidized, and coverslipped. Sections were photographed using a light microscope (Olympus Corp., Melville, NY; Model BX61) with Nomarski differential interference contrast and summation of 7–10 planes of focus ($\sim 1\text{-}\mu\text{m}$ steps; AnalySIS, Soft Imaging Systems, Lakewood, CO).

Corticosterone measurement

Corticosterone plasma concentration was measured by RIA. Plasma samples were diluted 1:50 in 0.01 M PBS and were heated at 75 C for 1 h to inactivate corticosteroid-binding globulin. Samples were incubated in duplicate overnight at 4 C with rabbit antiserum raised against corticosterone-21-hemisuccinate BSA (B21–42 or B3–163; Endocrine Sciences, Inc., Calabasas Hills, CA) and [1,2,6,7- ^3H]corticosterone (NEN Life Science Products). Antibody-bound corticosterone was separated from free steroid by centrifugation after addition of dextran-coated activated charcoal. Coefficients of variation between and within assays was less than 10%. Assay sensitivity was approximately 0.5 $\mu\text{g}/100$ ml.

Data analysis

Data for each experiment were analyzed using ANOVA and *post hoc* comparisons were made using the Tukey procedure ($\alpha \leq 0.05$). Data presented in figures are means \pm SEM.

Results

MR and GR protein up-regulation time-course 1 and 5 d after adrenalectomy

Animals ($n = 4$) were either sham-adrenalectomized, adrenalectomized and killed 24 h later, or adrenalectomized and killed 5 d later. Two of the sham-adrenalectomized rats were killed 24 h after surgery, and two were killed 5 d after surgery; data were pooled for all four sham-surgery animals. Separate Western blot assays were performed to quantify MR and GR on whole-cell extracts from hippocampus. Trunk blood was collected at the time of killing and assayed for corticosterone to confirm adrenalectomy.

Figure 2 shows a significant up-regulation of hippocampal

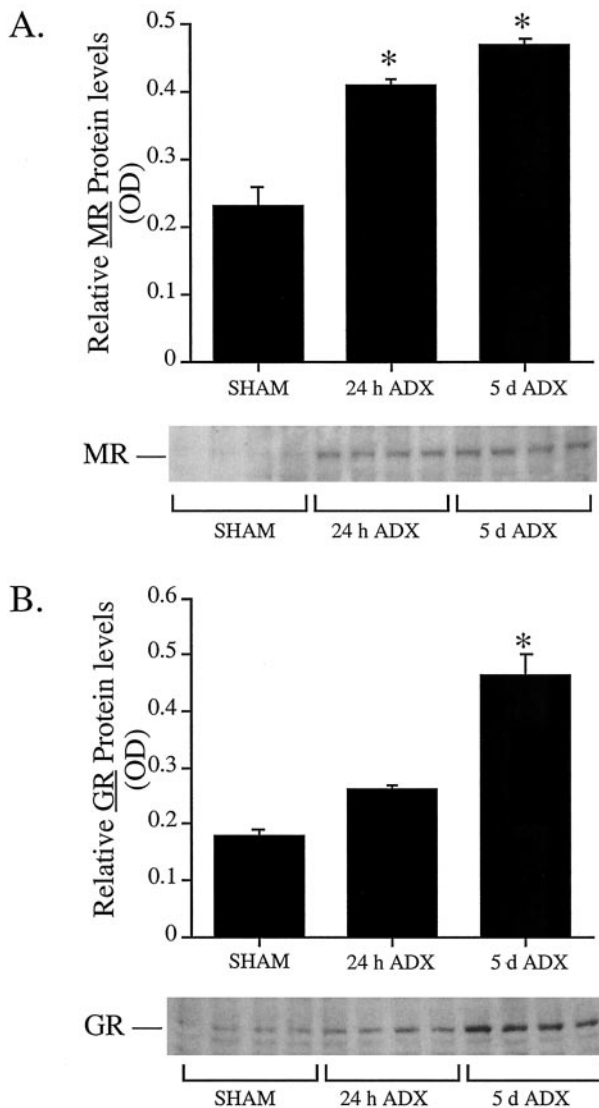


FIG. 2. MR and GR protein up-regulation time-course, 1 and 5 d after adrenalectomy. Relative hippocampal whole-cell MR (A) and GR (B) levels of sham-adrenalectomized, 24-h adrenalectomized, and 5-d adrenalectomized rats ($n = 4$) were determined by Western blot. The levels are labeled as "relative" reflecting that the OD levels were not standardized against a known quantity of receptor protein. Each lane of the blots contain a hippocampal sample from a different animal. *, Significant difference from sham group, $P < 0.05$, Tukey test.

MR protein after adrenalectomy ($P < 0.001$). *Post hoc* comparisons indicate that all of the significant up-regulation of MR occurred within 24 h after adrenalectomy. In contrast, whereas there was an overall increase in hippocampal GR protein with adrenalectomy ($P < 0.001$), the majority of the up-regulation occurred between d 1 and d 5 after adrenalectomy.

Brain region comparison of MR and GR protein up-regulation

The pattern of adrenalectomy-induced up-regulation of MR and GR protein was reexamined in hippocampus, as well as examined in parietal cortex and hypothalamus/diencephalon. For this experiment, separate groups of rats ($n = 6$) were adrenalectomized either 1, 3, or 5 d before they were killed. A sham-surgery control group ($n = 6$) was included; two rats each were killed 1, 3, or 5 d post surgery. Each tissue region was analyzed on separate sets of gels/blots; thus, this data set does not provide for a direct comparison of relative MR and GR protein levels between different brain regions but rather provides for a region by region comparison of the time-course of receptor up-regulation.

