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Pregnane Steroid Alphaxalone Attenuates Anxiogenic Behavioral Effects of Corticotropin Releasing Factor and Stress

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BRITTON, K. T., S. McLEOD, G. F. KOOB AND R. HAUGER. *Pregnane steroid alphaxalone attenuates anxiogenic behaviorial effects of corticotropin releasing factor and stress.* PHARMACOL BIOCHEM BEHAV **41**(2) 399–403, 1992.— The 3α-hydroxy A-ring-reduced steroid alphaxalone was examined for its ability to block stress-induced behavioral effects. Alphaxalone (3 and 6 mg/kg, IP) significantly antagonized the response-suppressing effects of corticotropin releasing factor (CRF) (0.5 μg, ICV) on punished responding in a conflict paradigm. In the plus maze, alphaxalone (3 and 6 mg/kg, IP) blocked the anxiogenic behavioral effects produced by a prior ambient-temperature swim stress. These doses of alphaxalone produced no intrinsic effects on plasma ACTH levels, nor did they attenuate CRF-induced increases in plasma ACTH. These findings support the hypothesis that some pregnane steroids may be involved in the modulation of an animal's behavioral response to stress and suggest that these effects may occur independently of the hypothalamic–pituitary–adrenocortical axis.

Steroids Stress Corticotropin releasing factor Alphaxalone Conflict Plus maze

ALPHAXALONE (3α-hydroxy-5α-pregnane-11,20-dione) is a steroid anesthetic that potentiates GABA-mediated inhibition in the rat brain (30). Behaviorally, alphaxalone and certain other naturally occurring reduced analogs of progesterone and deoxycorticosterone have been shown to induce sedation and anesthesia (31), reduce aggressive behavior (10,14), and possess anticonvulsant properties (1,34). In animal models of anxiety, such as a conflict paradigm and plus maze, alphaxalone and naturally occurring analogs produce behaviors similar to those produced by benzodiazepines and barbiturates and consistent with a reduction of anxiety (6,9).

There is evidence that progesterone, deoxycorticosterone, and their metabolites are altered in various physiological states. For example, stress is known to induce the release of progesterone (16) and deoxycorticosterone (29). Recently, Purdy, et al. (26) reported that plasma and brain levels of allopregnanolone, a metabolite of progesterone, and allotetrahydroDOC, a metabolite of deoxycorticosterone, rise markedly following exposure to swim stress. These findings in conjunction with the reported high affinity of these steroids for $GABA_A$ receptors (11,18,22,33) and evidence for the presence of specific (11,18,22,33), and perhaps multiple (21), recognition sites on the $GABA_A$ receptor complex have led to the speculation that these steroids may be endogenous modulators of the central $GABA_A$ receptors (11,18,21–23). While the physiological significance of these findings is unknown, it is interesting to hypothesize that some adrenal steroids may play a role in modulating the behavioral and physiological consequences of stress and anxiety.

The present experiments were designed to determine the effect of alphaxalone on stress-induced behavioral effects. We now report that alphaxalone dose dependently antagonizes the "anxiogenic-like" behavioral effects of corticotropin releasing factor (CRF) in a Geller–Seifter conflict paradigm modified for incremental shock (25). We also report that alphaxalone attenuates the behavioral effects of swim stress in an elevated plus maze model of anxiety (20).

METHODS

Subjects

Male albino Wistar rats (200–250 g) were group housed three per cage in a light- and temperature-controlled environment. For operant training, rats were food deprived to 85% of their free-feeding weight and then maintained on 15–18 g of food per day in addition to that earned during testing.

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Surgery

Rats were anesthetized with halothane and a 7-mm 23-ga stainless steel guide cannula was lowered within 1 mm of the lateral ventricle and secured with two stainless steel screws and dental cement. The stereotaxic coordinates, with the toothbar 5 mm above interaural zero, were: −0.6 mm posterior to bregma, ±2.0 mm lateral, and −3.2 mm below the skull surface at the point of entry. Cannulas were equally distributed between left and right ventricles. Rats were allowed at least 2 days to recover from surgery before resuming baseline operant testing.

Conflict Test Procedure

Eight sound-proof operant chambers were used. Each chamber was equipped with a lever and dispenser for obtaining 45-mg food pellets. Foot-shock was delivered through stainless steel bars on the floor of each chamber. Foot-shock consisted of a scrambled constant DC current, biphasic square-wave produced by a SGS-003 stimulator (BRS/LVE Div. Tech. Serv., Inc.).

