Nycthemeral rhythm in adrenal responsiveness to ACTH

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DALLMAN, MARY F., WILLIAM C. ENGELAND, JAMES C. ROSE, CHARLES W. WILKINSON, JEANETTE SHINSAKO, AND FREDERICK SIEDENBURG. Nycthemeral rhythm in adrenal responsiveness to ACTH. Am. J. Physiol. 235(5): R210-R218, 1978 or Am. J. Physiol.: Regulatory Integrative Comp. Physiol. 4(3): R210-R218, 1978. - Adrenal adenosine 3',5'-cyclic monophosphate (CAMP) and corticosterone responses to exogenous ACTH were found to be about 2.5 times greater in the evening (at lights off) than in the morning (at lights on) in rats. The rhythm in adrenal responsiveness to ACTH was found to persist in rats treated with dexamethasone 15 and 3 h before exogenous ACTH (in the presumed absence of a rhythm in endogenous ACTH). Treatment with p-chlorophenylalanine did not affect the daily rise in circulating ACTH levels but did abolish the rhythm in adrenal responsiveness to ACTH. The magnitude of the rhythm in adrenal responsiveness to ACTH is greater than the magnitude of the rhythm in ACTH. Because the rhythms are dissociable, we conclude that in vivo measurements of adrenal corticosteroid levels do not necessarily reflect ACTH levels.

ACTH rhythm; adrenal rhythm; PCPA; dexamethasone; adrenal cyclic AMP; corticosterone

MARKED CIRCADIAN FLUCTUATIONS in plasma corticosteroid levels have been thoroughly documented in man, other primates, and rodents (1, 9, 12, 17). There is general agreement that under normal conditions corticosteroids rise to a peak at the start of the daily activity cycle. Rats, which are active nocturnally, exhibit peak plasma corticosterone levels at, or just prior to, dark (9).

In a recent study of the plasma ACTH and corticosterone responses of the rat to stressful stimuli applied at the peak (lights off) or trough (lights on) of the corticosterone rhythm, we obtained evidence for a difference in adrenal responsiveness to endogenously secreted ACTH between the two times of the day (5). Evidence for changing adrenocortical responsiveness to exogenously administered ACTH as a function of the time of day when ACTH was given has been found in man (7, 14, 15, 18). In both man and the rat, it appears that the adrenal is maximally responsive to ACTH when circulating ACTH levels are at their circadian maximum. In studies of man the change in adrenal responsiveness to ACTH was ascribed to the influence of changing circulating levels of endogenously secreted ACTH (14, 15, 18).

Against the interpretation that circadian increases in circulating levels of ACTH increase adrenocortical responsiveness to ACTH, are reports that plasma corticosterone levels retain a circadian rhythm after hypophysectomy (and the presumed elimination of cyclical changes in circulating ACTH levels) in both the killifish (22) and the rat (11). These results strongly suggest that the rhythm in adrenocortical responsiveness to ACTH is determined by a mechanism that does not depend upon a daily rhythm in circulating ACTH levels.

In this study we have attempted to determine whether there is a nycthemeral change in the responsiveness of the rat adrenal gland to exogenously administered ACTH, and whether such a change includes alterations in the adenosine 3',5'-cyclic monophosphate (CAMP) response. In addition, we have attempted to dissociate the rhythms in ACTH and adrenal responsiveness to ACTH using either multiple treatments with dexamethasone to inhibit the ACTH rhythm, or treatment with p-chlorophenylalanine, a drug that has been reported to alter the nycthemeral rhythm in plasma and adrenal corticosterone (20, 24, 25), to inhibit the adrenal rhythm.

MATERIALS AND METHODS

Male or female Sprague-Dawley rats weighing 100-150 g at the time of the experiment were received from Simonsen suppliers 10-14 days before use and were placed two or three to a cage in hanging wire baskets. The animals were maintained in rooms with a controlled light cycle (12 h light:12 h dark) and had continuous access to food (Berkeley Diet rat and mouse chow) and water.