The pattern of adrenalectomy-induced corticosteroid receptor up-regulation was similar for all three brain regions (Fig. 3) and was similar to the pattern seen in the first experiment. Thus, for all three brain regions there was a large up-regulation of MR present by 24 h after adrenalectomy, and this degree of up-regulation was no greater after 5 d of adrenalectomy. An up-regulation of GR also occurred after adrenalectomy; however, the majority of the up-regulation occurred between d 1 and d 5 after adrenalectomy. There was some up-regulation of GR within 24 h after adrenalectomy, but this only reached statistical significance for the hypothalamus/diencephalon tissue.

Studies using receptor binding measures have consistently found that MR levels in the hippocampus are generally higher than levels in other brain regions (10, 12). To compare relative differences in hippocampal and cortical whole-cell homogenates, samples from sham or 5-d adrenalectomy groups were measured in a side-by-side fashion on the same Western blot. To consistently detect the low level of MR present in sham adrenalectomized rats, it was necessary to load double the normal amount of tissue extract protein on each lane ($150 \mu\text{g}/\text{lane}$). For both sham-adrenalectomized rats and adrenalectomized rats, there was a significantly greater amount of MR protein in hippocampal tissue than in cortical tissue (Fig. 4).

MR up-regulation within 24 h after adrenalectomy

Because a large degree of up-regulation of MR was observed 24 h following adrenalectomy, a more detailed early time-course was examined. Four groups of animals ($n = 5$) were included in this study. One group of animals was sham-adrenalectomized, and the three remaining groups were adrenalectomized and killed 12, 18, or 24 h later. Hippocampal tissue was processed for whole-cell extract and assayed for MR and GR levels by immunoblotting.

Figure 5 shows a significant effect of adrenalectomy on hippocampal MR protein ($P < 0.001$). *Post hoc* analysis in-

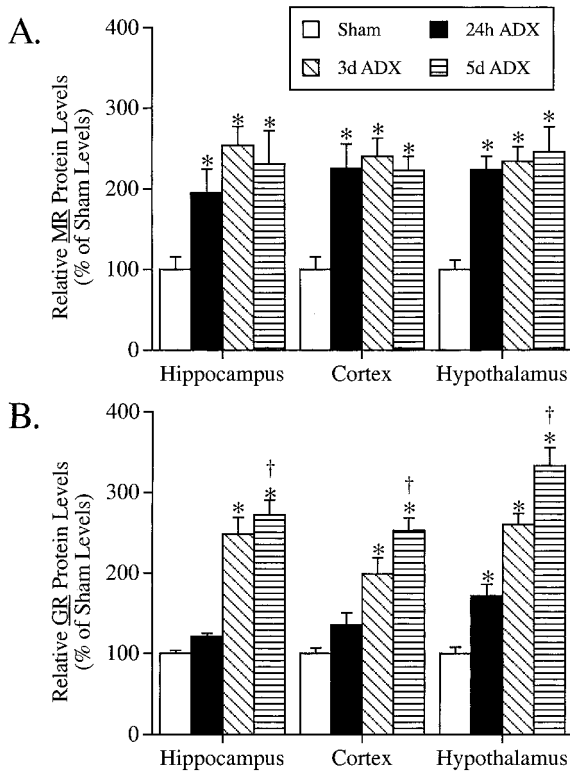


FIG. 3. Brain region comparison of MR and GR protein up-regulation. Relative whole-cell MR (A) and GR (B) levels of sham-adrenalectomized, 24-h adrenalectomized, 3-d adrenalectomized, and 5-d adrenalectomized rats ($n = 6$) were examined by Western blot in tissue taken from the hippocampal formation, parietal cortex and hypothalamus/diencephalon. All data for a particular brain region were expressed as a percent of the sham-adrenalectomized group mean for that brain region. *, Significant difference from sham group, $P < 0.05$, Tukey test. †, Significant difference from the 24-h adrenalectomized group, $P < 0.05$, Tukey test.

indicated that MR levels were significantly increased within 12 h following adrenalectomy and appeared to reach plateau levels by 18 h after adrenalectomy. GR protein levels, on the other hand, did not increase significantly within 24 h after adrenalectomy.

Effect of acute corticosterone treatment on MR protein levels and available MR receptor binding

To rule out the possibility that our Western blot procedure was not able to fully detect MR in the presence of corticosterone (*i.e.* the activated form of MR), relative hippocampal MR protein levels were examined in 24-h adrenalectomized animals that had been acutely treated with corticosterone before they were killed. Four groups of rats were examined: sham-adrenalectomy ($n = 6$), adrenalectomy + vehicle injection ($n = 6$), adrenalectomy + 10 min corticosterone ($n = 9$), and adrenalectomy + 45 min corticosterone ($n = 6$). A larger number of subjects were included in the 10 min corticosterone treatment group because it was thought that such an early postinjection interval might result in incomplete drug distribution in a few animals, thus excluding them from analysis based on low plasma corticosterone. However, all of the animals in this group had high plasma corticosterone, so

