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Evidence for Mineralocorticoid Receptor Facilitation of Glucocorticoid Receptor-Dependent Regulation of Hypothalamic-Pituitary-Adrenal Axis Activity*

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ABSTRACT

These studies further evaluated the relative role of mineralocorticoid (type I) and glucocorticoid (type II) receptors in mediating corticosteroid feedback regulation of the hypothalamic-pituitary-adrenal (HPA) axis. Acute treatment of rats with the selective mineralocorticoid receptor antagonist, RU28318 (50 mg/kg sc), produced elevated basal corticosterone levels in the morning, but had no effect on basal corticosterone levels in the evening or on restraint stress corticosterone levels at either time of day. Acute treatment with the selective glucocorticoid receptor antagonist, RU40555 (30 mg/kg sc) had no effect on basal or restraint stress corticosterone levels at either time of day. However, combined treatment with RU28318 and RU40555 produced an elevation of evening basal corticosterone levels (and morning basal on one occasion) and produced an increase in corticosterone levels during and after stress at both times of day. In a separate experiment conducted in the morning, the combined RU28318 and RU40555 treatment also produced elevated ACTH responses during restraint stress. Based on available corticosteroid receptor measures, the RU28318 treatment was estimated to selectively occupy approximately 85% of mineralocorticoid receptors in rat brain, whereas the RU40555 treatment was estimated to selectively occupy approximately 50% of glucocorticoid receptors in rat brain. We conclude that mineralocorticoid receptor activation is necessary and sufficient to maintain low basal corticosterone levels during the circadian trough, whereas glucocorticoid receptor activation is necessary to constrain corticosterone secretion during the circadian peak or during acute stress. However, even during the circadian peak or acute stress, mineralocorticoid receptor activation plays an important role in facilitating the glucocorticoid receptor dependent regulation of HPA axis activity by corticosterone. (*Endocrinology* **139:** 2718–2726, 1998)

ACTIVITY of the hypothalamic-pituitary-adrenal (HPA) axis is tightly and dynamically regulated by corticosteroid negative feedback (1). The inhibitory effects of corticosterone on HPA axis function are believed to be transduced primarily by intracellular steroid receptor proteins that function as hormone-activated transcription factors. The existence of two different corticosteroid receptors was first suggested in pharmacological studies nearly 20 yr ago (2) and has been confirmed with the more recent cloning of the complementary DNA for these two closely related receptors (3, 4). The lower affinity corticosterone receptor, the glucocorticoid receptor (type II corticosteroid receptor), is found in fairly high concentrations throughout the brain and the pituitary (5–7). The higher affinity corticosterone receptor, the mineralocorticoid receptor (type I corticosteroid receptor), also serves as the aldosterone receptor in the kidney (8). Interestingly, the highest concentration of mineralocorticoid receptors in the body appears to be in the hippocampal formation, with substantially lower levels of mineralocorticoid receptors scattered throughout the brain and pituitary (3, 9). Thus, the two receptor subtypes for corticosterone are located in target tissues believed to contribute importantly to negative feedback regulation of HPA axis function (*i.e.* throughout the brain and pituitary).

Two separate groups of investigators have been especially instrumental in defining the relative roles of these two receptor subtypes in mediating corticosteroid feedback regulation of the HPA axis. Dallman and colleagues have focused on the role of glucocorticoid and mineralocorticoid receptors in regulating the basal and stress-induced activity of the HPA axis and how those roles change across the circadian cycle of HPA axis basal activity (1, 10–14). de Kloet and colleagues performed many of the initial studies characterizing the distribution and hormone binding properties of mineralocorticoid and glucocorticoid receptors in brain and pituitary. They have also examined the role of corticosterone receptor subtypes in mediating corticosterone regulation of basal and stress-induced HPA axis activity during the circadian trough of activity (2, 15, 16).

Based primarily on the work of these two groups of investigators, a basic model of corticosteroid receptor mediation of HPA axis regulation by corticosteroids has been put forth in which: 1) the low levels of corticosterone present during the circadian trough act via the high affinity mineralocorticoid receptors to maintain low basal activity of the HPA axis; 2) the higher levels of corticosterone present during the circadian peak or acute stress act via the lower affinity glucocorticoid receptors to constrain HPA axis activity (1, 2, 15). A recent study indicates that mineralocorticoid receptors contribute to the corticosterone maintenance of basal HPA axis activity during the circadian peak, perhaps by potentiating corticosterone effects at glucocorticoid receptors (13).

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Whether mineralocorticoid receptors also contribute to feedback inhibition of the HPA axis by corticosterone during acute stress, however, is not clear. Ratka *et al.* (16) found that treatment of rats with the mineralocorticoid receptor antagonist, RU28318, prolonged stress-induced elevations of corticosterone, but Weidenfeld and Feldman (17) observed no effect of RU28318 treatment on corticosterone or ACTH responses to acute stress. Neither study examined the effects of blocking both mineralocorticoid and glucocorticoid receptors at the same time. In addition, studies have yet to address whether there are circadian differences in mineralocorticoid and glucocorticoid receptor involvement in corticosteroid feedback inhibition of the HPA axis acute stress response. Two studies have suggested that the shut-off of the HPA axis response to acute stress may not be as dependent on active corticosterone negative feedback in the evening as in the morning (18, 19).

The experiments reported in this paper were designed to further test this emerging model of corticosteroid receptor subtype mediation of corticosteroid feedback regulation of the HPA axis. The basic strategy of these experiments was to acutely administer selective mineralocorticoid and/or glucocorticoid receptor antagonists to rats and examine their effect on basal and acute stress corticosterone levels during both the time of trough and peak basal HPA axis activity.

For these studies, we used the mineralocorticoid receptor antagonist, RU28318, and the glucocorticoid receptor antagonist, RU40555. RU40555 has been described by its supplier as having similar receptor selectivity as the more widely used RU486 (both progesterone receptor and glucocorticoid receptor antagonist), but not as much potency (unpublished correspondence from Roussel Uclaf). The choice of RU40555 as glucocorticoid receptor antagonist over RU486 was 2-fold: 1) in preliminary studies we didn't see any evidence for a partial glucocorticoid receptor agonist effect of RU40555 (20), as has been described for RU486 (12, 16, 21); and 2) during this project RU486 was temporarily unavailable in the United States.