Adrenal responsiveness to ACTH. When used, pentobarbital sodium (Nembutal, Abbott) was given ip in doses of 4.5 mg/100 g body wt to females and 5.0 mg/100 g body wt to males; dexamethasone (Decadron, Merck Sharp & Dohme) was given 25 μg/100 g body wt sc. Synthetic α-24-ACTH (Cortrosyn, Organon) was diluted in 0.005 N HCl-0.9% saline and kept on ice for each experiment. When the individual experiments were separated by 12 h, ACTH was made up twice;
when several groups were to be injected within 12 h, the same solution, kept cold, was used. Several protocols were used to test adrenal responsiveness to ACTH. In the first experiments, female rats were anesthetized with pentobarbital 20 min before intravenous injections of acid saline or 7.5 or 15 ng ACTH into a lateral tail vein. Plasma corticosterone levels were measured 15 min after the injections at lights on and off. In the second experiment, female rats were treated with dexamethasone 2 h before pentobarbital. After 20 min, acid saline, 4 or 8 ng ACTH was injected intravenously and the rats were killed 15 min later for determination of adrenal and plasma corticosterone concentrations. Other, untreated rats were decapitated at the same time of day (6 times during a 24-h period) to determine resting plasma ACTH and corticosterone levels. In the third experiment, female rats were again treated with dexamethasone and pentobarbital and were injected with acid saline, 2 or 4 ng ACTH. Plasma and adrenal corticosterone levels were determined 5 and 10 min after the injections at lights on or at lights off. In the fourth experiment, male rats were treated with dexamethasone and pentobarbital and injected with 2 or 4 ng ACTH intravenously. Groups of rats were killed at zero time (before) and 3, 7, or 15 min after ACTH at lights on and lights off. Plasma corticosterone, and adrenal corticosterone and cAMP concentrations were determined. In the fifth experiment, male rats were pretreated either once at -3 h or twice at -15 and -3 h with dexamethasone. All rats were anesthetized with pentobarbital and injected intravenously at zero time with acid saline, 1, 2, or 4 ng ACTH. Groups of rats were killed at 3 and 15 min after injections at lights on and lights off. In the sixth, seventh, and eighth experiments, male rats were treated at -72 and -48 h with either p-chlorophenylalanine methyl ester (PCPA) 32 mg/kg body wt or 0.9% saline (0.6 ml/rat). Some rats were treated at -3 h with dexamethasone. At zero time, either at lights on or at lights off, rats not treated with dexamethasone were decapitated for measurement of circulating ACTH and corticosterone levels and adrenal corticosterone concentration under resting conditions. The dexamethasone-blocked rats were injected with saline, 50 or 100 ng ACTH/100 g body wt ip. Plasma and adrenal corticosterone concentrations were measured 15 min after ACTII at lights on and lights off.

A final, nonexperiment was performed in which rats were decapitated without prior treatment at lights on or lights off and plasma ACTH and corticosterone and adrenal corticosterone were determined. These resting levels were collected from not more than 8 rats/time (16 rats/day) over a period of 6 mo.

All rats were killed by decapitation and trunk blood was collected into iced, heparinized plastic tubes. Blood was centrifuged at 4°C, and plasma was separated and frozen. Left adrenal glands were removed and placed on filter paper saturated with 0.9% NaCl in dishes kept on ice. In the experiment in which adrenal cAMP content was determined, left and right adrenals from alternate rats were assigned to the cyclic nucleotide and corticosterone assays, so that there were equal numbers of left and right adrenals in each assay. The adrenal to be used for cAMP assay was removed within 60 s of decapitation and snap-frozen on dry ice until homogenized in 1.0 ml 5% trichloroacetic acid. After four extractions with ether the supernatant was lyophilized and processed as directed for measurement with the Amersham cAMP competitive protein binding kit (8). Adrenals collected for corticosterone determination were cleaned of fat and connective tissue, weighed to the nearest 0.01 mg and homogenized 1.500 (wt/vol) in 20% EtOH:0.9% saline (vol/vol). Plasma and adrenal corticosterone concentrations were measured by competitive protein binding (13) using human transcortin, and plasma ACTH was determined by radioimmunoassay (4, 19). All ACTH and corticosterone samples from a single experiment were distributed in the assays so that groups were run across assays and that representative samples from each group were run within each assay. The coefficient of interassay variation is 14% for the ACTH assay and 10% for the corticosterone assay.