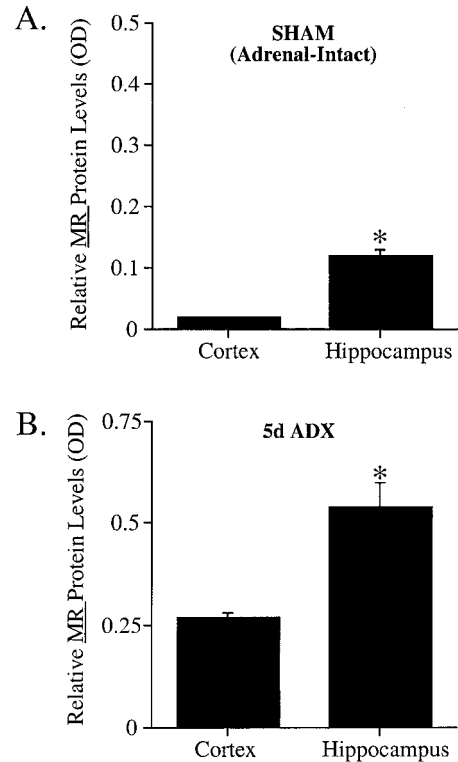


FIG. 4. Direct comparison of relative MR protein levels in cortex and hippocampus. Relative whole-cell MR in the parietal cortex and hippocampal formation of rats ($n = 6$) were compared side by side on Western blot. Tissue was collected from either sham adrenalectomized rats (A) or 5-d adrenalectomized rats (B). *, Significant difference from cortical levels, $P < 0.05$, Student's *t* test.

none were excluded. Hippocampus from one hemisphere per brain was processed for whole-cell extraction and MR protein levels measured with Western blot. The hippocampus from the other hemisphere was processed for cytosol extraction and available MR receptors measured by radioligand receptor binding.

As seen in the previous experiments, there was a significant increase in MR protein levels evident 24 h after adrenalectomy (Fig. 6A). Ten minutes of corticosterone treatment had no effect on the amount of MR detected by Western blot, whereas 45 min of corticosterone produced a significant decrease in MR levels, although the level of detected MR was still substantially greater than that in sham-adrenalectomized rats. Because whole-cell extracts were used for the Western blot procedure, this result suggests that MR protein levels were decreased within 45 min, but not 10 min after corticosterone treatment. Equivalent results were obtained regardless of whether blots were probed with the MCRN-17 antibody (Fig. 6A) or the MR 214 antibody (data not shown). Both 10 and 45 min of corticosterone treatment reduced available MR binding levels to nearly undetectable levels (Fig. 6B). Because the receptor binding assay measures only the unactivated form of MR present in tissue cytosol, this result suggests that nearly all of MR were activated both 10 and 45 min after corticosterone injection. The dose of corticosterone injected (2.5 mg/kg ip) produced plasma corticosterone levels (Fig. 6C) that after 10 min were superphysiologic (>75

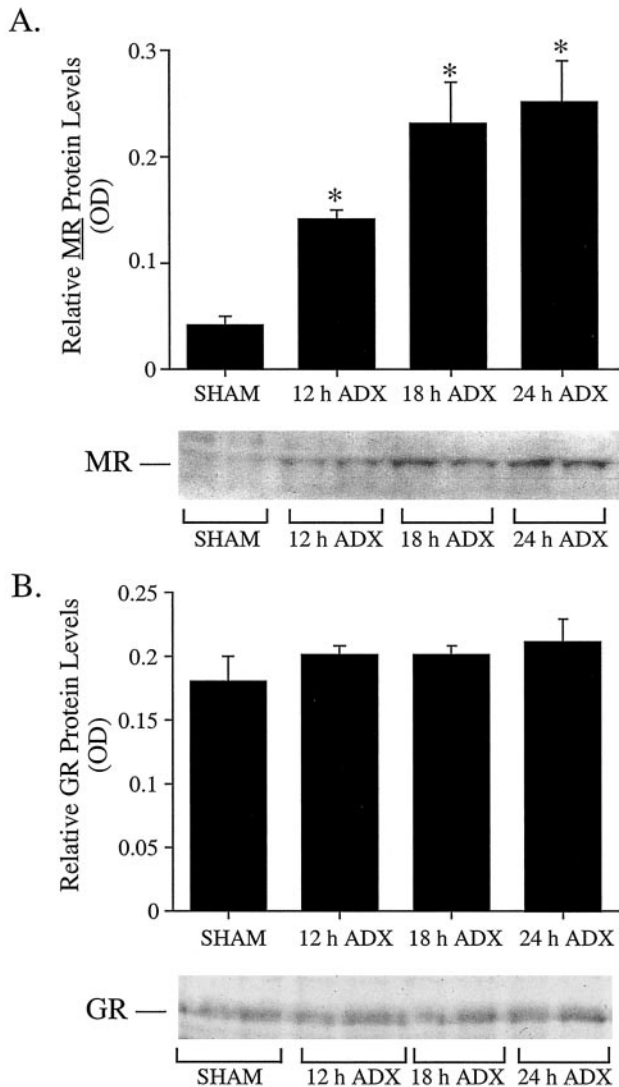


FIG. 5. MR up-regulation within 24 h after adrenalectomy. Relative hippocampal whole-cell MR (A) and GR (B) levels of sham-adrenalectomized rats or rats 12 h, 18 h, or 24 h after adrenalectomy (n = 5) were determined by Western blot. The representative blots show data from two animals in each group. *, Significant difference from sham group, $P < 0.05$, Tukey test.

$\mu\text{g}/100 \text{ ml}$) and by 45 min were in the high stress corticosterone secretion range ($\sim 50 \mu\text{g}/100 \text{ ml}$).

