Corticosteroids mediate fast feedback of the rat hypothalamic-pituitary-adrenal axis via the mineralocorticoid receptor

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Atkinson HC, Wood SA, Castrique ES, Kershaw YM, Wiles CC, Lightman SL. Corticosteroids mediate fast feedback of the rat hypothalamic-pituitary-adrenal (HPA) axis under basal conditions, in particular the role of the mineralocorticoid receptor. Blood samples were collected every 5 min from conscious rats at the diurnal peak, using an automated blood sampling system, and assayed for corticosterone. Feedback inhibition by rapidly increasing concentrations of ligand was achieved with an intravenous bolus of exogenous corticosteroid. This resulted in a significant reduction in plasma corticosterone concentrations within 23 min of the aldosterone bolus and 28 min of methylprednisolone. Evaluation of the pulsatile secretion of corticosterone revealed that the secretory event in progress at the time of administration of exogenous steroid was unaffected, whereas the next secretory event was inhibited by both aldosterone and methylprednisolone. The inhibitory effect of aldosterone was limited in duration (1 secretory event only), whereas that of methylprednisolone persisted for 4–5 h. Intravenous administration of canrenone (a mineralocorticoid receptor antagonist) also had rapid effects on the HPA axis, with an elevation of ACTH within 10 min and corticosterone within 20 min. The inhibitory effect of aldosterone was unaffected by pretreatment with the glucocorticoid receptor antagonist RU-38486 but blocked by the canrenone. These data imply an important role for the mineralocorticoid receptor in fast feedback of basal HPA activity and suggest that mineralocorticoids can dynamically regulate basal corticosterone concentrations during the diurnal peak, a time of day when there is already a high level of occupancy of the cytoplasmic mineralocorticoid receptor.

corticosterone; basal; pulsatility; ultradian; canrenone; RU-38486; mifepristone

CORTICOSTEROID FEEDBACK on the hypothalamic-pituitary-adrenal (HPA) axis is primarily mediated via the genomic actions of the transcription factors mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) (27). MR is understood to regulate tonic HPA activity, whereas GR mediates the recovery from a stress response (13). In addition to the classic genomic mechanisms of feedback, there is also evidence for rate-sensitive steroid feedback in a nongenomic time frame that was first identified in rodents in 1969 (10) and has subsequently been confirmed in humans (11). Despite numerous studies, there is as yet no clear site or mechanism for the fast feedback despite investigations at pituitary, hypothalamus, and other brain centers (1, 12, 16, 45, 49). There is, however, recent electrophysiological evidence in vitro that confirms a rapid inhibitory nongenomic action of corticosterone in hippocampal slices in mice that is dependent on a low-affinity membrane-associated MR (26).

To investigate the mechanisms underlying the rapid feedback response under conditions of basal HPA activity in vivo, it is necessary to obtain frequent blood samples from unstressed and unrestrained animals. This has been very difficult to achieve in the rodent, and the published in vivo data have therefore focused on the effects of exogenous steroids on stress-induced or corticotropin-releasing hormone (CRH)-stimulated secretion of corticosterone. Studies on rapid feedback have been much easier to perform in humans, and administration of exogenous cortisol, near the diurnal peak, has been shown to reduce basal ACTH within 15–30 min (7, 39). The time domain of fast feedback on basal HPA activity in humans (~15–60 min) differs from that of fast feedback inhibition of stress-induced activity in rats (<30 min). This may be due to different feedback mechanisms in rats and humans or to differences between feedback effects on basal and stress-induced HPA activity. Fast corticosteroid feedback may also have implications for health and well-being, since it is no longer evident in aged volunteers (7) and in depressed patients (52). There is a need, therefore, for an in vivo rat model in which we can better investigate the physiological mechanism underlying fast feedback in basal HPA activity.

The aim of the current study was to investigate fast feedback of the HPA axis under basal conditions in the conscious rat, in particular the role of MR. Using our frequent blood sampling system, we are able to measure circulating corticosterone both under basal conditions and following administration of exogenous corticosteroids. This has allowed us to develop a model of fast rate-sensitive corticosteroid, which we have been able to probe with selective MR and GR ligands.

MATERIALS AND METHODS

Animals

Adult female Sprague-Dawley rats were obtained from Harlan, UK and maintained under standard housing conditions with lights on at 0515 (14:10-h light-dark). Food and water were available ad libitum. Animals were allowed to adapt to our animal house conditions for at least 1 wk prior to surgery. All animal procedures were carried out in accordance with the UK Home Office animal welfare regulations.