Finally, in our studies we estimated the proportion of corticosteroid receptors occupied *in vivo* by our two antagonists. Based on these estimates we were able to verify the *in vivo* receptor selectivity profile for each compound. In addition, we obtained evidence that the dose of RU40555 used in these studies occupied only 50% of glucocorticoid receptors. The poor solubility of this compound made testing of the acute effects of higher doses of RU40555 impractical. This submaximal blockade of glucocorticoid receptors, appears, however to have been fortuitous in that it allowed for evidence of a cooperative effect between both mineralocorticoid and glucocorticoid receptors in regulation of HPA axis activity.

Materials and Methods

Subjects

All of the experiments used male Sprague-Dawley rats (Harlan Sprague-Dawley, Inc., Indianapolis, IN) weighing 285–300 g at the time of experimentation. Animals were housed in wire mesh hanging cages (3 rats per cage) and were given food (Purina laboratory rat chow) and tap water ad lib. The animal room was maintained on a 12-h light, 12-h dark cycle (lights on at 0700 h). All animals were given a 2-week acclimation period to the animal facilities before the onset of experimentation.

Corticosteroid receptor antagonist treatment

The mineralocorticoid receptor antagonist used for these studies was RU28318 ([7,17alpha]-17-hydroxy-3oxo-7-propyl-pregn-4-ene-21-carboxylic acid potassium salt) and the glucocorticoid receptor antagonist used was RU40555 (17-β-hydroxy-11-β-/4-/[methyl)-[1-methylethyl-]aminophenyl/-17[E0]-[prop-1-ynyl]estra-4,9-dien-3-one). Both compounds were donated by Roussel Uclaf (Romainville, France). The dose of RU28318 (50 mg/kg) used in our studies was based on separate studies in which this dose was found to produce near maximal decreases in available mineralocorticoid receptor binding without affecting glucocorticoid receptor binding (20). The dose of RU40555 (30 mg/kg) used in our studies was chosen based on our experience from other studies and manufacturer's information. We have found that sc injection of this dose of RU40555 completely blocked dexamethasone (50 μg/kg sc) suppression of stress-induced corticosterone secretion (20). Unpublished correspondence from Roussel Uclaf states that this dose of RU40555 when administered orally exhibited near total antagonist activity against dexamethasone's effects on a variety of measures, including plasma corticotrophic activity. Due to the poor solubility of RU40555, all drugs were dissolved in propylene glycol and the injections were given sc (0.9 ml per rat). For combined antagonist treatment a single injection containing RU28318 (50 mg/kg) and RU40555 (30 mg/kg) was given (0.9 ml per rat). Control rats were given an equal volume sc injection of propylene glycol (vehicle).

Antagonists were injected 1 h before the onset of restraint. Experiments conducted during the morning began with injections at 0800 h. Experiments conducted in the evening began with injections at 1900 h.

Restraint stress

Restraint stress consisted of placing rats in a clear Plexiglas tube (23.5 cm in length and 7 cm in diameter) that inhibited forward/backward and lateral movement, but did not interfere with breathing. The animals were placed into the restraint tubes for 1 h.

Blood sampling procedure

The tail clip method was used for serial blood sampling. For experiments in which only plasma corticosterone was measured, blood (approximately 100 μl) was collected into heparanized tubes, and plasma was stored at -20 C. For ACTH measurement, blood samples (approximately 300 μl) were collected into EDTA coated tubes and plasma was stored at -70 C. All rats within a cohort were sampled simultaneously and blood sampling was completed within 3–5 min of entering the animal room. Blood samples were taken immediately upon restraint (basal corticosterone determination), 30 and 60 min into restraint (restraint corticosterone response), and 60 min after the termination of restraint (recovery from restraint determination).

Plasma corticosterone and ACTH determination

Plasma corticosterone was measured by RIA. Plasma samples (20 μl) were diluted in 0.01 M PBS and heat inactivated for 1 h at 70 C. Samples and corticosterone standards (25–2000 pg/tube) were incubated overnight with antiserum (B21–42, Endocrine Sciences, Agoura Hills, CA) and [3-H] corticosterone (20,000 cpm/tube). Antibody-bound steroid was separated from free steroid with dextran-coated activated charcoal. The intra and interassay coefficients of variability were 7.3% and 13.1%, respectively. Assay sensitivity was approximately 0.5 μg/ml for a 20-μl plasma sample. Plasma ACTH was measured with a commercial kit (IncStar, Stillwater, MN). The ACTH assay had a sensitivity of 10 pg/ml for a 100-μl plasma sample.

Estimates of in vivo corticosteroid receptor occupancy by antagonists

The effect of a 1-h treatment with RU28318 (50 mg/kg sc), RU40555 (30 mg/kg sc), or a combination of both RU28318 (50 mg/kg sc) and RU40555 (30 mg/kg sc) on mineralocorticoid and glucocorticoid recep-

tor binding in brain tissue was assessed in adrenalectomized rats. Because only the form of the corticosteroid receptor that has not been occupied and activated by ligand can be measured in a cytosolic receptor binding assay (22, 23), we refer to our receptor binding values as available receptors. Rats were adrenalectomized (ketamine 50 mg/kg + xylazine 10 mg/kg anesthetic) 24 h before antagonist treatment to remove endogenous corticosteroids which would compete with the antagonists for *in vivo* receptor occupancy. Rats were killed (rapid decapitation) 1 h after antagonist treatment. The hippocampus was rapidly dissected, frozen, and stored at −70 C until subsequent corticosteroid receptor binding determination.