Effect of adrenalectomy and acute corticosterone treatment on hippocampal MR immunohistochemistry

To examine whether the MCRN-17 antibody is able to recognize both the unactivated and activated form of MR in the nondenatured form, immunohistochemistry was performed on brain sections taken from rats receiving the same treatments as described in the previous experiment. Prominent MR immunostaining was present in the principal cells of the dentate gyrus (granule cells) and the hippocampus (pyramidal cells). MR immunostaining was especially dark within the CA2 region of the hippocampus (Fig. 7). For tissue from adrenalectomized animals, MR immunostaining appeared prominent in both cytoplasm and nucleus of most

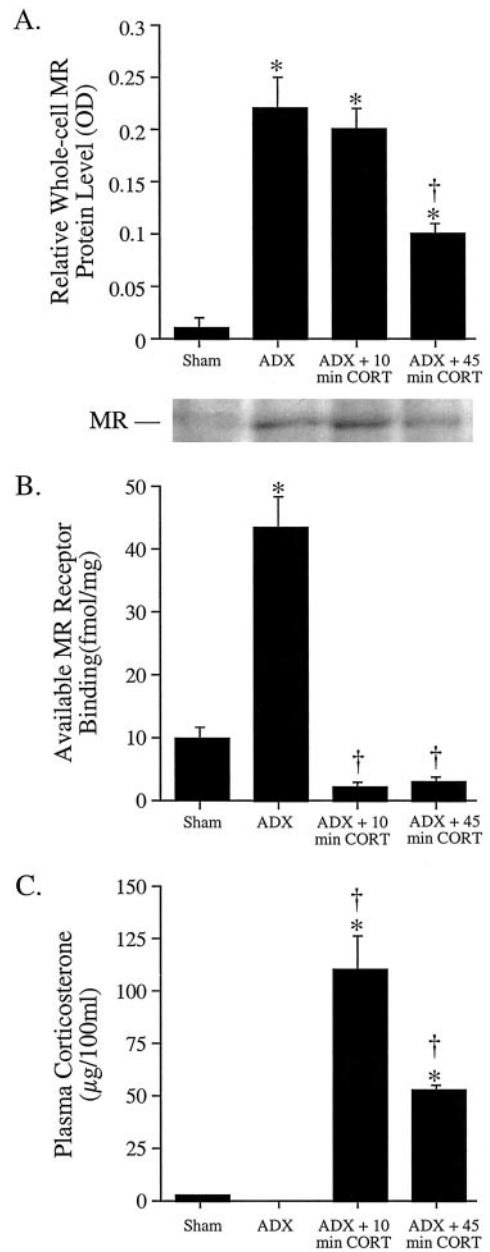


FIG. 6. Effect of acute corticosterone treatment on MR protein and available receptor binding levels. Relative hippocampal whole-cell MR levels of sham-adrenalectomized rats (n = 6), 24-h adrenalectomized rats (n = 6), or 24-h adrenalectomized rats treated with high dose corticosterone (2.5 mg/kg ip) 10 min (n = 9) or 45 min (n = 6) before death were determined by Western blot (A). The representative blot shows data from a single animal in each treatment group. Hippocampal available MR cytosolic binding levels were determined in the same animals (B). Corresponding plasma corticosterone levels in trunk blood is shown in C. *, Significant difference from sham group, $P < 0.05$, Tukey test. †, Significant difference from the 24-h adrenalectomized group, $P < 0.05$, Tukey test.

cells. This was most evident in the medial portion of CA1 and the CA2 region of the hippocampus (Figs. 7B and 8, A and B). Ten minutes after corticosterone treatment, MR immunostaining was predominantly nuclear and remained predominantly nuclear 45 min after corticosterone treatment (Fig. 8, C–F).

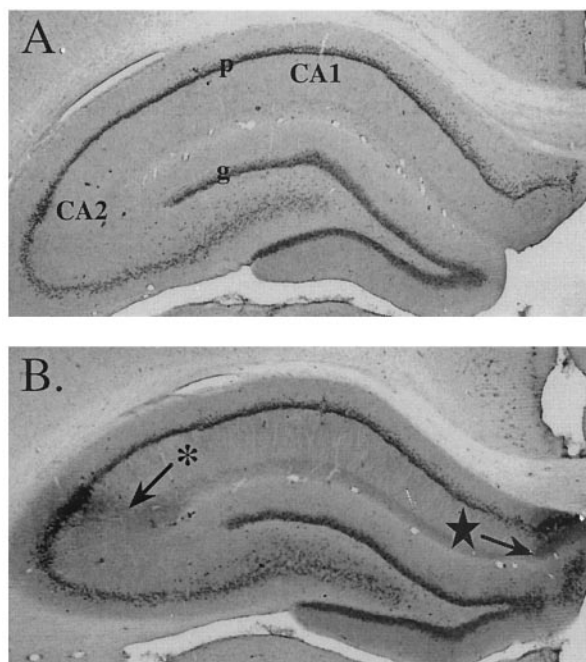


FIG. 7. Low magnification of MR immunohistochemistry in hippocampus of a sham-adrenalectomized or adrenalectomized rat. Representative photomicrographs ($\times 4$ magnification) are presented of MR immunostaining (MCRN-17 antibody) in hippocampal formation of a sham-adrenalectomized rat (A) or a 24-h adrenalectomized rat (B). Note the overall prominent immunostaining in pyramidal cells (p) of the hippocampus and granule cells (g) of the dentate gyrus, as well as the especially strong immunostaining of the CA2 region of the hippocampus (A). Also, note the pronounced cytoplasmic staining of medial CA1 (star) and CA2 (asterisk) pyramidal cell apical dendrites (arrows) in the adrenalectomized rat (B).