Experimental Procedures

Surgery. Animals were anesthetized with a combination of Hypnorm (0.32 mg/kg fentanyl citrate and 10 mg/kg fluanisone im; Jannsen...
Pharmaceuticals, Oxford, UK) and diazepam (2.6 mg/kg ip; Phoenix Pharmaceuticals, Gloucester, UK). The right jugular vein was exposed, and a Silastic-tipped (ID 0.50 mm, OD 0.93 mm, Merck) polyethylene cannula (Portex, Hythe, UK) was inserted into the vessel until it lay close to the entrance of the right atrium. The cannula was prefilled with pyrogen-free heparinized (10 IU/ml) isotonic saline. The free end of the cannula was exteriorized through a scalp incision and then tunnelled through a protective spring that was anchored to the parietal bones using two stainless steel screws and self-curing dental acrylic. Following recovery, animals were housed in individual cages in the automated blood sampling room. The end of the protective spring was attached to a mechanical swivel that rotated through 360° in a horizontal plane and 180° through a vertical plane, allowing the animals maximum freedom of movement. The cannulae were flushed daily with the heparinized saline to maintain patency. In addition to the jugular vein cannulation, some animals received bilateral adrenalectomy (ADX) at the time of surgery. Bilateral ADX was performed by the dorsal approach. At the time of surgery, a subcutaneous injection of dexamethasone (50 μg) in saline was administered. Following surgery, ADX rats were given 0.9% saline to drink supplemented with 250 μg/ml corticosterone (CORT). Some animals also received an additional subcutaneous cannula at the time of surgery. The subcutaneous cannula passed through the protective spring and was tunneled along a subcutaneous pocket above the scapular so that the tip of the cannula was ~3–4 cm distal to the scalp incision. The subcutaneous cannula was filled with saline, and a small amount of saline (0.1–0.2 ml) was flushed into the cannula daily. This cannula enabled the sc placement of a drug without handling or stressing the animal.

**Blood Sampling and Hormone Assays**

The collection of blood with an automated blood sampling system has previously been described in detail (6, 29, 50). Briefly, 4 or 5 days after surgery, the jugular vein cannula of each animal was connected to the automated blood sampling system. Blood samples were collected at 5- or 10-min intervals using the automated system for a period of up to 16 h. Blood samples were collected at a dilution of 1:3 in heparinized saline for corticosterone measurement. Each sample from the automated system contained no more than 40 μl of whole blood. Blood samples from separate groups of animals were also collected manually for the determination of ACTH. Concentrations of ACTH were determined by immunoradiometric assay (DiaSorin). Antisera were supplied by Prof G. Makara (Institute of Experimental Medicine, Budapest, Hungary), and [125I]corticosterone from OBI-Diagnostics (University of Oxford, UK). ACTH in plasma (100 μl per tube) was measured using a commercial immunoradiometric assay (DiaSorin).

**Experimental Protocols**

**Experiment 1: acute CORT response to MR agonist aldosterone or MR antagonist canrenoate.** Five days after the jugular vein was implanted, rats were connected to the automated blood sampling system. Blood samples were collected every 5 min from 1602 until 2202 and thereafter every 10 min until 0802 the following morning. At 1800 (during the 2- to 3-min quiescent period between blood sample collection), the jugular vein cannula was disconnected from the system, and a bolus of CAN (250 or 5,000 μg in 0.1 ml) was administered. The line was flushed with saline (0.15 ml) and reconnected to the system. Ten minutes later, all animals received a bolus of methylprednisolone (250 μg in 0.1 ml) via the jugular cannula, which was then flushed with 0.15 ml saline and reconnected to the system. CORT concentrations in diluted whole blood were determined by RIA.

**Experiment 2: cross-reactivity of exogenous steroids with CORT RIA.** Preliminary studies using whole blood spiked with canrenoate (CAN) revealed cross-reactivity of CAN with the CORT antisera (results not shown). In vivo, CAN is rapidly converted to canrenone, which may also cross-react with the CORT antisera; therefore, it was necessary to use an in vivo model to test cross-reactivity. Steroids used in this study were given to ADX rats so that overall cross-reactivity of the parent compound and its metabolites could be determined for the CORT assay. At the time of surgery, five rats underwent bilateral ADX, and the jugular vein was cannulated. On the day prior to blood sampling, the CORT-supplemented saline was withdrawn, and the animals were given 0.9% saline to drink. Blood samples were collected by the automated sampling system every 5 min commencing at 0802 for 13 h. The following steroids were injected intravenously: at 1000 ALDO (250 μg); at 1200 methylprednisolone (250 μg); at 1400 CAN (250 μg); at 1600 CAN (1,000 μg); and at 1800 CAN (5,000 μg). The apparent amount of CORT was determined in each blood sample by RIA.

**Experiment 3: acute ACTH response to MR antagonist CAN.** Five days after the jugular vein cannula was implanted, manual blood samples were collected from rats for ACTH determination. Blood samples were collected at 20, 10, 20, 30, 40, 50, and 60 min following an intravenous administration of CAN (5,000 μg) or saline at 1800. Plasma concentrations of ACTH were determined by immunoradiometric assay.

**Experiment 4: administration of MR antagonist CAN prior to methylprednisolone challenge.** Five days after the jugular vein cannula was implanted, rats were connected to the automated blood sampling system. Blood samples were collected every 5 min from 1602 until 2202 and thereafter every 10 min until 0802 the following morning. At 1800 (during the 2- to 3 min quiescent period between blood sample collection) the jugular vein cannula was disconnected from the system, and a bolus of CAN (250 or 5,000 μg in 0.1 ml) was administered. The line was flushed with saline (0.15 ml) and reconnected to the system. Ten minutes later, all animals received a bolus of methylprednisolone (250 μg in 0.1 ml) via the jugular cannula, which was then flushed with 0.15 ml saline and reconnected to the system. CORT concentrations in diluted whole blood were determined by RIA.

