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Corticotropin-Releasing Hormone Antagonists, Astressin B and Antalarmin: Differing Profiles of Activity in Rhesus Monkeys

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The present study compares the activity of two chemically distinct corticotropin-releasing hormone (CRH) antagonists at the level of the pituitary gland in rhesus monkeys, using exogenous CRH-stimulated increases in adrenocorticotropin (ACTH) and cortisol. Of chief interest was whether the CRH-R1-selective pyrrolopyrimidine, antalarmin, shown previously to have activity in the central nervous system (CNS), would differ in its antagonist profile from the CRH-R1- & 2-selective peptide, astressin B, which is unlikely to have access to the CNS following systemic administration. Nine rhesus monkeys (eight male), each with an indwelling venous catheter, were subjects in this study. Astressin B (0.001, 0.003, 0.03, 0.1, and 0.3 mg/kg) or antalarmin (1.0, 3.2, and 10 mg/kg) was administered as an intravenous (i.v.) pretreatment 15 min prior to administration of 1 or 10 μg/kg i.v. CRH. Antalarmin (10 mg/kg) was also administered alone on six occasions and its effects on behavior as well as on ACTH and cortisol levels were measured. Astressin B was assessed following i.v. and intracisternal (i.c.) administration. Astressin B dose-dependently abolished the CRH-stimulated ACTH and cortisol responses, with an antagonist effect lasting in excess of 24 h. Astressin B was approximately 300-times more potent when given i.c. than when it was administered via the i.v. route. By contrast, antalarmin antagonized the effects of CRH on ACTH but not cortisol at 1.0 and 3.2 mg/kg. At a larger dose, antalarmin stimulated ACTH and cortisol release and produced behavioral sedation. These latter effects diminished with repeated administration of antalarmin. The differences between astressin B and antalarmin may be due either to non-CRH receptor-mediated effects of antalarmin or to a complex interaction of antalarmin's effects at both central and peripheral CRH receptors.
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INTRODUCTION

Several recent publications have shown that hyperactivity of the hypothalamic–pituitary–adrenal (HPA) axis may often accompany a diagnosis of depression and anxiety (Nemeroff, 1996; Holsboer, 1998; McEwen, 2000). There is a growing body of evidence that implicates abnormal HPA functioning in the etiology of a variety of affective disorders. The HPA axis has also been suggested as a target for the treatment of substance abuse, as drug-related behaviors can be disrupted by treatment with corticotropin-releasing hormone (CRH) antagonists (Goeders, 1997; McCarthy *et al*, 1999). Treatment with the CRH antagonist CP-154,526 has been reported to reduce the amount of cocaine self-administered by rats (Goeders and Guerin, 2000), and attenuate the reinstatement of drug-related behaviors for cocaine (Shaham *et al*, 1998) and opioids (Lu *et al*, 2000). These findings have helped fuel the recent investigation of CRH antagonists for use in the treatment of these disorders.

A variety of CRH antagonists have been synthesized, beginning with the early modifications of CRH itself that resulted in α-helical $CRH_{(9-41)}$ (Rivier *et al*, 1984) and D-Phe $CRH_{(12-41)}$ (Hernandez *et al*, 1993; Menzaghi *et al*, 1994), and extending to the more recent analogs, astressin (Gulyas *et al*, 1995) and the longer-lasting astressin B (Rivier *et al*, 1999). Nonpeptidic compounds, the so-called 'small molecule' CRH-R1 selective antagonists, such as CP-154,526 (Schulz *et al*, 1996), its methyl analog, antalarmin (Webster *et al*, 1996; see also review by McCarthy *et al*, 1999), and SSR125543A (Griebel *et al*, 2002) have been investigated

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preclinically using animal models of depression and anxiety. Currently, this literature is dominated by rodent studies. One exception to this was a study carried out in rhesus monkeys (Habib *et al*, 2000) using antalarmin. In this paper, the pharmacokinetic profile of antalarmin following intravenous (i.v.) and oral administration was assessed, as were the effects of oral administration of antalarmin on both the HPA and behavioral responses to a stressor. Antalarmin was detected in cerebrospinal fluid (CSF) following oral administration, indicating that antalarmin has access to the central nervous system (CNS) when given peripherally. A social stressor that involved placing two unfamiliar males in a single cage separated only by a transparent wall was used to measure the anxiolytic effects of antalarmin. Following antalarmin pretreatment, Habib *et al* (2000) reported a significant reduction in anxiety-related behaviors observed during the 30 min that monkeys were placed in close proximity. Significant reductions in both adrenocorticotropin (ACTH) and cortisol levels in plasma were reported following the social stressor.