Data were analysed using one-, two-, or three-way analysis of variance (ANOVA), for either time of day, time of day vs. dose of ACTH or vs. treatment, time of day vs. dose of ACTH vs. time after ACTH, or time of day vs. dose of ACTH vs. treatment.

RESULTS

Effect of time-of-day on adrenal response to ACTH in pentobarbital-anesthetized female rats without or with dexamethasone pretreatment. In the first experiment, female rats (n = 6/group) were given pentobarbital 20 min before injecting acid saline, 7.5 or 15 ng ACTH iv at the time of lights on, or 12 h later at lights off. Pentobarbital anesthesia was used because it has been reported to decrease plasma corticosterone levels in the evening to levels observed in the morning (27). Comparison of the corticosterone levels in saline-treated rats in the morning and the evening using an unpaired t test revealed that there was no difference in these levels (t = 1.73, P > 0.2). There were significant effects of treatment with ACTH (F = 45.07, P < 0.001), and of time of day (F = 90.25, P < 0.001) on plasma corticosterone levels obtained 15 min after these treatments (Fig. 1). However, there were no differences between corticosterone responses to 7.5 or 15 ng ACTH, either in the morning or in the evening, suggesting that both doses of ACTH had produced a maximal adrenal response 15 min later.

In the second experiment, groups of six rats were decapitated without prior treatment at 4-h intervals during the 24-h day and plasma ACTH and corticosterone, and adrenal corticosterone levels were measured. Other groups of 14 rats were treated with dexamethasone 2 h before death, and 20 min after pentobarbital these animals were injected intravenously with saline (n = 2), 4 ng (n = 6), or 8 ng (n = 6) ACTH. The rats were killed 15 min after the injections at the same time as the untreated groups and plasma and adrenal corticosterone levels were measured. Figure 2 shows that
the lowest plasma ACTH and corticosterone levels in resting rats and those stimulated with ACTH were observed at lights on (0600 h), and that peak resting levels and responsiveness to ACTH were observed 12 h later at lights off (1800 h). One-way ANOVA revealed significant time-of-day effects for all parameters measured except for plasma ACTH (resting ACTH: $F = 2.09, P > 0.1$; resting adrenal B: $F = 7.14, P < 0.001$; resting plasma B: $F = 9.52, P < 0.001$; after 4 ng ACTH: adrenal B: $F = 3.00, P < 0.05$ (data not shown); plasma B: $F = 14.09, P < 0.001$). The results after 8 ng ACTH were similar to those after 4 ng (data not shown); it appeared that 4 ng ACTH was a dose that was adequate to saturate the adrenal corticosterone response 15 min later. These results show clearly that adrenal responsiveness to ACTH is lowest at lights on and highest at lights off. Additionally, because the effect was observed at the adrenal gland as well as in the plasma, the effect is not simply a consequence of altered distribution, binding, or metabolism (DBM) of corticosterone.

In the third experiment, rats were treated with dexamethasone 3 h before pentobarbital anesthesia and injection of saline, 2 or 4 ng ACTH iv (8/group) at lights on or at lights off. The rats were killed 5 or 10 min after the injections and plasma and adrenal corticosterone levels were measured. The results are shown in Fig. 3. An additional group of rats was killed at zero time to determine initial levels. The injection of saline did not change adrenal or plasma corticosterone levels at 5 or 10 min compared to the zero time controls ($F = 1.31, P > 0.7$). In contrast, there were main effects of both ACTH and time of day on both adrenal and plasma corticosterone levels (adrenal: $F = 21.71, P < 0.001$; $F = 23.15, P < 0.001$, respectively; and plasma: $F = 36.90, P < 0.001$; $F = 22.36, P < 0.001$, respectively). There was, however only a suggestion of an increased effect of the 4-ng dose above that observed after 2-ng dose.

Nycthemeral difference in the adrenal cAMP response to ACTH in male rats. To determine whether the daily change in adrenal responsiveness to ACTH occurred before or after the step of activation of adrenal adenylate cyclase, male rats were treated in the morning or the evening with dexamethasone 2.5 h before pentobarbital anesthesia and injection with 2 or 4 ng ACTH (8/group). Adrenal cAMP and corticosterone levels and plasma corticosterone levels were measured 3, 7, and 15 min later (Fig. 4). The results of the three-way ANOVA (dose of ACTH vs. time after ACTH vs. time of day) show that there is a highly significant effect of all three variables on adrenal cAMP and plasma corticosterone levels and of dose of ACTH and time of day on adrenal corticosterone levels (Table 1). The time course of all three responses changes significantly between morning and evening with a more prolonged response accompanying the greater amplitude of the response in the evening (time-time of day interactions, Table 1).