**Experiment 5: administration of MR antagonist CAN prior to ALDO challenge.** Five days after the jugular vein cannula was implanted, rats were connected to the automated blood sampling system. Blood samples were collected every 5 min from 1532 until 0457 the following morning, a total of 13.5 h. At 1800 (during the 2- to 3 min quiescent period between blood sample collection), the jugular vein cannula was disconnected from the system and a bolus of CAN (250 or 5,000 μg in 0.1 ml) or saline was administered. The line was flushed with saline (0.15 ml) and reconnected to the system. Ten minutes later, all animals received a bolus of ALDO (250 μg in 0.25 ml 2% EtOH in saline) or saline via the jugular vein cannula, which was then flushed with 0.15 ml of saline and reconnected to the system. CORT concentrations in diluted whole blood were measured by RIA.

**Experiment 6: administration of GR antagonist mifepristone prior to aldosterone challenge.** At the time of surgery, rats received both jugular vein and subcutaneous cannulae. Five days later, rats were connected to the automated blood sampling system via the jugular cannula. Blood samples were collected every 5 min from 1532 until 0457 the following morning, a total of 13.5 h. The GR antagonist [100 mg/kg mifepristone (RU-38486) in 0.20–0.25 ml] or vehicle [20% DMSO in polyethylene glycol (PEG 300)] was administered via the subcutaneous cannula at 1705, and each line was flushed with 0.1 ml of PEG 300. When the subcutaneous cannula for drug delivery was being used, it was not necessary to handle the animal, or to interrupt blood sampling via the jugular vein cannula. With this vehicle, mifepristone remained in solution until it came into contact with an aqueous environment. Immediately after administration, mifepristone-treated rats briefly scratched the area under the skin where the drug had precipitated. At 1810 (during the 2- to 3 min quiescent period between blood samples), the jugular vein cannula was disconnected from the system, and a bolus of saline or ALDO (250 μg in 0.25 ml 2% EtOH in saline) was administered. The line was flushed with saline (0.15 ml) and reconnected to the system. CORT concentrations
in diluted whole blood were measured by RIA. At the completion of blood sampling, the presence of residual mifepristone was visually confirmed in the subcutaneous pocket of all treated animals.

**Drugs**

The following were obtained from Sigma: dexamethasone, CORT, ALDO, CAN, mifepristone (RU-38486 or RU), and DMSO. PEG 300 was obtained from FLUKA. Methylprednisolone sodium succinate (SoluMedrone) was obtained from Pharmacia (Milton Keynes, UK) and prepared according to the manufacturer’s instructions. All drugs were prepared immediately prior to injection. Mifepristone was first dissolved in the smallest possible volume of DMSO prior to dilution with PEG 300. ALDO was first dissolved in a small volume of ethanol prior to dilution in pyrogen-free saline. CAN dissolved readily in saline.

**Statistical Analyses**

Data are presented as individual profiles from a single animal, group means only, or group means ± SE. For all statistical tests, significance at \( P < 0.05 \) was used. Within each group, the effect of steroid treatment over time was determined by one-way repeated-measures analysis of variance (RM-ANOVA) whereas comparisons between treatment groups was analyzed using two-way RM-ANOVA. The post hoc tests used for each analysis are cited in the text. Some of the CORT profiles were separated into smaller regions, and the areas under the curves (AUCs) for each animal’s profile was determined. The AUCs for each treatment were then compared by either one-way or two-way ANOVA with post hoc tests as appropriate.

The onset of CORT pulsatility was determined using the PULSAR algorithm (31) with differences among group parameters analyzed using the nonparametric Kruskal-Wallis test. Deconvolution analysis (47, 48) of the CORT time series was used to determine the mass of secretory episodes of CORT before and after ALDO and methylprednisolone treatment. For this analysis, the half-life of CORT was fixed at 10 min on the basis of published data in female rats (19, 51).

**RESULTS**

**Experiment 1: Acute CORT Response to MR Agonist ALDO or MR Antagonist CAN**

Neither of the VEH injections (saline or 2% EtOH in saline) caused a CORT stress response; therefore, data from both groups was combined. Figure 1 shows the blood CORT concentra-
centrations after a single intravenous bolus of CAN, ALDO, or VEH just prior to lights off.

VEH-treated rats have discrete pulses of CORT (Fig. 1C) similar to those observed in our previous studies. These pulses were not synchronized between animals; thus, group means for these animals are relatively straight lines with high variation. Comparisons of separate time points in each treatment group when compared with the VEH controls often failed to show significance because of the high degree of variation that is to be expected with asynchronous pulsatile data. Within each treatment group, CORT values were therefore compared with baseline values by RM-ANOVA prior to each injection. There was no effect of time within the VEH group. Treatment with the MR antagonist CAN produced two CORT peaks; the earliest peak at 2–3 min postinjection was subsequently shown to be the result of cross-reactivity of the CAN with the CORT RIA (see experiment 2). In the second peak, CORT concentrations were elevated by the 18-min sample and remained elevated for the following 45 min ($P < 0.05$, RM-ANOVA), before the resumption of CORT pulsatility.

Acute administration of the MR agonist ALDO resulted in a decline in CORT concentrations, which was significant by 23 min and remained low for a further 15 min before pulsatility resumed ($P < 0.05$; RM-ANOVA).