Corticosteroid receptor binding

Mineralocorticoid and glucocorticoid receptors were measured in the cytosolic fraction of tissue according to the procedure described by Spencer *et al.* (7, 19). A homogenization/incubation buffer was used that was comprised of 10 mM Tris, 1 mM EDTA, 20 mM molybdic acid, 5 mM dithiothreitol, and 10% glycerin, pH 7.4, at 4 C. Frozen tissue was thawed, homogenized, and then centrifuged for 30 min at 100,000 × *g*, and the resulting supernatant was used as cytosol. Cytosol (0.5–1.5 mg/ml) was incubated overnight (4 C) with [^{3}H]dexamethasone ± unlabeled steroids. Bound steroid was separated from unbound steroid by gravity filtration over Sephadex LH-20 (Pharmacia LKB Biotechnology, Piscataway, NJ) minicolumns in triplicate. Glucocorticoid receptor binding was determined by the amount of total [^{3}H]dexamethasone (10 nM) binding that was displaced by the selective glucocorticoid receptor ligand RU28362 (0.5 μM). Mineralocorticoid receptor binding was determined by the amount of [^{3}H]dexamethasone (10 nM) in the presence of RU28362 (0.5 μM) that was displaced by the mineralocorticoid and glucocorticoid receptor ligand, corticosterone (10 μM). We have found that [^{3}H]dexamethasone *in vitro* is as effective as [^{3}H]aldosterone for measuring mineralocorticoid receptors (7) and has the advantage over [^{3}H]aldosterone and [^{3}H]corticosterone in that there is no potential cross-reaction of [^{3}H]dexamethasone with corticosteroid binding globulin. A single saturating concentration of [^{3}H]dexamethasone was used so that available mineralocorticoid and glucocorticoid receptors could be measured in the hippocampus from each individual animal. Specific binding was expressed as femtomoles of steroid bound per mg cytosolic protein. Proteins were measured by the method of Bradford (24), with use of BSA as protein standard.

Data analysis and statistics

Each experiment was analyzed by the appropriate multiway ANOVA. To determine differences between individual groups at a particular time point a one-way ANOVA was conducted, and in each case was followed by both a conservative (Tukey's test) and a more powerful (Fisher's least significant difference test) posthoc test. Significant results from the Fisher's least significant difference test are indicated only in the cases where there was not a significant difference as determined by the Tukey's test. Data presented in text and figures are the means ± SEM.

Results

Exp 1: RU28318 effects on morning and evening plasma corticosterone levels

The first experiment examined the effects of the selective mineralocorticoid receptor antagonist, RU28318, on basal and stress-induced plasma corticosterone levels (Fig. 1). The vehicle treated group, as expected, exhibited relatively low basal corticosterone levels in the morning (5.7 ± 1.6 μg/100 ml) and high basal corticosterone levels in the evening (23.2 ± 4.4 μg/100 ml). RU28318 treatment produced a significant elevation of basal corticosterone levels relative to vehicle treatment in the morning (15.2 ± 3.5), but not in the evening (23.4 ± 4.2).

Restraint when administered in the morning or evening produced a rise in plasma corticosterone levels that returned to basal levels by 1 h after the end of restraint. There was no difference between vehicle- and RU28318-treated rats in the magnitude of the plasma corticosterone response to restraint. In addition, there was not a significant effect of RU28318 treatment on poststress corticosterone levels, although in this experiment there was considerable variability in the morning poststress corticosterone levels of RU28318-treated rats.

The maximum corticosterone levels attained during stress (occurring 30 min after restraint) were higher for the vehicle rats in the evening than in the morning. However, the acute stress response in the morning for vehicle-treated rats was lower in this experiment than in Exp 2–5, and this is the only experiment of this study in which there was a diurnal difference in stress corticosterone levels for vehicle rats.

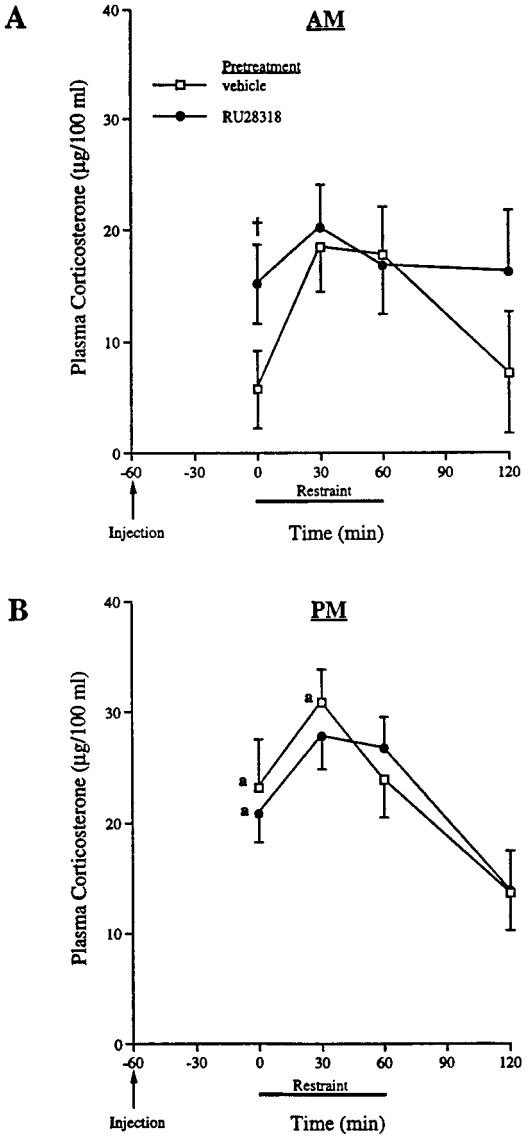

FIG. 1. Effect of mineralocorticoid receptor blockade on corticosterone response to restraint stress. RU28318 (50 mg/kg sc) or vehicle (0.9 ml/rat propylene glycol sc) were injected in rats after the beginning of the light (A) or dark (B) period (n = 6). Rats were placed in restrainers 1 h after injection. †, Significant difference from the vehicle group at the same time point and same time of day, $P < 0.05$, Student's t test. [a], Significant difference from the morning vehicle group at the same time point, $P < 0.05$, Tukey's test.