Nycthemeral difference in adrenal responses to ACTH in male rats after single or multiple treatments with dexamethasone. Rats were treated with dexamethasone 3 h before pentobarbital anesthesia and were injected with saline, 1, 2, or 4 ng ACTH iv (n = 8/group). Adrenal and plasma corticosterone levels were determined in the saline-treated group at 3 min and in
the ACTH-treated groups at 3 and 15 min. The adrenal corticosterone response to ACTH was similar to earlier experiments in that ACTH given in the evening was significantly more effective than the same dose given in the morning (Fig. 5) (time of day: $F = 339.47$, $P < 0.001$). Plasma corticosterone levels 3 min after ACTH were not different from saline-treated controls either in the morning or in the evening. In agreement with the adrenal measurements, the two lower doses of ACTH did not result in increases of plasma corticosterone above control levels at 15 min in the morning; the 4-ng dose of ACTH resulted in plasma corticosterone levels of $6.8 \pm 0.4 \mu g/100$ ml (mean $\pm$ SE) at 15 min, significantly above the control level of $1.5 \mu g/100$ ml.

Fifteen minutes after 1, 2, and 4 ng ACTH given in the evening plasma corticosterone levels were $4.3 \pm 0.8, 7.0 \pm 1.3$, and $24.3 \pm 1.2 \mu g/100$ ml, respectively.

To test whether changing levels of ACTH during the preceding 12 h were necessary for the altered adrenal responsiveness to ACTH observed between morning and evening, additional groups were injected twice with dexamethasone 15 and 3 h before pentobarbital treatment and injection with 2 ng ACTH. There were significant main effects of time of day and of dexamethasone treatments when the adrenal and plasma corticosterone responses to 2 ng ACTH were compared in rats treated with dexamethasone either once or twice (Fig. 5) (adrenal: $F = 112.24$, $P < 0.001$, and $F = 28.06$, $P < 0.001$, respectively; and plasma: $F = 31.92$, $P < 0.001$ and $F = 7.01$, $P < 0.01$, respectively). Adrenal corticosterone at 3 min and plasma corticosterone at 15 min are shown in Fig. 6. The adrenal corticosterone response to 2 ng ACTH at 3 min was significantly greater in the evening than in the morning following 15-h pretreatment with dexamethasone; there was no difference between groups treated at $-3$ or at $-15$ and $-3$ h with dexamethasone. However, the 15-min response in adrenal (Fig. 5) and plasma (Fig. 6) corticosterone was affected by longer duration dexamethasone treatment.

**Effect of treatment with PCPA on plasma ACTH and plasma and adrenal corticosterone rhythms.** The results of two similar experiments were pooled and are

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**FIG. 3.** Adrenal and plasma corticosterone levels obtained 5 or 10 min after injection of saline (dotted portion of bars), 2 or 4 ng ACTH at lights on (0600 h) or lights off (1800 h). Female rats (8/group) were pretreated with dexamethasone and pentobarbital before iv injection.

**FIG. 4.** Adrenal cAMP and adrenal and plasma corticosterone responses to 2 ng (A) and 4 ng (B) ACTH given at 0600 h (lights on) and 1800 h (lights off). Male rats (8/group) were pretreated with dexamethasone and pentobarbital before iv injection of ACTH.
Examination of Table 2 reveals that although testosterone levels were significantly affected both by PCPA treatment and by time of day (adrenal: F = 4.93, P = 0.028, and F = 49.91, P < 0.001). In contrast, adrenal and plasma corticosterone levels were not affected by treatment with PCPA, but no time-of-day effect was noted in the adrenal.