The AUCs were calculated for an hour after the cross-reactivity peaks (1812–1912) were compared by Kruskal-Wallis one-way ANOVA with LSD post hoc test ($P < 0.05$). The AUC for ALDO (1,637 ± 204) was significantly lower than that for VEH (5,205 ± 1,016), whereas that for CAN (14,793 ± 1,923) was significantly higher than for VEH. The transient effects of the acute injection of ALDO were also analyzed using deconvolution analysis for the ALDO and VEH group for the period 2 h before and 4 h after injection. The first complete secretory event after the one during which the injection occurred was significantly lower ($P < 0.01$, Mann-Whitney test) by mass in the ALDO (30 ± 12; $n = 5$) compared with the VEH (207 ± 73; $n = 5$)-treated rats. The timing of this secretory event was not different for either group, with the midpoint of this event occurring 40 ± 14 min after injection. There were no differences between the two groups in the masses of the secretory events immediately before or after this event.

Experiment 2: Cross-Reactivity of Steroids With CORT RIA

The apparent concentrations of CORT in ADX rats given intravenous bolus injections of ALDO, methylprednisolone, and CAN are presented in Fig. 2. Significant elevations of CORT above baseline were determined by RM-ANOVA with post hoc pairwise comparisons. Compared with baseline values, there was no significant increase in CORT after ALDO or low-CAN (250 μg). For the methylprednisolone and the mid dose of CAN (1,000 μg), only the first time points (3 min after bolus) were significantly greater than baseline, yielding respective values of 22 and 18 ng/ml greater than baseline values. For the high dose of CAN (5,000 μg) CORT values were greater than baselines for the first two samples (i.e., 3 and 8 min). At these time points, cross-reaction of CAN with the CORT antibody resulted in an apparent elevation of CORT by 81 and 11 ng/ml, respectively. At the third time point after the highest dose of CAN (i.e., 12 min) CORT concentrations were not significantly different from baseline CORT values prior to intravenous injection.

Experiment 3: Acute ACTH Response to MR Antagonist CAN

Concentrations of plasma ACTH were low for both groups prior to injection [CAN 53 ± 11 pg/ml, VEH (saline) 59 ± 14 pg/ml, mean ± SD]. There was a rapid and robust ACTH response to CAN (Fig. 3). For the ACTH data there was a significant effect of treatment (CAN vs. VEH, $P < 0.001$), time ($P < 0.001$), and interaction between the two by two-way RM-ANOVA. Individual differences were determined by Fisher’s LSD post hoc test ($P < 0.05$). Within the VEH group there was no effect of time, indicative of the lack of a stress response to injection and blood sampling. For the CAN-treated rats, however, ACTH concentrations at 10, 20, and 30 min were significantly higher than those of the VEH-treated rats.

Experiment 4: Administration of MR Antagonist CAN Prior to Methylprednisolone Challenge

The CORT profiles of rats pretreated with saline, low-CAN, (250 μg), or high-CAN (5,000 μg) prior to the intravenous methylprednisolone feedback challenge are presented in Fig. 4. An example of the suppressive effects of methylprednisolone on endogenous CORT is shown in Fig. 1D. From visual inspection of the profiles, it appears that any pulse that has been initiated at the time of methylprednisolone injection will be completed; however, subsequent pulses are suppressed for a period of 4–5 h. This was confirmed by deconvolution analysis. Rats exhibited either no new secretory events or a few small secretory events (typically <15% by mass). The last point in a major secretory event ranged from 8 to 40 min (mean ± SD 20 ± 11) after methylprednisolone injection depending on whether the methylprednisolone injection occurred near the end or the beginning of an endogenous pulse.

Early Effects of CAN Pretreatment (1800–1930)

To determine when CORT concentrations were affected by the steroid injections, values within each treatment group were compared with baseline values prior to each injection by
one-way RM-ANOVA with pairwise multiple comparisons using the Holm-Sidak method. For the group treated with saline then methylprednisolone, CORT concentrations were significantly ($P < 0.05$) lower than baseline by the sample collected 33 min after methylprednisolone injection. For animals pretreated with low-CAN the decline in CORT concentrations was significant ($P < 0.05$) one sample earlier i.e., at 28 min after methylprednisolone injection. The effects of low-CAN on CORT decline is not likely to be different from that of saline because the time difference is not greater than twice the blood sampling interval, which in this case is 5 min. For the animals treated with high-CAN then methylprednisolone, CORT concentrations were significantly elevated ($P < 0.05$) 13 min after the CAN was administered and remained high for 45 min. To compare treatment effects among the groups, the CORT AUCs were subdivided into smaller regions and the AUCs for each region calculated (see Table 1). The basal region included all time points prior to the first injection (1602–1757). The region for acute effects included time points from 1812 until 1932. The first two points after the CAN injection were excluded due to a brief period of cross-reactivity of CAN with the CORT. This was based on the results in experiment 2. The results of the CAN-only treatment in experiment 1 were also included. Preinjection basal values were not different among the four groups (Kruskal-Wallis one-way ANOVA). The methylprednisolone-induced inhibition of circulating CORT was not affected by prior treatment with low-CAN compared with saline pretreatment (AUC 3,152 ± 363 vs. 1,990 ± 274). Both of these groups, however, had significantly lower CORT levels than both groups that received high-CAN ($P < 0.05$; Table 1). Methylprednisolone treatment after the higher dose of CAN did not affect the CORT response compared with the CAN-only group (AUC 14,873 ± 1,657 vs 16,201 ± 2,282).