Adrenalectomy-induced up-regulation of MR protein is prevented by MR selective agonist treatment

This experiment examined whether treatment of rats at the time of adrenalectomy with a selective MR agonist (aldosterone) and/or a selective GR agonist (RU28362) would be able to prevent the increase in hippocampal MR protein levels. Five groups of rats ($n = 5$) were included in this experiment. One group of rats was sham-adrenalectomized, and the other four groups were adrenalectomized. At the time of adrenalectomy, three groups of rats were given replacement treatments consisting of miniosmotic pumps (sc) that delivered either aldosterone (10 $\mu\text{g}/\text{h}$), RU28362 (10 $\mu\text{g}/\text{h}$), or both aldosterone and RU28362 (10 μg each/h). All animals were killed 5 d after surgery. Hippocampal tissue was processed for whole-cell extract and assayed for MR levels by immunoblotting.

Replacement treatment with aldosterone or aldosterone and RU28362 prevented the adrenalectomy-induced up-regulation of MR protein (Fig. 9). Treatment with RU28362 alone was ineffective in preventing MR protein up-regulation.

Down-regulation of MR protein with long-term high-dose corticosterone treatment

This experiment examined whether long-term treatment with a high dose of corticosterone could produce a down-regulation of MR protein levels as has been suggested pre-

viously from receptor binding studies (21). Two groups of rats were studied ($n = 6$), one that was sham adrenalectomized and one that was adrenalectomized and given high dose corticosterone replacement (four 100-mg corticosterone pellets, sc) at the time of surgery. All animals were killed 5 d after surgery. Hippocampal tissue was processed for Western blot analysis of MR as described in the above experiments. Twice the normal concentration of protein was used (150 $\mu\text{g}/\text{lane}$) to maximize observation of the low MR levels in adrenal-intact and chronic corticosterone-treated rats. Spleen and thymus tissue were removed and weighed at the time the rats were killed as an indirect measure of the extent of corticosteroid excess produced by the corticosterone pellet treatment. Five-day treatment with corticosterone pellets produced nearly a 50% reduction in spleen and thymus weights relative to sham adrenalectomized rats (data not shown). This treatment also produced a significant decrease in hippocampal MR protein levels (Fig. 10).

Discussion

Western blot on whole-cell tissue extracts revealed that both MR and GR undergo significant up-regulation in brain tissue after adrenalectomy. However, the time-course of this response was very different for the two receptor types. There was a substantial increase in MR, but not GR protein levels by 12 h after adrenalectomy (Fig. 5). No additional up-regulation of MR protein was seen after 24 h of adrenalectomy. In contrast, there was very little GR up-regulation within the first day after adrenalectomy, but GR levels increased considerably over the subsequent 4 d after adrenalectomy (Figs. 2 and 3).

In the case of MR, this adrenalectomy-induced up-regulation of receptor protein levels is contrary to reports of receptor binding studies that consistently conclude that MR does not up-regulate after adrenalectomy (16, 20, 21). This discrepancy can be explained in light of the limitations of the corticosteroid receptor binding methodology. Because only the unactivated form of corticosteroid receptors can be measured in a cytosolic receptor binding assay (12, 15, 31), assessment of total tissue receptor protein levels requires adrenalectomy of the animal to provide clearance of endogenous corticosterone. Previous studies have assumed that any increase in MR binding within the first 24 h after adrenalectomy is due simply to recycling of existing receptors to an unactivated state as endogenous corticosterone clears from the system (16). This conclusion seemed reasonable because of the observation that the closely related molecule, GR, shows an increase in receptor binding level that is only first clearly evident more than 24 h after adrenalectomy. In addition, GR binding levels progressively increase for several days following adrenalectomy (21, 32).

The rapid up-regulation of MR protein observed in this study was evident across several different brain regions (Fig. 3). Also, consistent with results from MR receptor binding assays and MR mRNA expression studies (9, 10, 12, 33), we observed a greater level of MR within the hippocampus than the cortex (Fig. 4). This was true regardless of whether the levels were measured in the adrenal-intact or adrenalectomized animal.