Exp 2: RU40555 effects on morning and evening plasma corticosterone levels

The second experiment examined the effects of the glucocorticoid receptor antagonist, RU40555, on plasma corticosterone levels under the same diurnal and stress conditions as were used in the first experiment (Fig. 2). As was the case in the first experiment, vehicle-treated rats had low basal corticosterone levels in the morning (4.6 ± 1.4 μg/100 ml) and high basal corticosterone levels in the evening (19.0 ± 1.9 μg/100 ml). RU40555 treatment had no effect on basal corticosterone levels at either time of day.

Restraint produced a rise in plasma corticosterone both in the morning and evening, and in this experiment the maximum stress-induced corticosterone levels did not differ in the morning or evening. There was no statistically significant effect of RU40555 treatment on plasma corticosterone levels during or after stress in either the morning or evening.

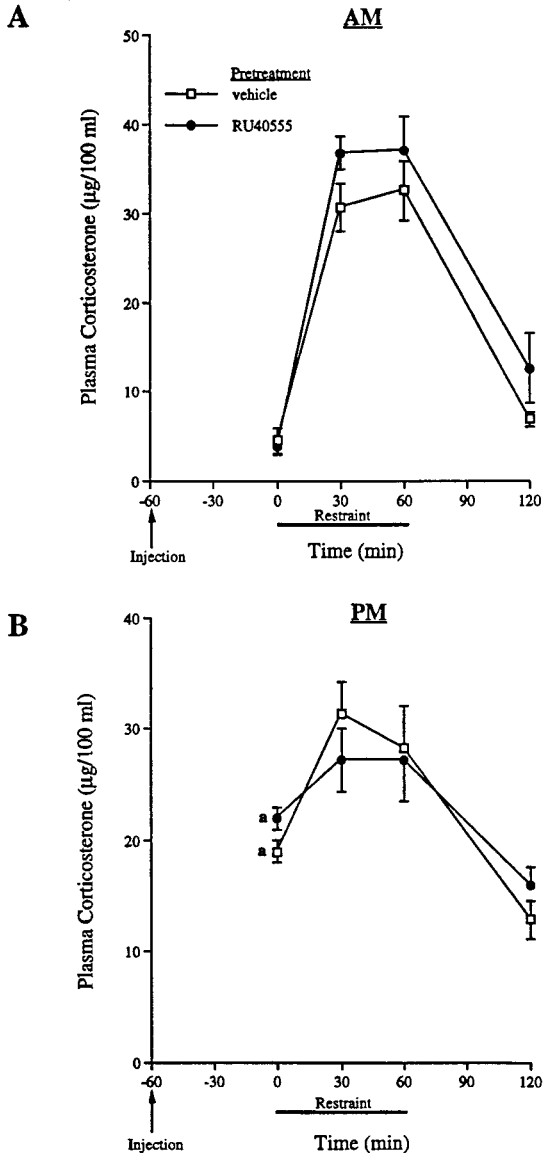

FIG. 2. Effect of glucocorticoid receptor blockade on corticosterone response to restraint stress. RU40555 (30 mg/kg sc) or vehicle (0.9 ml/rat propylene glycol sc) were injected in rats after the beginning of the light (A) or dark (B) period (n = 6). Rats were placed in restrainers 1 h after injection. [a], Significant difference from the morning vehicle group at the same time point, $P < 0.05$, Tukey's test.

Exp 3: combined RU28318 and RU40555 effects on morning and evening plasma corticosterone levels

The third experiment examined the effects of combined treatment with RU28318 and RU40555 on plasma corticosterone levels (Fig. 3). For this experiment, two separate cohorts of rats were tested on separate occasions. Although there was an overall statistically significant cohort effect due to higher stress-induced corticosterone levels of all treatment groups in one cohort compared with the other cohort, there was not a significant interaction effect of cohort and time of day or cohort and drug on plasma corticosterone levels. Consequently, for presentation purposes we have pooled the data from the two cohorts.

Again, as expected, vehicle-treated rats had low basal corticosterone levels in the morning (2.4 ± 0.6 μg/100 ml) and high basal corticosterone levels in the evening (19.2 ± 2.2 μg/100 ml). Stress produced an elevation of plasma corticosterone levels which attained a similar maximum at both times of day.

Treatment with a combination of both RU28318 and RU40555 produced an overall drug effect on plasma corticosterone levels, $F(1,44) = 15.8$, $P < 0.001$. Post hoc analysis indicates that the combined antagonist treatment produced an elevation of basal corticosterone levels in the evening. In addition, there was a significant elevation of corticosterone levels during stress both in the morning and evening. Finally, both in the morning and evening, corticosterone levels of the receptor antagonist treated group were significantly elevated compared with the vehicle group 1 h after the termination of restraint.

Exp 4: within experiment comparison of RU28318, RU40555, or combined RU28318 and RU40555 effects on morning plasma corticosterone levels

The fourth experiment was conducted to determine the reproducibility of the effects observed in the first three experiments. In addition, the fourth experiment provided for a within experiment comparison of the three drug conditions examined in the first three experiments. Because there were very few diurnal differences in the drug effects observed in the first three experiments, the fourth experiment was conducted only in the morning.

The vehicle-treated rats exhibited basal corticosterone levels (3.1 ± 0.8 μg/100 ml) that were similar to morning basal levels observed in the three previous experiments (Fig. 4). The stress response of the vehicle-treated rats was also similar to that observed in the previous experiments.