In a third experiment, treatment with PCPA appeared to be ineffective. Rats were treated with dexamethasone, and saline or 10, 50, or 100 ng ACTH were injected ip 3 h later at lights on and lights off; animals were killed 15 min after the injection. ANOVA revealed no significant effect of PCPA treatment on adrenal corticosterone (F = 3.38, P = 0.065), however, the usual effects of dose of ACTH (F = 21.17, P < 0.001) and of time of day (F = 4.30, P = 0.038) were observed. In Table 2 the results of the first two experiments with PCPA are expressed as a ratio of the mean levels obtained in the evening divided by those in the morning. Examination of Table 2 reveals that although similar excursions occurred in plasma ACTH levels in both saline- and PCPA-treated rats, the normal rhythm in adrenal responsiveness to ACTH is abolished in rats treated with PCPA.

Plasma ACTH and adrenal corticosterone levels obtained from resting rats in the morning or evening. Plasma ACTH and adrenal corticosterone levels were determined at lights on or at lights off in 152 rats under resting conditions. The results are presented as a histogram with mean adrenal corticosterone levels plotted against 10-20 pg/ml increments in ACTH concentration (Fig. 9). For the same range of plasma ACTH levels (between 0 and 49 pg/ml) adrenal corticosterone levels in the morning were significantly lower than in the evening (P < 0.001). Individual comparisons between mean morning and evening adrenal corticosterone levels were made for the ranges of ACTH 0-19, 20-39, and 40-49 pg/ml; Student's t values are indicated at the top of Fig. 9.

**DISCUSSION**

The results of these studies provide unequivocal evidence that adrenal responsiveness to ACTH changes between morning and evening in the rat as it is reported to in man (7, 14, 18, 18). The change in responsiveness observed between lights on and lights off was initially inferred by us to occur from a study of the nycthemeral responses of the unanesthetized rat to ACTH-releasing stimuli. In that study we found that the plasma ACTH response to histamine injected intraperitoneally was greater in the morning whereas the plasma corticosterone response in the same rats was probably greater in the evening. Moreover, although basal circulating ACTH levels were only found to double between morning and evening, plasma corticosterone levels increased ninefold (5). Because plasma corticosterone levels represent the net result of adrenal corticosterone secretion rates and corticosterone distribution-binding and metabolism rates, it was possible in that study (in which only plasma corticosterone levels were measured) that a marked nycthemeral decrease in corticosterone clearance rate and/or distribution volume might have explained the apparent increase in adrenal responsiveness to ACTH. Yauda et al. (27) have also reported a greater ACTH response to ether in the morning than in the evening. However, these authors concluded from changes in plasma corticosterone levels (from different initial levels) that the adrenal response to ACTH was greater in the morning than in the evening.

In the present study, both adrenal and plasma corticosterone responses to ACTH administered at different
times of the day were measured. In every experiment, the rat adrenal was found to be at least 2.5 times more responsive to ACTH at lights off than at lights on. This was true when exogenously administered ACTH was given to rats treated a) only with pentobarbital (Fig. 1), b) only with dexamethasone (Fig. 8), and c) with dexamethasone and pentobarbital (Figs. 2–6). Further-

more, mean adrenal corticosterone concentration was approximately 2.6 times higher in the evening than in the morning in untreated resting rats whose plasma ACTH levels ranged between 0 and 48 pg/ml (Fig. 9). Thus, the nycthemeral rhythm in adrenal responsiveness to ACTH appears to be independent of these experimental conditions.

Not only were the adrenal as well as plasma corticosterone responses to ACTH increased at lights off compared to lights on, but the magnitude and time course of the adrenal cAMP response to ACTH was increased and prolonged at lights off (Fig. 4, compare response to 4 ng ACTH at lights on with that to 2 ng ACTH at lights off). Because the nycthemeral change in adrenal responsiveness to ACTH is evident at the level of the cAMP response, the data suggest that the altered responsiveness may involve either the number or affinity of ACTH receptors on the adrenal cortical cell membrane or the transduction step between receptor occupancy and activation of adenylate cyclase. Because the levels of cAMP are elevated for the entire 15-min observation period at lights off, whereas they have returned to baseline by 15 min at lights on, it is possible that the nycthemeral change in adrenal responsiveness to ACTH also involves changes in phosphodiesterase activity or in the kinetics of the reaction between ACTH and its receptor.