The CRH-R1 receptor specificity of antalarmin is based on *in vitro* work in which antalarmin displaced ^{125}I-oCRH binding in rat pituitary, frontal cortex, and cerebellum where CRH-R1 receptors predominate, but not in heart where CRH-R2 receptors predominate (Webster *et al*, 1996). However, the *in vivo* evidence that antalarmin produces a CRH-R1 blockade at the pituitary level in rats is weak, as the CRH-antagonist effects of antalarmin do not appear to be dose-dependent, nor was HPA antagonism demonstrated following application of stressors whose behavioral effects antalarmin has been reported to attenuate (Deak *et al*, 1999; Wong *et al*, 1999). In non-human primates, however, antalarmin produced a significant attenuation of stress-induced ACTH and cortisol release in monkeys as well as a reduction in the behavioral signs of stress (Habib *et al*, 2000).

Although there are fewer publications to date concerning astressin B, these nevertheless provide convincing evidence that acute administration of astressin B results in a time- and dose-dependent blockade of ACTH release in adrenalectomized rats (Rivier *et al*, 1999), as well as in rats exposed to inescapable footshock or treated with h/r CRH, lipopolysaccharide or intragastric alcohol (Rivier *et al*, 2003). Although astressin B, a peptidic CRH antagonist, is unlikely to cross the blood–brain barrier following peripheral administration, it clearly produces a robust blockade of pituitary CRH_1 receptors.

To date, there have been no direct comparisons of nonpeptidic and peptidic CRH antagonists at the level of the HPA axis. Blocking CRH-stimulated increases in ACTH and cortisol is a fundamental test of the pituitary activity of CRH antagonists. It is predicted that the differences in chemical structure as well as in distribution between the CNS and periphery following i.v. administration of antalarmin or astressin B will result in divergent profiles of pituitary CRH-receptor antagonism. In the present study, the effects of antalarmin and astressin B were compared in rhesus monkeys using i.v. injection of CRH as the stimulus and ACTH and cortisol levels as the dependent measures. The effects that antalarmin treatment had on behavioral and HPA axis activity were also measured, as were the effects of repeated treatment with antalarmin. Astressin B was evaluated for its duration of antagonist activity, as well as for its effectiveness using central and systemic routes of administration.

METHODS

Subjects

Eight adult male rhesus monkeys (*Macaca mulatta*), seven intact and one castrate (Monkey 2595), weighing between 10.0 and 15.1 kg, and one intact female monkey, weighing 6.3 kg, were the subjects for this study. Most subjects had a drug self-administration history with two or more classes of drug, including cocaine and methohexital. The duration of drug-taking history averaged 2.5 years, with a range from 3 months to 5 years. The monkeys continued to self-administer drug or saline during the course of the present study. Small doses of drug (saline, 0.03 mg/kg/inj cocaine for a maximum of 13 injections, or 0.1 mg/kg/inj methohexital for a maximum of between 90 and 140 injections) were available during each of two, 2-h daily sessions. Saline was substituted for drug during half of the self-administration sessions, and apart from a decrease in drug-maintained behavior, no behavioral disruption was evident when drug was not available. The monkeys were randomly assigned to the different treatment groups, so any effect of their different histories was most likely negated. Cocaine and methohexital have short half-lives, averaging 0.8 ± 0.2 and 4 ± 2 h respectively in humans. Each test day was preceeded by at least a 24 h drug-free period. Three of the monkeys were antalarmin-naive at the time of this study.

Monkey Housing

Each monkey was individually housed in a stainless steel cage measuring $83.3 \times 76.2 \times 91.4\,cm^3$ deep (Bryan Research Equipment Corporation, Bryan, TX) located in a laboratory that contained a total of 24 similarly housed monkeys. The temperature in the room was maintained at $21 \pm 1^{\circ}C$, and lights were illuminated from 0630 until 1930 daily. The monkeys were fed eight to 12 Purina Monkey Chow biscuits twice daily to maintain normal adult weight and water was freely available.