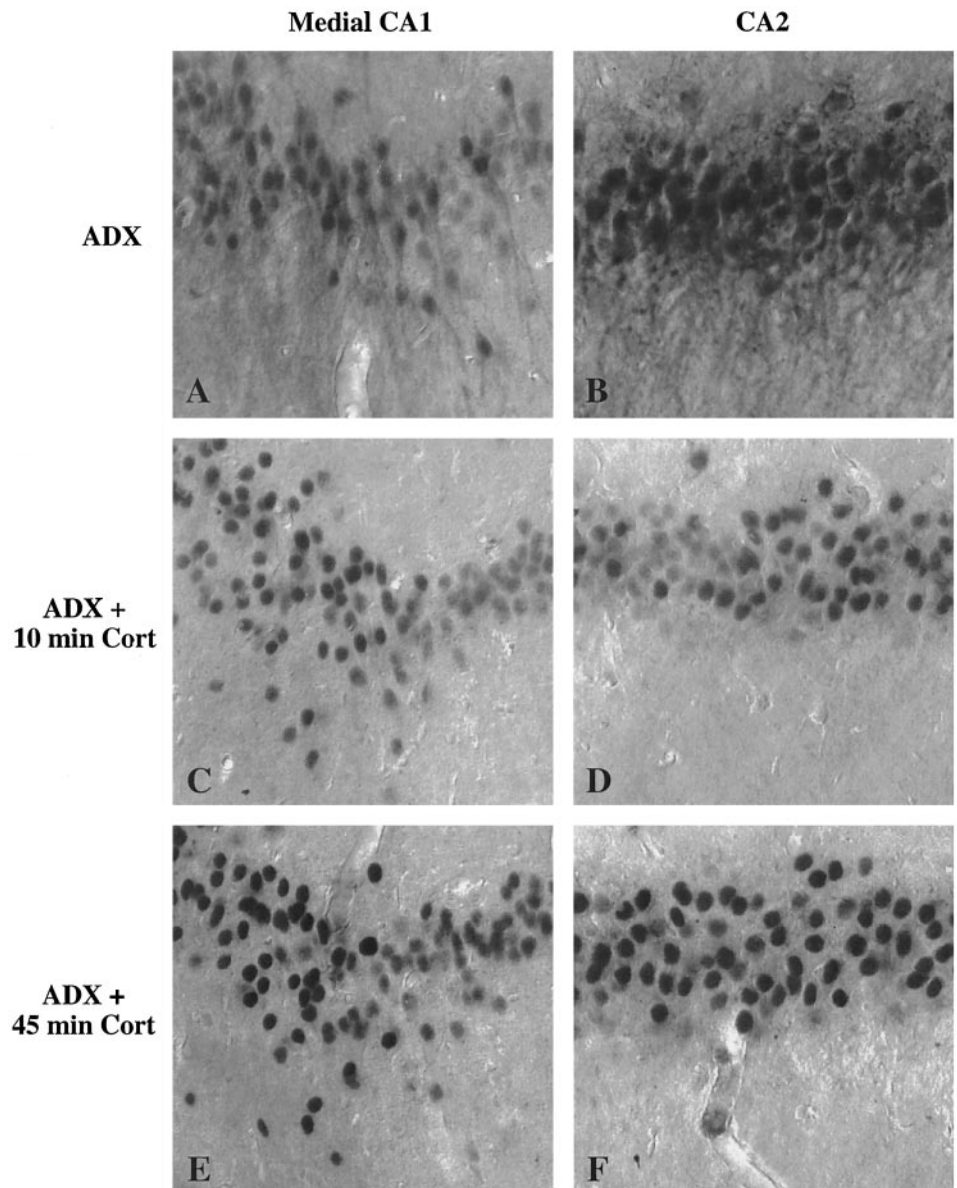


FIG. 8. Higher magnification of MR immunohistochemistry in hippocampus of adrenalectomized rats \pm 10 min or 45 min of corticosterone treatment. Representative photomicrographs ($\times 20$ magnification) are presented of MR immunostaining (MCRN-17 antibody) in medial CA1 (A, C, and E) or CA2 (B, D, and F) of a 24-h adrenalectomized rat (A and B), a 24-h adrenalectomized rat treated with corticosterone (2.5 mg/kg ip) 10 min before death (C and D), or a 24-h adrenalectomized rat treated with corticosterone (2.5 mg/kg ip) 45 min before death (E and F). Note both the prominent nuclear and cytoplasmic staining (especially evident in apical dendrites) of pyramidal cells in the adrenalectomized rat (A and B), and the almost exclusive nuclear pattern of immunostaining in both of the corticosterone-treated rats (C–F). Images were captured under Nomarski differential interference contrast conditions. The final image is a collapsed composite of 7–10 planes of focus (approximately 1- μ m steps).

The up-regulation of MR protein appears to be a response to the absence of MR activation by endogenous adrenal steroids (corticosterone and/or aldosterone) because the MR selective agonist aldosterone prevented MR up-regulation, whereas a GR-selective agonist, RU28362, did not (Fig. 9). This dose of RU28362 has been found in a previous study to occupy the majority of hippocampal GR (34).

Chronic high levels of corticosterone also led to a down-regulation of adrenal-intact levels of MR (Fig. 10). Thus, endogenous corticosterone via MR appears to tonically inhibit MR protein expression levels, but there is room for further down-regulation of MR with chronically elevated corticosterone exposure. There also appears to be a rapid down-regulation (within 45 min) of MR protein that occurs after a high bolus dose of corticosterone (Fig. 6; see below).

The adrenalectomy-induced up-regulation and chronic corticosterone treatment-induced down-regulation of MR are likely to be a result of autologous regulation of MR gene

expression. Several studies have observed increases in MR mRNA after adrenalectomy that are prevented by adrenal steroid replacement treatment (35, 36). Moreover, adrenalectomy-induced increases in MR mRNA in the hippocampus have been observed as early as 6 h after adrenalectomy (37). A down-regulation of MR mRNA has also been observed after chronic treatment with high doses of corticosterone (38). However, neither MR mRNA or protein expression in epithelial cells appear to be dependent on adrenal steroid levels (39), so autoregulation of MR gene expression may vary depending on cell phenotype. A number of studies have also found changes in MR gene expression in rat brain with various stress paradigms (40–43). Whether gene expression in these cases is mediated primarily by stress-induced alterations in corticosteroid levels or depends on other factors remains to be determined. Interestingly, progesterone has been shown to increase MR mRNA levels in rat hippocampus independent of direct interactions with MR