Beyond the 2.5-fold amplification of the ACTH rhythm by the nycthemeral increase in adrenal responsiveness to ACTH, our results suggest that further amplification of the plasma corticosterone rhythm may be effected by DBM elements of the adrenocortical system. Low adrenal corticosterone levels at lights on are usually reflected by low to undetectable plasma corticosterone concentration, and high adrenal corticosterone levels at lights off are usually reflected by much higher plasma corticosterone levels. Because plasma corticosterone levels ranged between ≤1.5 and approximately 30 µg/100 ml, it is likely that corticosterone was bound primarily to transcortin and was held within
the plasma compartment (10). It is unlikely, therefore, that the distribution volume changed markedly between morning and evening, since transcortin levels have been reported to remain constant throughout the day (10). Assuming that the system is linear over the ranges examined and that adrenal corticosterone concentration is a direct reflection of secretion rate, a 50% reduction in the rate of corticosterone metabolism between morning and evening would account for our observations that overall, ACTH doubles between morning and evening, adrenal responsiveness to ACTH increases by a factor of 2.5, and plasma corticosterone concentration increases 9- to 10-fold (e.g., see Table 2; Ref. 5).

From these experiments, it seems to us unlikely for several reasons that the increased levels of ACTH that are found just before or at lights out are responsible for the increased adrenal responsiveness to ACTH in the evening. First, the relative increase in responsiveness persisted when rats were treated twice at -15 and -3 h with dexamethasone, although the adrenals of these rats were less responsive to ACTH than those of rats treated only once with dexamethasone (Figs. 5 and 6). Treatment with dexamethasone 15 h before injection with ACTH probably effectively inhibited ACTH secretion for at least 12 h, thus the normal increase in circulating ACTH levels that occurs during the lights-on hours was prevented in the rats injected with ACTH at lights off. In contrast, the rats injected with ACTH at lights on had experienced most of the previous day's increase in ACTH levels before dexamethasone treatment (see Fig. 2). We suspect that prolongation of the treatment with dexamethasone for periods longer than 15 h would result in such profound adrenocortical atrophy and unresponsiveness (6) that testing differential adrenal responsiveness under these conditions would not be useful.

Second, in two of three experiments, treatment of rats with PCPA appeared to abolish the rhythm in adrenal responsiveness to ACTH without affecting the rhythm in plasma ACTH (Table 2). Plasma ACTH was twice as high in the evening as in the morning in both saline- and PCPA-treated rats. Adrenal corticosterone concentration was also twice as high in the evening as in the morning in PCPA-treated rats, and the response to ACTH was the same at both times of day. In contrast, in saline-treated rats, adrenal corticosterone concentration was 4.5 times greater in the evening as was the adrenal response to 50 ng ACTH.

We have no certain explanation for the failure of PCPA to affect the adrenocortical system in the third experiment. In that experiment, failure of PCPA treatment was strongly suspected before the corticosterone results were obtained. In two experiments, rats treated with PCPA appeared to be unusually jumpy during the 48 h after initiation of treatment than the saline-injected controls. Furthermore, in the first two experiments, adrenal weight of PCPA-treated rats was significantly greater than that of saline-treated controls at 72 h. Similar effects of PCPA have also been noted by Van Delft et al. (24). In the third experiment, none of these differences was noted and, from the apparent lack of effect of PCPA treatment on these variables as well as on adrenal function, it seems possible to us that the lot of PCPA used was impotent.

The present study demonstrates a rhythm in adrenal responsiveness to ACTH that is dissociable from the rhythm in ACTH. We have presented preliminary results elsewhere showing that rhythms in adrenal and plasma corticosterone levels may persist in the absence of a rhythm in ACTH levels either 24-48 h after changing the light cycle by 180° (21), or by limiting access to food to a period of 2 h/day either at lights on or lights off (26). Although rhythmic secretion of corticosterone in vitro has been reported to occur from adrenals in organ culture (2, 3, 23) but not from monolayers of primary cultures of adrenal cells (16), it seems necessary to postulate the existence of another factor (or factors) that changes in response to environmental cues and that drives the rhythm in adrenal responsiveness to ACTH.

The magnitude of the rhythm in adrenal responsiveness to ACTH is large, and the results of the present
experiments demonstrate that measurement of adrenal corticosteroid production in vivo is not a reliable reflection of ACTH levels except under very strictly controlled conditions such as those in bioassays for ACTH.

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