There was an overall drug treatment effect, $F(3,20) = 7.4$, $P = 0.002$. The profile of drug treatment effects was very similar to that observed in the first three experiments. As was observed in Exp 1, treatment with RU28318 produced a significant elevation in basal corticosterone levels but had no effect at any other time point. As was observed in Exp 2,

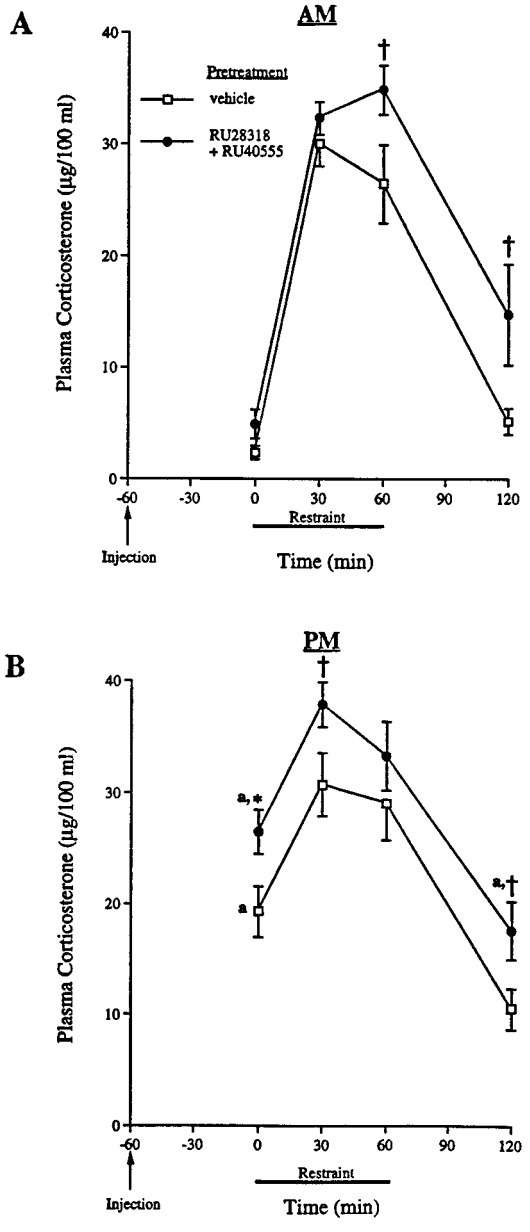

FIG. 3. Effect of combined mineralocorticoid and glucocorticoid receptor blockade on corticosterone response to restraint stress. RU28318 (50 mg/kg sc) + RU40555 (30 mg/kg sc) or vehicle (0.9 ml/rat propylene glycol sc) were injected in rats after the beginning of the light (A) or dark (B) period (n = 12). Rats were placed in restrainers 1 h after injection. †, Significant difference from the vehicle group at the same time point and same time of day, $P < 0.05$, Student's t test. *, Significant difference from the vehicle group at the same time point and same time of day, $P < 0.05$, Tukey's test. [a], Significant difference from the morning vehicle group at the same time point, $P < 0.05$, Tukey's test.

treatment with RU40555 had no significant effect at any time point. As was observed in Exp 3, treatment with a combination of RU28318 and RU40555 produced an elevation of corticosterone levels during restraint, and corticosterone levels remained elevated compared with vehicle treated rats one h after removal from restraint. In contrast to Exp 3, in this experiment the combined antagonist treatment produced a significant elevation of basal corticosterone levels in the morning.

Exp 5: combined RU28318 and RU40555 effects on morning plasma ACTH and corticosterone levels

The fifth experiment examined the effects of the combined RU28318 and RU40555 treatment on both plasma ACTH levels and plasma corticosterone levels in the morning. This experiment was conducted to verify that the increased corticosterone response observed after treatment with RU28318 and RU40555 reflects also increased ACTH levels. Although it seems unlikely that acute treatment with corticosteroid receptor antagonists could alter corticosterone levels independent of alterations in ACTH secretion, there is evidence for neural alteration of adrenal activity independent of changes in other components of the HPA axis (25). Because plasma ACTH levels are capable of rising more rapidly after stress onset than are plasma corticosterone levels, in this experiment blood samples were taken at 15 min after restraint onset in addition to the later time points used in the previous experiments (30 and 60 min).

Restraint stress produced a small rise in plasma ACTH levels in the vehicle-treated rats, which reached its maximum 30 min after restraint onset. There was an overall drug treatment effect on plasma ACTH levels, $F(2, 21) = 10.6$, $P = 0.004$ (Fig. 5). Posthoc analysis indicated that treatment with the combined corticosteroid receptor antagonists produced an enhanced ACTH response to restraint that reached its peak at 15 min after stress onset and remained significantly elevated 30 and 60 min after stress onset. In this experiment, the combined RU28318 and RU40555 treatment produced a significant elevation in plasma corticosterone levels only at the 60 min restraint time point.

Exp 6: effect of RU28318, RU40555, or combined RU28318 and RU40555 treatment on available corticosteroid receptors

Available mineralocorticoid and glucocorticoid receptor binding were measured in hippocampal tissue of adrenalectomized rats that had been treated with vehicle or corticosteroid receptor antagonists 60 min before the rats were killed (Fig. 6). Treatment with RU28318 produced a selective 85% decrease in available mineralocorticoid receptors and no effect on available glucocorticoid receptors relative to vehicle treatment. Treatment with RU40555 produced a selective 50% decrease in available glucocorticoid receptors relative to vehicle treatment, and an unexpected increase in available mineralocorticoid receptors. Treatment with the combination of RU28318 and RU40555 produced decreases in both mineralocorticoid (80%) and glucocorticoid (40%) receptors relative to vehicle treatment.

Discussion

The results of this study largely support the emerging model of the relative roles of mineralocorticoid and glucocorticoid receptors in mediating corticosteroid regulation of HPA axis activity (1, 2, 13, 16). In this study, acute blockade of mineralocorticoid receptors with RU28318 produced an