Delayed Effects of CAN Pretreatment (1930–0800)

Following an injection of methylprednisolone, CORT concentrations were suppressed for several hours before CORT pulsatility resumed. To determine the duration of CORT pulse inhibition, the time of the first point in a peak (as detected by PULSAR algorithm) was determined for each animal. Pulsatility resumed at 2248 ± 0026 h after methylprednisolone treatment; prior treatment with low-CAN (Fig. 1E) did not affect the timing of pulse resumption (2304 ± 0026 h), whereas pretreatment with high-CAN significantly delayed the onset of CORT pulsatility ($P < 0.01$, Kruskal-Wallis AOV with Dunn’s multiple pairwise comparisons) an addition 4–5 h to 0319 ± 0113 h the next morning. For some of the animals treated with high-CAN although pulsatility had not resumed by the end of sampling at 0800 (Fig. 1G), for statistical analysis a value of 0800 was used for these animals, therefore the additional delay may be an underestimate. The high dose of CAN alone did not suppress pulsatility in experiment 1, because pulsatility resumed at 2001 ± 0034 h (mean ± SD; $n = 7$) in that group, which was immediately after CORT concentrations declined following the CAN injection.

Experiment 5: Administration of MR Antagonist CAN Prior to ALDO Challenge

CORT profiles before and after CAN and/or ALDO challenge are presented in Fig. 5. The means only for all four groups is presented in Fig. 5A, and for comparison the mean of the VEH-treated rats from experiment 1 is also included. Visual inspection of the data suggests that the main effects of these steroids occur in the first hour after administration. The effects of the intravenous ALDO challenge were similar to those described in experiment 1. CORT concentrations within the saline-then-ALDO group were analyzed by RM-ANOVA with pairwise multiple comparisons by the Holm-Sidak method. CORT concentrations after the ALDO challenge were significantly lower ($P < 0.05$) than preinjection concentrations at the 28-min sample and remained low for a further 20 min before returning to preinjection values. An example of this rapid and transient suppression of circulating CORT by an intravenous bolus of ALDO is presented in Fig. 5B. To compare among the four treatment groups, the CORT profiles were divided into sections and analyzed by AUC and one-way ANOVA. The baselines (values prior to iv injection) were not different among the groups. For the first hour following the second intravenous injection, the groups means ± SE are presented in Fig. 5C, with the corresponding AUC in Fig. 5D. The higher dose of CAN clearly elevated circulating CORT with the AUC highest for both groups given high-CAN; these values were significantly higher than for animals that received low-CAN or saline as a first injection ($P < 0.05$, one-way ANOVA with post hoc tests). For the two groups that received high-CAN, the AUC for the first hour was not affected by subsequent ALDO vs. saline treatment. There were no longer-term effects of ALDO or CAN treatment detectable from 2100 until the end of sampling at 0500, i.e., in the period 3–11 h after steroids were administered. The AUCs for this period were not different among the four groups (one-way ANOVA); neither were there any differences in the number of pulses detected by the PULSAR algorithm among the groups (one-way ANOVA) with the mean number of pulses ranging from 11 to 13.

Experiment 6: Administration of GR Antagonist Mifepristone Prior to ALDO Challenge

Group means ± SE for the CORT profiles for all four groups (VEH$_s$/VEH$_i$, VEH$_s$/ALDO$_i$, RU$_i$/VEH$_s$, RU$_i$/ALDO$_i$) are presented in Fig. 6. The CORT profiles were analyzed by AUC for four separate regions of the profile. First, the basal region from 1532 to 1702; second, the hour immediately after the subcutaneous injection of mifepristone or VEH (1707 to 1812). In these comparisons the null hypothesis was not rejected for any region. This was based on the results in experiment 1, where pulsatility was not detectable from 2100 until the end of sampling at 0500, i.e., in the period 3–11 h after steroids were administered. The AUCs for this period were not different among the four groups (one-way ANOVA); neither were there any differences in the number of pulses detected by the PULSAR algorithm among the groups (one-way ANOVA) with the mean number of pulses ranging from 11 to 13.
Fig. 4. CAN treatment prior to Mpred challenge. Profiles of blood CORT in animals sampled at 5- to 10-min intervals for 16 h. Animals were given an iv bolus of VEH (n=9), low-CAN (n=6), or high-CAN (n=10) 10 min prior to the iv bolus of Mpred (250 μg). Vertical bars or arrows represent times of iv injections. Horizontal bar, period of lights off.

A: group means only of the 3 groups for the entire sampling period. Group means for high-CAN only and VEH only from Fig. 1 are also included for comparison.

B: group means ± SE around the times of injection.

C: duration of pulse suppression by Mpred for each treatment, as determined by PULSAR analysis. *Significant differences (P < 0.05) by Kruskal-Wallis ANOVA.

D–G: representative profiles to show suppression of CORT pulsatility by Mpred and enhanced suppression associated with prior high-CAN treatment.
third, the period immediately after the intravenous challenge with ALDO or VEH until lights off (1812 to 1917); and fourth, the full duration of the dark phase (1922 to 0458). There were no differences among the four groups during the basal period or in the first hour after the subcutaneous injection of the mifepristone by one-way ANOVA. The AUCs for the four groups immediately after the intravenous challenge were analyzed by two-way ANOVA with Tukey’s test. There was a significant effect of the intravenous challenge (ALDO vs. VEH) \( F(1,34) = 19.6, P < 0.001 \) and the effect of subcutaneous injection.