Rats were first trained on a continuous reinforcement schedule. After stable baseline responding was achieved, a multiple-schedule conflict test was initiated consisting of three components: an unpunished or reward component, a time-out component, and a punished or conflict component. Responding in the unpunished component was reinforced on a random-interval 30-s schedule in a darkened chamber. The chamber was illuminated with a houselight during the time-out component and responses were not reinforced. The punished component was signaled by three flashing lights above the lever and responses were both reinforced with food and punished with foot-shock on a continuous reinforcement schedule. The current was increased by 0.15 mA with each successive shock during the conflict component to a maximum of 3.3 mA, where it remained constant for the duration of the conflict period regardless of subsequent lever presses. A biphasic square-wave DC current of 1.2 mA is equivalent to approximately 0.3 mA of 60 HZ AC.

A testing session consisted of two cycles of a 5-min reward period, 2-min time-out, and 2-min conflict period presented in succession, giving a total session time of 18 min. Animals were tested 5 days a week at the same time of day and were allowed access to food only during the test period and for approximately 1 h following testing. After establishing a stable response rate (±10% on three successive days), rats were anesthetized and implanted with lateral ventricular cannulae.

For testing, rats (N = 77) were trained until they achieved stable responding. Using a between-subject design, rats were divided into the following groups: saline/vehicle, CRF (0.5 μg, ICV)/vehicle (IP), saline/alphaxalone (1.5, 3.0, and 6.0 mg/kg, IP), and CRF (0.5 μg, ICV alphaxalone (1.3, 3.0, and 6.0 mg/kg, IP). The particular dose of CRF used was chosen because it consistently produces "proconflict" effects in our laboratory (5).

Plasma Hormone Procedure

The effect of alphaxalone on CRF-induced increases in plasma ACTH concentrations was examined in 29 rats. The doses of alphaxalone were chosen on the basis of preliminary experiments showing no effect by alphaxalone on baseline levels of ACTH except at doses exceeding 10 mg/kg (see Table 1). Rats were randomly divided into five groups: saline ICV/vehicle (IP), CRF (0.5 μg, ICV)/vehicle (IP) and CRF (0.5 μg, ICV)/alphaxalone (1.5, 3.0, and 6.0 mg/kg, IP). One half-hour following injection, animals were removed from their cages and sacrificed by decapitation. Truncal blood samples were collected in plastic tubes containing 200 μl of a solution of 5 mg/ml EDTA and 500 KIU aprotinin (Sigma) and plasma prepared by centrifuging at 4°C. Plasma was frozen at −70°C until time of assay. Plasma concentrations of ACTH were measured with a sensitive immunoradiometric assay (Allegro IRMA) developed at Nichols Institute (San Juan Capistrano, CA) using synthetic rat $ACTH_{1\text{-}39}$ as a standard (27,35). The ACTH IRMA has a 1 pg/ml limit of detection and the intra- and interassay coefficients of variation are 3 and 7%, respectively.

TABLE 1

EFFECT OF ALPHAXALONE ON BASELINE LEVELS OF ACTH

Alphaxalone (mg/kg)	ACTH (pg/ml)
0	93 ± 5.8
2.5	97 ± 6.5
5.0	108 ± 3.6
10.0	238 ± 55.0*

*Significantly different from control, $p < 0.05$.

Swim Stress and Plus Maze Procedure

The swim stress procedure consisted of placing rats in a cylindrical plastic container containing 15 cm water maintained at 25°C. After 2 min in the water, they were removed and briskly towel dried for 30 s. Animals were then injected with alphaxalone (3 or 6 mg/kg, IP) and immediately tested on the plus maze according to the procedure described by Pellow et al. (24). The plus maze consisted of two open arms and two enclosed arms and was elevated 50 cm above the floor. For testing, naive rats (N = 67) were placed in the center of the maze and observed over a 5-min period by a rater blind to the drug treatments. The number of entries into open and closed arms and the time spent in open and closed arms were scored. The total number of arm entries provided a measure of general activity. Data are reported as the percentage of time spent in the open arms during the 5-min session.