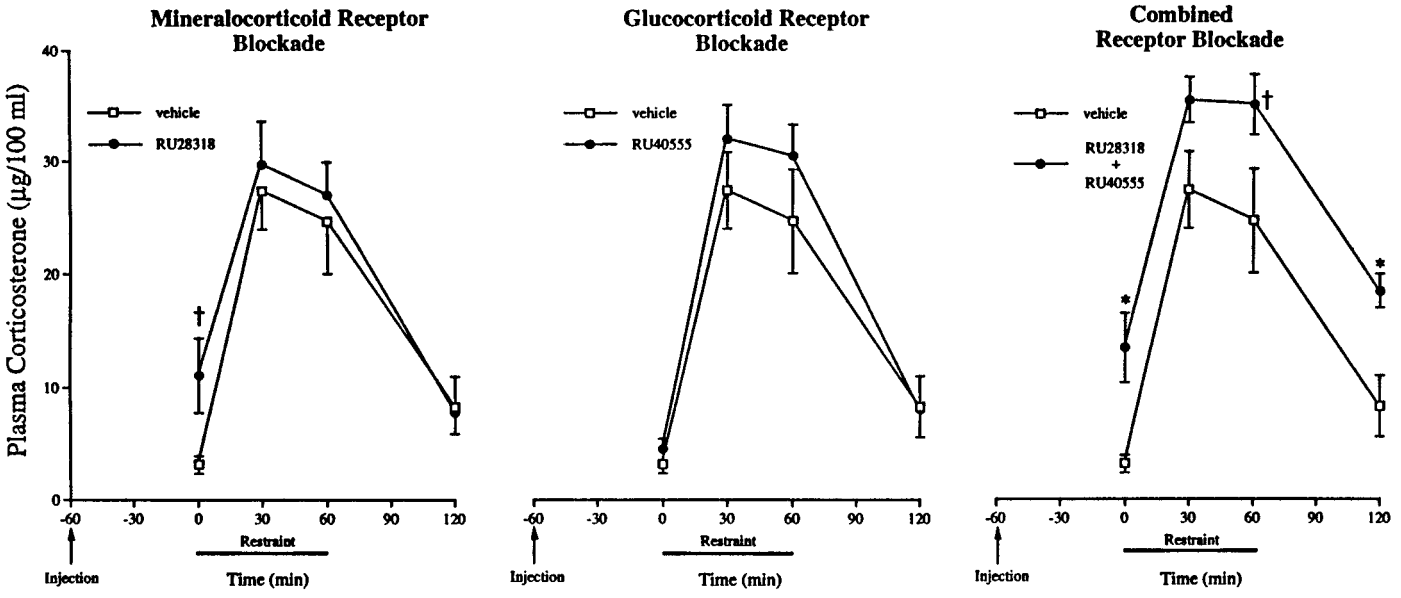

FIG. 4. Within experiment comparison of mineralocorticoid and glucocorticoid receptor blockade on corticosterone response to restraint stress. RU28318 (50 mg/kg sc), RU40555 (30 mg/kg sc), combined RU28318 (50 mg/kg sc) + RU40555 (30 mg/kg sc), or vehicle (0.9 ml/rat propylene glycol sc) were injected in rats 1 h after the beginning of the light period (n = 6). Rats were placed in restrainers 1 h after injection. †, Significant difference from the vehicle group at the same time point, $P < 0.05$, Student's t test. *, Significant difference from the vehicle group at the same time point, $P < 0.05$, Tukey's test.

increase in basal corticosterone levels in the morning, but not in the evening, and had no effect on stress-induced corticosterone levels. Treatment with the glucocorticoid receptor antagonist, RU40555, had no effect on basal or stress-induced corticosterone levels at either time of day. On the other hand, combined treatment with RU28318 and RU40555 increased basal corticosterone levels in the evening (and morning, Exp 4) and increased peak and poststress corticosterone levels at both times of day. As expected, an elevation of ACTH levels that parallels altered corticosterone levels was seen when rats were treated in the morning with both antagonists at once, suggesting that the combined receptor antagonism produced increased HPA axis activity not only at the level of the adrenal but also at the pituitary.

Estimates of corticosteroid receptor occupancy by antagonists

Receptor binding studies indicate that RU28318 treatment produced a selective 85% decrease in available mineralocorticoid receptors in rat hippocampal tissue, whereas RU40555 treatment produced a selective 50% decrease in available glucocorticoid receptors. Several studies have demonstrated that corticosteroid receptor antagonists, in addition to agonists, lead to translocation of corticosteroid receptors from the cytoplasm to the nucleus (26–28). Thus, the decrease in available corticosteroid receptor binding in the cytosolic/soluble tissue fraction seen in this study after acute antagonist treatment most likely reflects the occupancy and translocation of receptors by the antagonist before the rats were killed. We have found in previous studies that changes in systemic steroid levels produce changes in available corticosteroid receptor levels that are similar across each brain area examined (7, 19). Consequently, we infer from this study that our treatment with RU28318 produced approximately 85% occupancy of mineralocorticoid receptors throughout the rat brain, and treatment with RU40555 produced approximately 50% occupancy of glucocorticoid receptors in rat brain. We unexpectedly found that acute treatment with RU40555 treatment alone produced an increase in available mineralocorticoid receptors relative to vehicle treated rats. We note, however, that this result was not reproduced in other similar studies, and therefore may be spurious (20).

Regulation of basal HPA axis activity

Our study is consistent with the proposed role of corticosteroid receptors in regulation of HPA axis basal activity. We found that mineralocorticoid receptor activation was necessary for maintaining the low levels of corticosterone secretion normally seen in the morning, whereas activation of both mineralocorticoid and glucocorticoid receptors contributed to the normal evening basal corticosterone levels. This result supports other studies in which treatment of adrenalectomized rats with a low level of corticosterone, estimated to occupy only mineralocorticoid receptors, was able to maintain basal ACTH levels in the morning, whereas treatment with higher levels of corticosterone, estimated to occupy both mineralocorticoid receptors and some glucocorticoid receptors, was required to maintain basal ACTH levels in the evening (11). The interactive role of mineralocorticoid and glucocorticoid receptor regulation of ACTH levels in the evening was further demonstrated in a study in which treatment with low levels of corticosterone was able to potentiate the ability of dexamethasone (a relatively selective glucocorticoid receptor agonist) to maintain ACTH levels in the evening (13).