### Table 1. AUC for corticosterone concentrations in animals pretreated with CAN prior to Mpred challenge

<table>
<thead>
<tr>
<th>AUCs, times</th>
<th>Basal 1602 to 1757</th>
<th>Acute 1812 to 1932</th>
<th>Delayed 1932 to 0802</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline then Mpred</td>
<td>9,367 ± 1,579 (9)</td>
<td>3,152 ± 636 (9)*</td>
<td>17,149 ± 2,819 (9)</td>
</tr>
<tr>
<td>Low-CAN then Mpred</td>
<td>8,319 ± 1,549 (6)</td>
<td>1,990 ± 274 (6)*</td>
<td>15,365 ± 3,680 (6)</td>
</tr>
<tr>
<td>High-CAN then Mpred</td>
<td>10,838 ± 1,684 (10)</td>
<td>14,873 ± 1,657 (10)*</td>
<td>15,345 ± 3,260 (10)</td>
</tr>
<tr>
<td>High CAN only (experiment 1)</td>
<td>10,542 ± 1,727 (7)</td>
<td>16,201 ± 2,282 (7)*</td>
<td>26,397 ± 4,767 (6)</td>
</tr>
</tbody>
</table>

*P Value

| Value | 1NS (P = 0.73) | 2NS (P = 0.16) |

Values are means ± SE; nos. in parentheses, n. CAN, canrenoate; Mpred, methylprednisolone. Superscripts differ significantly, by Dunn’s pairwise multiple comparisons (P < 0.05).
neous drug treatment (RU vs. VEH) just reached significance \( F(1,34) = 4.2, P = 0.048 \); there was no significant interaction between the two treatments. The AUCs for the four groups during the dark phase were also analyzed by two-way ANOVA (Fig. 2C); however, the only significant effect was that of subcutaneous (RU vs. VEH) treatment \( F(1,34) = 5.1, P < 0.05 \), with the AUC for RU-treated rats 33% greater than for the rats treated subcutaneously with VEH (29,387 ± 2,145 vs. 22,031 ± 2,218). There was no significant effect of intravenous (ALDO or VEH) treatment on the CORT AUC during the dark phase.

**DISCUSSION**

The mechanisms underlying fast feedback within the HPA axis have puzzled physiologists ever since rate-sensitive fast feedback was first described by Dallman and Yates in 1969 (10). Demonstration of the disruption of normal fast feedback in aging humans (7) and in depressed patients (52) implies a role for fast feedback in the maintenance of homeostasis. Fast rate-sensitive feedback of the HPA axis in the rat has primarily been demonstrated by using a rapid presentation of CORT to the circulation in the anesthetized animal and to date has looked at these inhibitory effects only on subsequent stress responses. In the current study we have used our automated blood-sampling system to investigate fast feedback in conscious, freely behaving animals in their home cage. Moreover, with this system we have shown inhibition of basal, i.e., unstressed, HPA activity. Rapid presentation of exogenous steroids by intravenous bolus was used as a tool to investigate the steroid selectivity of this inhibition. For these studies we have focused on the role of the MR, which has been shown, in vitro, to be critical for rapid nongenomic actions of GRs in the hippocampus of the mouse (26). To clarify the importance of individual components of the fast feedback mechanism, we first needed a robust model of HPA feedback. This was achieved by rapid administration via an intravenous bolus of methylprednisolone, a corticosteroid with both MR and GR activity and of ALDO, a predominantly MR agonist (20). The intravenous injection resulted in a rapid increase in the circulating concentration of exogenous corticosteroid with resultant inhibition of basal HPA activity. This experimental model is analogous to previous models of the CORT-mediated fast rate-sensitive feedback of stress-induced HPA activity described in the rat in which a rising concentration of steroid is induced by intraperitoneal or intravenous administration (2, 10, 25, 27).

Administration of the MR agonist ALDO resulted in a rapid but transient inhibition of endogenous CORT, with a reduction

![Fig. 6. Glucocorticoid receptor (GR) antagonist treatment prior to ALDO challenge. Animals were treated with a sc bolus of mifepristone (RU-38486; RU_sc) or VEH (VEH_sc) at 1705 and then with an iv bolus of ALDO (ALDO_iv) or VEH (VEH_iv) at 1810 (vertical line). Horizontal bars, period of lights off. A: profiles of blood CORT in rats sampled at 5-min intervals across the full sampling period. Values are means ± SE. B: detailed view of the same data around the time of ALDO challenge. C: AUC for the period between iv challenge and lights out. AUCs were analyzed by 2-way ANOVA. Significance values of reduced CORT levels following ALDO, for both sc treatments are indicated (Tukey’s post hoc test).](http://ajp.endo.physiology.org/)
of CORT concentrations within 23 min and continuing low for a further 15 min before the resumption of pulsatility. Resolving the CORT profile into secretory events by deconvolution analysis demonstrated that the predominant effect of ALDO was a short-lived reduction in the magnitude of secretory events. Any secretory event that had commenced at the time of the ALDO injection was completed, but the subsequent secretory event had a substantially smaller mass (15%) compared with preinjection values. This was the only secretory event significantly affected by ALDO. To our knowledge, this is the first report of the efficacy of an MR agonist (ALDO) to induce a fast-feedback signal on basal HPA activity. Previous studies of fast feedback on basal HPA activity have been performed in humans and have used either cortisol or dexamethasone (7, 11, 17, 18). Fludrocortisone, a potent MR agonist, has been used to inhibit basal HPA activity in humans; this was, however, shown to be effective only at the nadir of the rhythm when MR had been depleted of endogenous ligand by prior treatment with metyrapone to reduce circulating cortisol concentrations; furthermore, the effect became apparent only after at least 1 h (35). In a rat model of stimulated HPA activity, one study has shown that ALDO is as effective as cortisol or dexamethasone on fast feedback of CRH-induced ACTH secretion (24).