Drugs

Alphaxalone was a gift from Glaxo Research Group, UK. Alphaxalone was suspended in saline with 2 drops of Tween 80 added and administered IP immediately prior to testing. Corticotropin releasing factor was generously provided by J. Rivier (The Salk Institute). CRF was dissolved in saline and administered ICV by gravity 15 min prior to testing. ICV administration by gravity is accomplished by holding the tubing above the rat and allowing the dissolved compound to flow into the ventricle. Vehicle injections consisted of equivalent amounts of the designated drug vehicle.

Data Analysis

For the conflict test, raw data for each drug were expressed as a percentage of the previous two baseline days to reduce within-group variance. The scores for each dose were sub-

jected to a two-factor analysis of variance (ANOVA) with drug (saline, CRF) as one factor and alphaxalone or vehicle as the other. Individual means comparisons were made using Newman-Keuls a posteriori test.

For the plus maze, the ratio of open to total arm entries was calculated for each rat individually and the mean of these values for each treatment group is presented as the percentage of open arm entries. Data from the elevated plus maze were analyzed using the Student's independent *t*-test.

RESULTS

Effects of CRF and Alphaxalone on Responding in the Conflict Test

Effects of CRF and alphaxalone on responding in the conflict test are shown in Fig. 1. Average baseline responding for control rats on the unpunished component of the conflict test was 41.5 lever presses/min, that is, an average of 415.4 ± 31 lever presses per 10-min (total time) session, and on the punished component was 5.2 lever presses/min, that is, an average of 20.8 ± 0.6 lever presses per 4-min (total time) session. Alphaxalone administered alone produced a significant increase in punished responding, $F(3,69) = 27.4$, $p < 0.01$. These increases in punished response rate reached significance at the 3- and 6-mg/kg doses in the conflict test.

ANOVA revealed a significant suppressive effect of CRF treatment on both unpunished, $F(1,69) = 24.49$, $p < 0.01$, and punished, $F(1,69) = 38.42$, $p < 0.01$, responding. Alphaxalone produced a dose-dependent antagonism of the rate-suppressing effects of CRF in the punished component of the conflict test. These effects were significant at the 3- and 6-mg/kg doses of alphaxalone. The antagonism of CRF by alphaxalone was not evident in the unpunished component of the conflict test.

Effect of Alphaxalone on CRF-Induced Release of ACTH and Corticosterone

Initial experiments revealed no intrinsic effects of alphaxalone on ACTH secretion except at the highest dose (10 mg/kg), which produced significant increases in plasma ACTH levels (Table 2) and mild behavioral sedation. The effect of alphaxalone pretreatment on CRF-induced activation of the pituitary-adrenal axis is displayed in Fig. 2. Infusion of CRF (0.5 μg, ICV) significantly increased circulating ACTH levels ($p < 0.05$). Pretreatment with alphaxalone (1.5–6.0 mg/kg) did not alter the ability of CRF to activate ACTH secretion at any of the doses tested.

Effect of Alphaxalone on Swim Stress

Results produced by swim stress on plus maze performance are displayed in Table 2. Two minutes of swim stress prior to plus maze testing produced a significant decrease in the percentage of time spent on the open arms ($p < 0.05$), as well as the percentage of entries made into the open arms ($p < 0.05$). These behavioral effects are consistent with an "anxiogenic" behavioral profile. Although swim stress produced a modest decline in the total number of arm entries, this effect was not significant. Alphaxalone (3 and 6 mg/kg) significantly blocked the stress-like behavioral effects of prior swim stress on plus maze performance at both doses tested ($p < 0.05$).