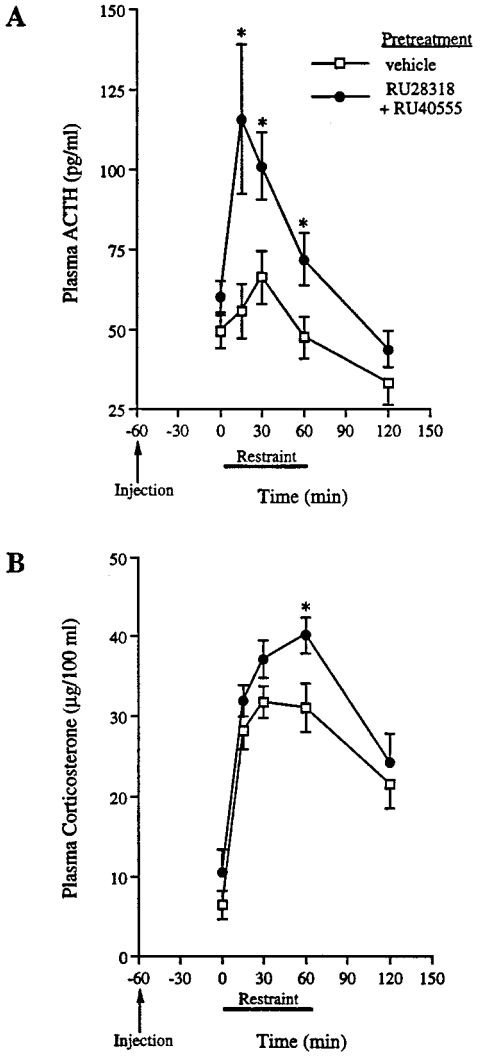

FIG. 5. Effect of combined mineralocorticoid and glucocorticoid receptor blockade on ACTH (A) and corticosterone (B) response to restraint stress. RU28318 (50 mg/kg sc) + RU40555 (30 mg/kg sc) or vehicle (0.9 ml/rat propylene glycol sc) were injected in rats 1 h after the beginning of the light period (n = 12). Rats were placed in restrainers 1 h after injection. *, Significant difference from the vehicle group at the same time point, $P < 0.05$, Student's t test.

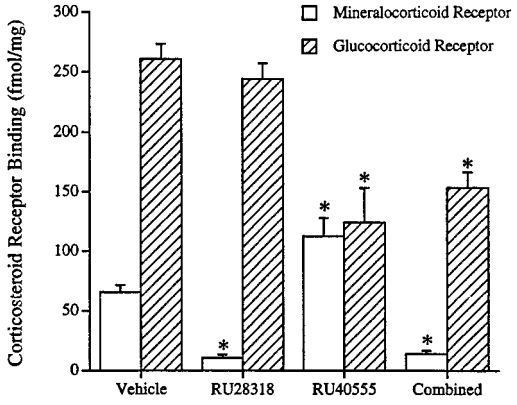

FIG. 6. Effect of mineralocorticoid and glucocorticoid receptor blockade on available corticosteroid receptor binding. RU28318 (50 mg/kg sc), RU40555 (30 mg/kg sc), combined RU28318 (50 mg/kg sc) + RU40555 (30 mg/kg sc), or vehicle (0.9 ml/rat propylene glycol sc) were injected in adrenalectomized rats 1 h before the rats were killed (n = 5–6). Hippocampal tissue was rapidly dissected, frozen, and stored at −70 C. Available cytosolic mineralocorticoid and glucocorticoid receptors were measured by a competitive radioligand binding assay (see *Materials and Methods*). *, Significant difference from the vehicle group for the same receptor subtype, $P < 0.01$, Tukey's test.

Regulation of acute-stress-induced HPA axis activity

Our study provides some refinement to the prior proposed exclusive role of glucocorticoid receptors in mediating acute corticosteroid negative feedback on HPA axis activity during and after stress (2). Thus, in our study it appears that activation of both mineralocorticoid and glucocorticoid receptors contributes to a corticosteroid feedback inhibitory effect that limits the magnitude and duration of stress-induced HPA axis activity. Moreover, this receptor contribution appears to be similar in both the morning and the evening.

The inability of the mineralocorticoid receptor antagonist alone to affect stress- and post stress-induced corticosterone levels is consistent with the results of Weidenfeld and Feldman (17). Of importance in our study was the finding that treatment with the glucocorticoid receptor antagonist alone was also insufficient to affect corticosterone levels during or following stress. This contrasts with the results of Weidenfeld and Feldman (17) and Ratka *et al.* (16) in which treatment with another glucocorticoid receptor antagonist, RU486, produced either an increase in peak stress levels of corticosterone (17) or sustained elevations of corticosterone after stress (16). The discrepancy between the effectiveness of RU40555 *vs.* RU486 most likely pertains to their difference in potency (*i.e.* magnitude of glucocorticoid receptor blockade). As discussed above, we have evidence that the dose of RU40555 used in our studies occupied approximately 50% of glucocorticoid receptors. Consequently, it appears that blockade of only 50% of glucocorticoid receptors is not sufficient to increase HPA axis responses during and after stress. On the other hand, blockade of greater than 50% of glucocorticoid receptors, as apparently was the case in studies using the more potent RU486 compound, may result in increased HPA axis responses during and after stress.

Importantly, our results indicate that the combined blockade of 50% of glucocorticoid receptors and a majority of mineralocorticoid receptors is a condition sufficient to significantly increase HPA axis responses during and after acute stress. Based on this result, we suggest that mineralocorticoid receptors contribute to the corticosterone active inhibition of the HPA axis during acute stress. This contribution may be a result of a direct facilitatory effect of mineralocorticoid receptors on glucocorticoid receptor function within the same corticosteroid target cells. Precedence for such an effect has been provided by recent studies demonstrating in corticosteroid receptor transfected cell lines a potentiating effect between mineralocorticoid receptors and glucocorticoid receptors on activation of a reporter gene. In those *in vitro* studies, the potentiating effect appears to be a result of the formation of heterodimers between the two subtypes of corticosteroid receptors (29). Such a potentiating effect may be limited to enhancer-like function of corticosteroid receptors. Whether the negative feedback effects of corticosteroids on HPA axis activity includes enhancement

of the transcription of certain target genes remains to be determined.