The effects of the bolus administration of the MR agonist ALDO were mirrored by the administration of an MR antagonist. Although CAN has limited affinity for MR, in vivo it is rapidly converted to canrenone, with which it is in equilibrium. Canrenone is a major metabolite of spironolactone with between one-half and one-tenth of its MR activity (9, 42). Following the administration of the water-soluble CAN, there was a rapid activation of the HPA axis that was short in duration. CORT concentrations were elevated within 23 min and remained elevated for about 1 h. Confirmation that the CAN was acting centrally rather than at the level of the adrenal glands was provided by studies on the ACTH response. Within 10 min of the administration of CAN (the first blood sample we collected after CAN), plasma ACTH had already reached peak levels, and concentrations declined to basal values by 40 min. The rapid effects of MR antagonist administration on HPA activity is not consistent with previous studies using peripheral administration in rats. This is most likely due to our mode of administration (intravenous) as well as our more frequent blood sampling. Although it is widely accepted that MR is important for regulation of basal CORT (13, 40, 41), particularly at the diurnal trough, administration of MR antagonists do not always result in the elevation of basal HPA activity. Intracerebroventricular administration of the MR antagonist RU-23818 elevate ACTH and CORT at both the diurnal trough and the diurnal peak (34, 38, 46), whereas peripheral administration (subcutaneous) of mifepristone using doses known to reduce the amount of available MR in the hippocampus by more than 80% elevates CORT in some (43), but not all, morning studies (36, 38). In the studies where peripheral administration of the MR antagonist did elevate basal CORT, the earliest time point studied was typically 1 h, whereas in the studies using intracerebroventricular administration basal CORT levels were elevated by 30 min but not at 15 min, and the effects lasted about 1 h (38). Therefore, in rats, intracerebroventricular administration of an MR antagonist has a more robust effect than peripheral administration on elevating basal CORT, and where it was measured these effects were all shown to be mediated by ACTH. A slightly different picture emerges from human studies using the MR antagonists spironolactone (given orally) and CAN (given as a bolus and then a continuous infusion). There is a consistent elevation of circulating cortisol at both the diurnal trough and peak after administration of the MR antagonist, but this effect takes at least 1–2 h even though blood samples are collected frequently and early enough to detect rapid changes (5, 21).

More importantly, these elevations in cortisol were not associated with any changes in circulating ACTH concentrations, with the exception of one study, in which there was an increase in ACTH 2–3 h after that of cortisol (5).

Methylprednisolone has both MR and GR potency (20). Our laboratory has previously used a water-soluble form of this steroid, methylprednisolone sodium succinate (4), to characterize rapid inhibition of endogenous CORT. The sodium succinate is rapidly hydrolyzed in vivo with the concentration of methylprednisolone peaking 5 min after the injection of methylprednisolone sodium succinate (8, 23). Although the onset of the timing of CORT inhibition by ALDO (23 min) was similar to that of methylprednisolone (28–33 min), the duration of inhibition was very different (1 pulse vs. 4–5 h). This may be due to their relative affinities for MR and GR rather than the different half-lives of the steroids: ALDO has a $t_{1/2}$ of 10–15 min (33) and methylprednisolone 25 min (23). ALDO is predominantly an MR agonist, whereas methylprednisolone activates both MR and GR, albeit with greater affinity for the GR than for the MR (20). To test whether the rapid inhibition of CORT by methylprednisolone is mediated by its MR activity, rats were pretreated with the MR antagonist CAN prior to methylprednisolone injection. The CORT profile in rats treated with the higher dose of CAN prior to methylprednisolone was no different to that seen in rats given CAN alone. The lack of inhibition of CORT by methylprednisolone in these CAN-treated rats suggests that the effects of methylprednisolone are not downstream of those of CAN (e.g., pituitary rather than hippocampus or hypothalamus). The fact that CAN was effective only at the higher dose probably reflects its lower affinity for the MR (9, 42). Our results do not allow us to deduce the site of action of these compounds since, although MR and GR are abundant in the hippocampus and GR is the main GR receptor in the PVN and pituitary, MR is also present, albeit at much lower levels in both the PVN and pituitary (22, 44).