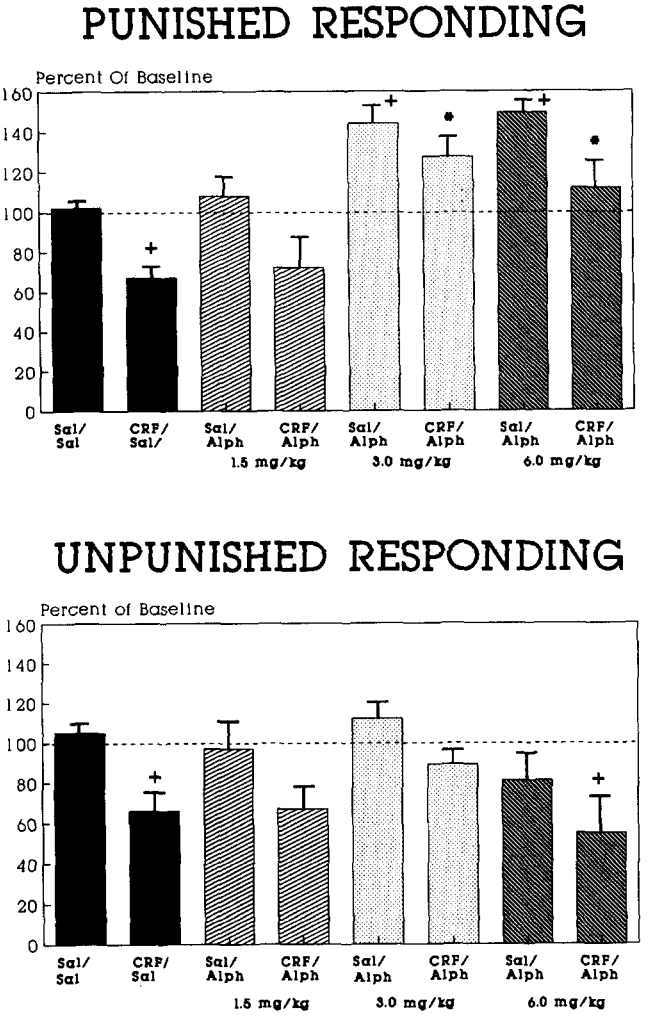

FIG. 1. Effect of alphaxalone (1, 3, or 6 mg/kg, IP) on CRF-induced (0.5 μg, ICV) suppression of punished and nonpunished responding in the conflict test. Black bars, 1.5 mg/kg alphaxalone; hatched bars, 3.0 mg/kg alphaxalone; stippled bars, 6.0 mg/kg alphaxalone. Values are expressed as percent of baseline responding from previous 3 days (mean ± SEM). Sal/Sal ($n = 21$), CRF/Sal) ($n = 16$), Sal/Alph 1.5 mg/kg, CRF/alph 3.0 ($n = 6$), Sal/alph 3.0 and 6.0, CRF/alph 1.5 and 6.0 ($n = 7$). * Significantly different from CRF/Sal groups; $^+$significantly different from vehicle control. ANOVA, Newman-Keuls, $p < 0.05$.

DISCUSSION

Previous investigations have shown that alphaxalone and other naturally occurring steroid metabolites possess anxiolytic properties similar to those observed with benzodiazepines and barbiturates (6,9). The present study extends these findings by examining the effects of alphaxalone in two animal models of stress, infusion of CRF and swim stress. In the conflict test, alphaxalone (3 and 6 mg/kg, IP) antagonized the rate-decreasing effects of CRF (0.5 μg, ICV) on punished lever pressing in a dose-dependent fashion. No significant effects on CRF-induced decreases in nonpunished responding were observed. In the plus maze, comparable doses of alphaxalone blocked the anxiogenic-like behavioral profile produced by a prior short, ambient-temperature swim test. These

TABLE 2
EFFECT OF ALPHAXALONE ON PLUS MAZE PERFORMANCE FOLLOWING SWIM STRESS

Treatment	n	Total entries	% Open Arm Entry	% Time Open Arm
Control/saline	8	22.4 ± 4.0	46.9 ± 6.5	62.9 ± 8.4
Swim stress/saline	8	31.1 ± 3.2	30.4 ± 8.4	27.8 ± 6.7†
Swim stress/alphaxalone (1.5 mg/kg)	8	26.8 ± 3.6	44.1 ± 6.3	34.5 ± 6.0
Control/saline	8	12.8 ± 1.3	42.8 ± 8.1	51.5 ± 9.7
Swim stress/saline	8	8.0 ± 1.1	31.7 ± 3.1	18.2 ± 6.1†
Swim stress/alphaxalone (3.0 mg/kg)	8	13.5 ± 1.6	55.7 ± 8.0*	57.7 ± 12.1*
Control/saline	6	10.9 ± 1.7	44.2 ± 4.7	53.3 ± 10.2
Swim stress/saline	6	8.3 ± 1.7	20.0 ± 7.0†	19.1 ± 8.6†
Swim stress/alphaxalone (6.0 mg/kg)	7	7.1 ± 3.1	55.9 ± 11.6*	60.4 ± 14.2*

*Significantly different from swim stress saline, $p < 0.05$.
†Significantly different from control, $p < 0.05$.

findings support the hypothesis that pregnane-related steroids may play a role in modulating an animal's behavioral response to aversive stimuli.