Alternatively, mineralocorticoid receptor facilitation of glucocorticoid receptor dependent corticosteroid negative feedback could be a result of an interaction between separate cells/pathways converging on the paraventricular nucleus of the hypothalamus (PVN). Thus, an indirect negative feedback facilitatory effect may result from a mineralocorticoid receptor sensitive input to the PVN (*e.g.* from the hippocampus) that adjusts the sensitivity of PVN neurons to the inhibitory effects of corticosterone mediated by glucocorticoid receptors. Although, there is no direct support for such a mechanism, it is worth noting that 1) the hippocampus expresses a high level of mineralocorticoid receptors, and 2) lesions of the hippocampus have been demonstrated to decrease the sensitivity of the HPA axis to the inhibitory effects of glucocorticoids (30).

Revised model of the dependence of HPA axis activity on corticosteroid receptor mediated function

Based on this study, in conjunction with the results from previous studies, we propose that mineralocorticoid receptor activation alone is sufficient to maintain normal HPA axis basal activity at the circadian trough. On the other hand, normal levels of HPA axis activity during the circadian peak or during times of stress requires activation of glucocorticoid receptors. However, mineralocorticoid receptor activation at these other times may contribute to HPA axis regulation by facilitating glucocorticoid receptor function, either directly, or indirectly. Thus, mineralocorticoid receptor activation may decrease the threshold of glucocorticoid receptor activation necessary to restrain the HPA axis during these circadian or stress circumstances.

This proposed potentiating effect of mineralocorticoid receptors on glucocorticoid receptor mediated function is essentially the same process proposed by Bradbury *et al.* (13) to be operative during the regulation of peak basal HPA axis activity by corticosterone. Our results extend this potentiating effect to corticosteroid regulation of the HPA axis during acute stress.

An implication of this proposal is that complete blockade of glucocorticoid receptors in the absence of mineralocorticoid receptor blockade would be expected to impair corticosteroid feedback inhibition of the HPA axis during acute stress or the circadian peak, but not during the circadian trough. Studies in both humans and rats found that treatment with RU486 produced elevated basal corticosteroid levels during the circadian peak but not circadian trough (31–34). As already noted above, two studies have also found that RU486 treatment of rats produced either increased stress or poststress corticosterone levels (16, 17). On the other hand, partial blockade of glucocorticoid receptors in the absence of mineralocorticoid receptor blockade, as occurred in our study, may not affect HPA axis function due to the potentiating effect of mineralocorticoid receptors on the remaining available glucocorticoid receptors.

Another implication of this proposal is that complete blockade of mineralocorticoid receptors may also have effects on HPA axis activity during acute stress or the circadian peak, but only in cases where glucocorticoid receptor activation alone is not sufficient to maintain normal HPA axis levels of activity. In an example of mineralocorticoid receptor blockade having an effect on stress-related HPA axis activity, Ratka *et al.* (16) found that RU28318 treatment resulted in elevated poststress corticosterone secretion. Those authors suggested that RU28318 interfered with a normal mineralocorticoid receptor-mediated suppressive effect of corticosterone on limbic function and stress responsivity. But it is also possible that in the Ratka *et al.* study there was not enough glucocorticoid receptor activation during acute stress to act alone to inhibit poststress HPA axis activity.

An example of mineralocorticoid receptor blockade having an effect on basal HPA axis activity at a time other than the circadian trough is provided in a study in which men were treated with the relatively selective mineralocorticoid receptor antagonist, canrenoate (35). Canrenoate treatment produced elevated basal cortisol levels both during the night, when basal cortisol levels were at their circadian trough, and early in the morning (0700 h), as cortisol levels approached their circadian peak (35). Because later time points were not examined, it is unknown whether the canrenoate treatment also increased cortisol levels later in the morning at the height of the circadian peak.

Time domains for corticosteroid regulation of HPA axis activity

The necessary time-relationship between corticosteroid receptor activation and subsequent effects on HPA axis activity were not systematically evaluated in this study. However, some inferences about that relationship can be determined from our experimental paradigm. Because mineralocorticoid and glucocorticoid receptors function as hormone activated transcription factors, their effects on cell activity are believed to depend on the alteration of cellular levels of specific proteins. It is generally accepted that the production of new functional proteins following initiation of gene transcription requires a minimum of 20 min to several hours. In our study, rats were treated with antagonists for 1 h before any blood samples were collected. We have found that our antagonist treatment paradigm produces maximal decreases in available hippocampal corticosteroid receptors (*i.e.* receptor occupation) by 60 min after injection (20). Thus, it is likely that we are measuring HPA axis activity at a time point in which steady state levels of proteins that are regulated by mineralocorticoid receptors and/or glucocorticoid receptors are just beginning to be altered. An implication of our results is that basal HPA axis activity is actively regulated by circulating corticosteroids and blockade of those effects for as little as 30–60 min is capable of disinhibiting HPA axis activity. Our treatment paradigm does not allow us to determine whether the increased stress and poststress corticosterone levels produced by corticosteroid receptor antagonism was a result of receptor blockade during the hour before the onset of stress or was a result of receptor blockade during stress. However, there is support from another study for the surge in corticosterone that accompanies a stress response to contribute a negative feedback effect on the magnitude of ACTH secretion present during that stress response (36).

Concluding remarks

In summary, this study further illustrates the dynamic dependence of HPA axis function on corticosteroid negative feedback. Short-term pharmacological blockade of corticosteroid receptors leads to increased corticosterone levels during basal and acute-stress conditions. Both mineralocorticoid receptors and glucocorticoid receptors contribute importantly to corticosteroid feedback inhibition of the HPA axis. Activation of mineralocorticoid receptors by the very low levels of corticosteroid present at the trough of the circadian cycle appears to be sufficient to maintain low basal HPA axis activity. On the other hand, glucocorticoid receptor activation is necessary to maintain normal levels of HPA axis activity at the peak of the circadian cycle and during acute stress. However, even under these circumstances, mineralocorticoid receptor activation appears to play an important role in potentiating the glucocorticoid receptor mediated effects of corticosterone on HPA axis function.

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