The delayed actions of methylprednisolone in CAN-pretreated rats were unexpected. If MR also mediates the delayed actions of methylprednisolone, then the blockade of the MR with CAN should have reduced the duration of CORT inhibition. Contrary to this, we actually observed that methylprednisolone inhibition of CORT pulsatility was significantly prolonged (8–9 vs. 4–5 h) when MRs had been blocked by CAN pretreatment. It would appear that that inhibition of CORT, when only GRs are available, is more prolonged than when both GR and MR are available. This may be related to greater duration of inhibition following DNA binding by GR homodimers than GR/MR heterodimers. Reduced clearance of methylprednisolone in CAN-pretreated rats is unlikely to explain our observation, since methylprednisolone does not bind to rat corticosteroid-binding globulin (28) and is therefore rapidly cleared from the circulation. Moreover, in humans, prior treatment with CAN does not alter the pharmacokinetics of glucocorticoids (3).
Although ALDO is a selective MR agonist, the high concentrations achieved after an intravenous bolus could be sufficient to activate GRs as well as MRs (30). To confirm that the effect of ALDO was mediated through an MR rather than a GR mechanism, rats were pretreated either with the MR antagonist CAN (experiment 5) or with the GR antagonist mifepristone (experiment 6) prior to the ALDO challenge. The GR antagonist was administered subcutaneously at a dose in excess of that required to access the brain and block classic GR-mediated feedback (35, 37). Although ALDO inhibited CORT in the saline- and the low-CAN-treated rats, there was no inhibition of CORT in the rats previously treated with the higher dose of CAN. Furthermore, blockade of GR by pretreatment with high-dose mifepristone 1 h earlier did not block the inhibition of CORT by ALDO. Taken together, these data provide strong evidence that the feedback effect of ALDO on HPA activity occurs through MR- and not GR-mediated mechanisms. Administration of mifepristone itself did elevate CORT concentrations but not within the first hour. The mean concentration of CORT was increased by 33% during the dark phase; this was achieved without disrupting pulsatility or altering the number of pulses.

Our data provide strong evidence that MR dynamically regulates CORT concentrations under basal conditions, even at a time of day when the receptor is at its highest level of occupancy by the endogenous ligand CORT. The question of how the presumably “always occupied” MR can still respond to rapid changes in circulating corticosteroids is not new (32). Although tissues such as the kidney utilize the enzyme 11β-hydroxysteroid dehydrogenase to inactivate cortisol or CORT, this cannot explain HPA responses to corticosteroid feedback.

In rodents, the MR does have a higher affinity for ALDO than for CORT, and it is conceivable that the transiently high ALDO concentrations used in our study might have differential effects on the kinetics of MR translocation and binding to DNA and thus result in altered signal transduction. Indeed, the effects of corticosteroids can display a U-shaped variation in the responsiveness of the hypothalamic-pituitary-adrenal axis (13). Given the time frame of the effects observed in our studies, a more likely explanation is that the MR-mediated inhibition of CORT is not via the classical intracellular receptor but occurs through a putative membrane-associated form of the MR that has similar ligand specificity but signals through more rapid, nongenomic mechanisms. In keeping with this suggestion, an in vitro study in mice (26) has demonstrated an MR-mediated fast-onset, rapidly reversible, and nongenomic enhancement by CORT of glutamate transport in the hippocampus, an area important in HPA feedback. This response occurred through a receptor mechanism that showed MR pharmacology with respect to its interaction with agonists/antagonists but a lower affinity for CORT and ALDO suggesting a difference in MR conformation. The necessity for a 10- to 20-fold higher concentration of ligand for activation of the putative membrane-bound MR compared with the classical intracellular receptor would enable a rapid response to CORT at times of the day when the intracellular MRs are fully occupied by endogenous CORT. Further studies that measure free steroid concentrations in the brain (such as microdialysis) and simultaneously in the blood are needed to elucidate the relationship between the rise in circulating steroid and whether the concentrations achieved are able to activate membrane-associated receptors in the hippocampus.

Our data characterizing the role of MR in mediating fast feedback does not exclude the possibility of other rapid feedback mechanisms. Indeed, ALDO, cortisol, and dexamethasone were equally effective at inhibiting the CRH-induced stimulation of ACTH (28), suggesting a possible additional role for GR, and there is also in vitro evidence for a membrane-associated GR at hypothalamic level that can activate an endocannabinoid signaling system (15).

The physiological significance of fast feedback is an important question. Previous studies on CORT fast feedback in the rat have suggested that it is the rate of the rise in the steroid, rather than its absolute concentration, that is important (27). Under physiological conditions, a rapid rise in circulating CORT occurs with the initiation of a stress response and is also a fundamental feature of basal CORT pulsatility. Fast feedback is thought to be important for the termination of stress responses (14) or to limit stress responsiveness when stressors occur in rapid succession (27). We propose that MR-mediated fast feedback of basal HPA activity might also be an important factor in the generation of CORT pulses, as the inhibitory response is rapid enough to function within the time span of ultradian CORT pulsatility. This could explain the alternating phases of activation and inhibition that characterize CORT pulses (50). Thus during the secretory phase of a pulse, the rapid rise in CORT produces a fast feedback signal that results in the subsequent inhibitory phase of a pulse during which CORT is cleared from the circulation. This inhibitory phase is short in duration thus allowing initiation of a new pulse.

In conclusion, our study provides the first in vivo evidence for a role of MR in fast feedback regulation of basal HPA activity. Circulating CORT concentrations can be dynamically regulated by MR at a time when these receptors are predominantly occupied by endogenous ligand. We propose that MR-mediated fast feedback of the HPA axis may be important for homeostasis and in the generation of CORT pulsatility.

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