Alphaxalone was effective in blocking the response suppression produced by CRF in the conflict test only at doses of alphaxalone that by themselves produced an increased rate of punished lever pressing. A possible interpretation of these results is that the observed effects are merely additive effects produced by the two compounds. However, the fact that similar effects were also observed in plus maze performance argues against a simple rate-dependency interpretation.

A number of investigators have shown that alphaxalone and related A-ring-reduced pregnane steroids bind with high affinity to $GABA_A$ receptor recognition sites. The relatively high affinity of these steroids for the $GABA_A$ receptor (11, 18,22,33), and the presence in brain of 3α-hydroxysteroid oxidoreductase and 5α-steroid reductase (2,13,28), have led to the hypothesis that these steroids are endogenous ligands of $GABA_A$ receptors. Consistent with this hypothesis is the recent finding that allopregnanolone and allotetrahydroDOC are present in the brain, and that swim stress produces rapid elevations of these steroids in the cortex, hypothalamus, and plasma (26). Furthermore, the peripheral-type benzodiazepine receptor has recently been implicated in the regulation of some aspects of steroidogenesis (15).

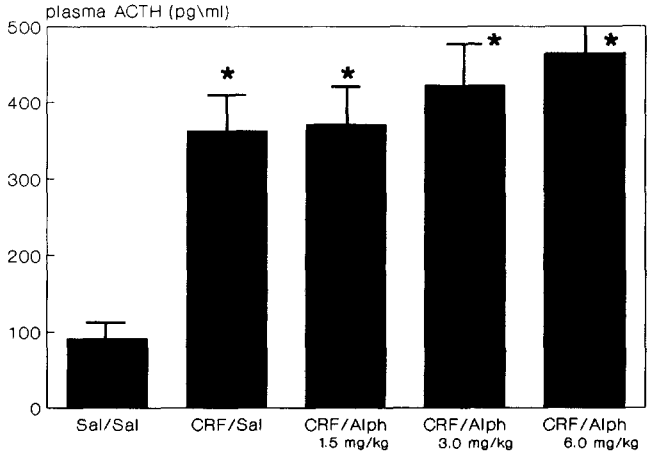

FIG. 2. Effect of alphaxalone on CRF-induced increases in plasma ATCH. *Significantly different from saline control. $n = 6$ for groups except Sal/CRF, where $n = 5$. ANOVA, Newman-Keuls, $p < 0.05$.

Classical anxiolytic compounds consistently block the behavioral effects of CRF in a number of behavioral paradigms. Chlordiazepoxide and ethanol antagonize the rate-suppressing effects of CRF in the conflict test (3,5). Benzodiazepines also block the CRF potentiation of the acoustic startle response (32) and reduce the stress-like behavioral effects associated with infusion of CRF using a modified open-field paradigm (17). These findings, coupled with the current investigation, raise the question of whether interactions exist between the $GABA_A$ and CRF receptor systems.

Immunohistochemical evidence suggests direct GABAergic synaptic contact to CRF neurons in the PVN (19), and GABA has been shown to inhibit the stimulated release of CRF from the hypothalamus in vitro (8). Chronic benzodiazepine treatment has been reported to significantly decrease CRF receptor binding in the frontal cortex and hippocampus (12), and the triazolobenzodiazepine alprazolam reportedly increases hypothalamic CRF concentrations and reduces the concentration of CRF in the locus coeruleus and amygdala (23). Although these effects provide indirect evidence for an interaction between brain CRF and GABA systems, no evidence is available showing binding of CRF to the $GABA_A$ receptor complex.

In contrast to alphaxalone's dose-dependent antagonism of the behavioral effects of CRF in the conflict test, alphaxalone pretreatment did not attenuate the pituitary-adrenal responses to centrally administered CRF. Consequently, the anxiolytic effects of alphaxalone do not involve the suppression of ACTH secretion. This finding is consistent with previous studies demonstrating that the behavioral effects of CRF are mediated by its action at central sites and not via an action of the pituitary-adrenocortical system (4,7).

In summary, the synthetic steroid alphaxalone produced a dose-dependent attenuation of stress-induced behavioral effects in two animal models of anxiety, the conflict test and the plus maze. These findings support the hypothesis that adrenal steroids may have a role of physiological significance in the modulation of an organism's response to stressful stimuli. These data also have potential clinical relevance for study of the etiology and specific treatment of anxiety disorders.

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