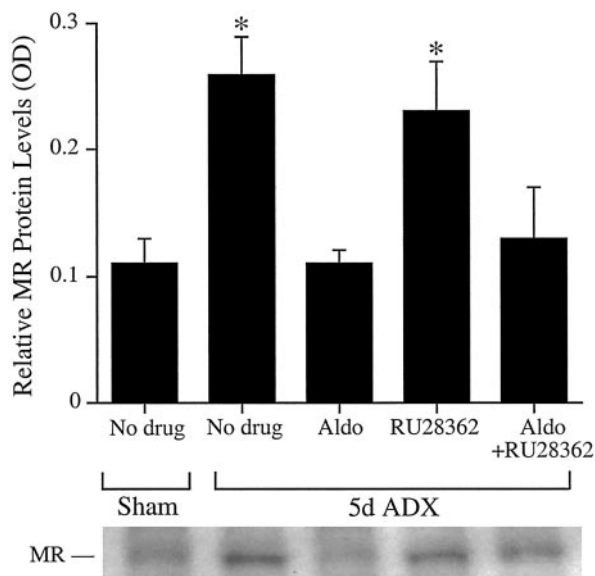


FIG. 9. Adrenalectomy-induced up-regulation of MR protein is prevented by MR selective agonist treatment. Relative hippocampal whole-cell MR levels of sham-adrenalectomized rats or 5-d adrenalectomized rats given replacement treatment at the time of surgery with either the MR selective agonist aldosterone (10 μ g/h, miniosmotic pump, sc), the GR selective agonist RU28362 (10 μ g/h, miniosmotic pump, sc), or combined aldosterone and RU28362 treatment were determined by Western blot (n = 5). The representative blot shows data from a single animal in each treatment group. *, Significant difference from sham group, $P < 0.05$, Tukey test.

(44). Moreover, Gesing *et al.* (25) have found that forced swimming leads to an increase in MR protein. This effect appears to depend on CRH and may be independent of changes in MR mRNA.

The autologous regulation of MR contrasts somewhat with GR, which exhibits both GR- and MR-dependent corticosteroid regulation. Thus, in the case of GR, the adrenalectomy-induced up-regulation of GR binding levels or mRNA can be blocked not only with the use of GR selective agonists for replacement treatment (20, 34), but also to some extent by very low doses of corticosterone or moderate doses of aldosterone that are believed to primarily activate MR (21, 35, 45). In addition, chronic treatment of rats with the selective MR antagonist spironolactone produced an increase in hippocampal GR mRNA (46). Consequently, GR expression appears to be inhibited by corticosterone acting through both MR and GR, whereas MR expression appears to be inhibited by corticosterone through an exclusive MR-dependent mechanism.

We also observed in this study a rapid decrease in MR protein levels after an injection of high-dose corticosterone. Due to the rapidity of this effect, it is not likely to be a result of decreased MR gene expression. Instead, the rapid decrease in MR protein levels suggests the possibility that this is an example of ligand-induced rapid degradation of MR. There is growing precedence for intercellular signals to induce rapid breakdown of specific target cell proteins through ubiquitination and proteasome-mediated degradation (47). Recent studies have found that another nuclear hormone receptor, estrogen receptor α , undergoes rapid ligand-

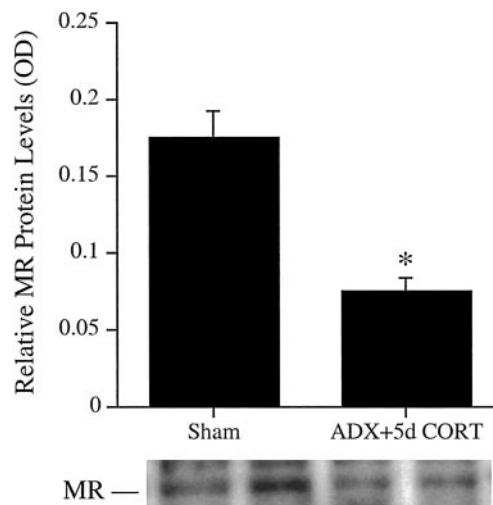


FIG. 10. Down-regulation of MR protein with long-term high dose corticosterone treatment. Relative hippocampal whole-cell MR levels of 5-d sham-adrenalectomized rats or 5-d adrenalectomized rats given replacement treatment at the time of surgery with high dose corticosterone (four pellets containing approximately 100 mg corticosterone, sc) were determined by Western blot (n = 6). The representative blot shows data from two animals in each treatment group. *, Significant difference from sham group, $P < 0.05$, Student's *t* test.

induced ubiquitination, and proteasomal degradation (48, 49). This degradative process may also account for the instability of MR that has been noted by others (50).

The potential for rapid ligand-induced degradation of MR may indicate that MR in general has a more rapid turnover rate than GR. This is an interesting possibility given the discrepancy reported between MR and GR mRNA levels and MR and GR binding levels. Levels of MR mRNA in the hippocampus are typically higher (up to five times) than the level of GR mRNA (51). This is in contrast to receptor binding studies that consistently observe the opposite relationship (*i.e.* concentrations of GR 2–3 times higher than MR; Refs. 10 and 12). One possible explanation is that the MR protein may have such a short half-life in the presence of corticosterone that the rate of transcription (or the life of the transcript) has to be very high to maintain a sufficient expression of MR protein.

An alternative explanation for the rapid corticosterone-induced decrease in MR protein levels that we observed is that some or all of the activated MR was not detected by our Western blot procedure. For several reasons, this does not appear to be a likely possibility. First, our tissue preparation uses homogenization in the presence of detergent. We have previously demonstrated for GR that these tissue homogenization conditions are sufficient to extract receptor from both the nucleus and cytoplasm, regardless of activation state (14). Second, because electrophoresis was conducted under denaturing conditions, it is expected that our primary antibody was able to recognize the receptor protein present on Western blot membranes, regardless of the protein's conformation before denaturation. Moreover, we obtained the same result when probing blots with two different primary antibodies directed against two different regions of MR. Third, we did not see a decrease in MR protein levels with

Western blotting 10 min after corticosterone treatment, although receptor binding measures (Fig. 6B) and immunohistochemistry (Fig. 8, C and D) indicate that the majority of MR had already become activated and accumulated within the nucleus at this time-point. Our immunohistochemistry results are consistent with other (52–55) but not all reports (17, 56–58), indicating that in the absence of ligand MR is distributed in both the cytoplasm and nucleus but in the presence of ligand MR is localized predominantly in the nucleus. The strong nuclear MR immunoreactivity evident in the hippocampus of adrenalectomized rats treated acutely with a saturating dose of corticosterone (Fig. 8) further supports the ability of the MR N-17 antibody to be able to recognize both the activated and unactivated form of MR.

The significance of determining that MR undergo up-regulation following adrenalectomy has important implications for current beliefs concerning the concentration of corticosteroid receptors present and the percentage of receptors occupied under different hormonal contexts. We and others have noted that the amount of MR binding detected in adrenal-intact animals is quite low relative to the binding levels measured in adrenalectomized animals. Even if tissue is obtained from animals killed with very low circulating corticosterone levels (*i.e.* unstressed animals at the trough of their HPA axis circadian cycle), we obtain a level of MR binding (10–30 fmol/mg tissue protein) that is substantially less than 24-h adrenalectomy levels (75–125 fmol/mg protein; Refs. 12 and 13). These relative available MR binding levels are the primary basis for concluding that the majority of MR (upwards of 90%) are occupied by endogenous corticosterone, even under conditions of the lowest levels of hormone secretion (10, 12). However, if the receptor binding levels present in animals 24 h after adrenalectomy reflect a substantial degree of receptor up-regulation, then the previous estimates of receptor occupancy are largely inflated. Based on our Western blot measures we see approximately a 2- to 3-fold increase in MR protein levels after adrenalectomy. Thus, the proportion of MR occupied by very low basal levels of corticosterone may be approximately half as much as previously estimated. It is interesting to note that we were able to measure as much as 50 fmol/mg protein of MR binding in the hippocampus of adrenal-intact rats that were acutely treated with dexamethasone (12). Because dexamethasone potently shuts down endogenous HPA axis activity at the level of the pituitary, this treatment produces a virtual absence of endogenous corticosterone in brain tissue (*i.e.* pharmacological adrenalectomy; Ref. 59). Thus, the MR binding levels that we measured under this condition may be more representative of the total amount of MR present in the hippocampus of the adrenal-intact rat. This would suggest that, at the trough of the circadian cycle, the proportion of MR that are unoccupied is more on the order of 50%.

It should be pointed out that a less than near maximal occupancy of MR under low basal corticosterone secretion is consistent with measures of the relative affinities of rat hippocampal MR and GR for corticosterone. Studies report a 4- to 10-fold difference in affinity between MR and GR for corticosterone (10, 11). We and others see very little difference in the amount of available GR binding in the brains of adrenal-intact rats killed at a time when basal corticosterone

levels are very low compared with 24-h adrenalectomized rats (10, 12, 13). Thus, it appears that under these low hormone conditions that less than 10% of GR are activated by endogenous corticosterone (17). However, based on the equation of mass action kinetics adapted for ligand-receptor interactions (60), an 81-fold difference in dissociation constant between MR and GR would be required in order for MR to be 90% occupied by a concentration of corticosterone that occupies only 10% of GR (see also Ref. 61).

The prospect that a significant proportion of MR are unoccupied during the trough of HPA axis activity allows for the possibility that MR can transduce phasic changes in corticosterone. This may explain the ability of acute treatment with a selective MR antagonist at this time of day to produce an enhanced magnitude of a corticosterone response to acute stress. We have observed such an effect when challenging rats with either a low intensity stressor (novel environment) or with a stressor (restraint) to which they have developed habituation (62, 63). We have also found that acute MR antagonist treatment combined with GR antagonist treatment led to an enhanced HPA axis response in rats exposed for the first time to a moderate intensity stressor, restraint stress (64). Ratka *et al.* (65) also observed that acute treatment with an MR antagonist produced a sustained corticosterone response to brief openfield exposure. Thus, there may be an acute phasic corticosterone negative feedback signal that is at least partially transduced by MR. This MR-mediated feedback component may be especially important under conditions of mild stress when the total amount of corticosterone secreted is relatively low and results in minimal GR activation (62).

We also note that if the majority of MR are always occupied by endogenous ligand, then it is hard to explain how chronic exogenous corticosterone treatment is able to lead to an additional down-regulation of MR gene and protein expression. Further, it has been difficult to explain the evolution and functional significance of a receptor that is putatively always occupied to near saturating levels by endogenous ligand. Based on the results of this study, we suggest that MR under some hormonal conditions may play a more dynamic role than previously believed in mediating phasic corticosterone effects during acute stress, such as the negative feedback effects of corticosterone on HPA axis activity.

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