Surgical Procedures

Each monkey had an indwelling venous catheter in a femoral, internal, or external jugular vein. Catheters were inserted during aseptic surgery under ketamine (10 mg/kg) and xylazine (2 mg/kg) anesthesia. Following placement in the vein, the catheter was guided subcutaneously to the midscapular region where it was externalized. The external portion of the catheter was protected inside the cage by a flexible stainless steel tether, with one end attached to a double layer polyester jacket (Lomir, New York, NY) worn by the monkey and the other bolted to the rear of the cage. Monkeys were treated postoperatively with the analgesic, buprenorphine, as needed. In addition, they were treated with the broad-spectrum antibiotic, chloromycetin (Chloramphenicol), at the time of surgery and twice daily for 3 days postsurgery. Behavior and food intake were monitored regularly throughout each day, a physical examination was

performed under ketamine anesthesia every 2 weeks, no experiments were conducted and expert veterinary care was available on an on-call basis if illness was suspected, or while monkeys were being treated for any illness. Test treatments were administered and blood samples were obtained by accessing the i.v. catheter from the rear of the cage with minimal disturbance to each monkey. Each monkey was tested in its home cage. All studies reported here were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Study Design

HPA response following CRH injection. A total of seven male monkeys received i.v. CRH (1.0 and/or 10 μg/kg; eg Broadbear *et al*, 1999b). Three monkeys were selected to receive pretreatment with antalarmin (1.0, 3.2, and 10 mg/kg; eg Broadbear *et al*, 2002) and the remaining four received pretreatment with astressin B (0.001, 0.003, 0.03, 0.1 ($n = 3$), and 0.3 mg/kg; eg Broadbear *et al*, 1999a). The order in which antagonist doses were administered was randomized. The pretreatment and blood sampling times for the antagonist + CRH studies were based on the above studies (Table 1).

Samples continued to be drawn, first at 10, then 15, 30, and 60 min intervals for the next 4–7 h. The first blood sampling took place between 0900 and 1000. The details regarding sample collection and processing for the measurement of ACTH and cortisol levels in plasma are described below.

Procedure for the intracisternal (i.c.) administration of astressin B. Three monkeys were selected to receive i.c. injections of astressin B. The monkeys were briefly anesthetized with 4 mg/kg i.v. methohexital followed by booster injections of 2 mg/kg methohexital. Each booster injection produced anesthesia lasting 5–8 min during which the animals were motionless and unresponsive to touch. Following anesthetization with methohexital, the area on the back of the head and neck was shaved and the skin was cleaned by alternating with povidone–iodine (Vedco Inc., St Joseph, MO) and alcohol-soaked gauze squares. While an assistant held each animal securely, a spinal needle (22 gauge, $1\frac{1}{2}$inch; Becton Dickenson, Franklin Lakes, NJ) was slowly directed toward the cisterna magna until CSF returned through the needle. A syringe containing 0.1 μg astressin B was then attached to the needle and injected in a volume of 0.5 ml. Each monkey recovered normal function within 30 min.

In order to evaluate the effect of methohexital anesthesia on the subsequent ACTH and cortisol response to i.v. CRH, five male monkeys, including the three that received i.c. astressin B injections, were anesthetized and treated exactly the same way as described in the previous paragraph, except that a spinal need was not inserted and i.c. astressin B was not administered. After 40 min, 3.2 μg/kg CRH was administered i.v.. Blood was sampled prior to methohexital administration, following methohexital administration, prior to CRH administration, and then over 4 h following CRH administration.

HPA response following repeated treatment with antalarmin. To evaluate the effects of frequent administration of antalarmin alone or as a pretreatment before the injection of CRH, two experiments were conducted. In the first, four monkeys were tested using the following protocol (Table 2).

In the second experiment, an additional three antalarmin-naive monkeys, two of which were male, received 10 mg/kg i.v. antalarmin on six occasions at weekly intervals. The effects of saline and vehicle treatments on ACTH and cortisol were tested before and after the 6 weeks of antalarmin tests. These animals did not receive CRH injections. Blood samples in both of these studies were obtained at the times described above and were used for the measurement of ACTH and cortisol levels.

Blood collection and handling. Prior to drawing each blood sample, a 3 cm^3 syringe was used to empty the contents of the catheter and this fluid was discarded. Blood samples (1.1–1.4 ml) were placed in a 2 ml Vacutainer (Becton

Table 2 Chronic Antalarmin Experiment

Experimental days	Treatment (each administered i.v.)
0 and 21	10 μg/kg CRH
1, 4, 7, and 12	3.2 mg/kg antalarmin
2, 9, and 14	3.2 mg/kg antalarmin+10 μg/kg CRH

Table 1 Experiment Timeline for Antagonist + CRH Studies

Time (min) and drug	-25	-20	-15 (or 24 h for astressin B)	-10	-2	0	Next 4–7 h
I.v. pretreatment with antalarmin or astressin B							
I.v. antalarmin	Blood sample	Blood sample	Antalarmin pretreatment	Blood sample	Blood sample	CRH treatment	Blood sampling
I.v. astressin B	Blood sample	Blood sample	Astressin B pretreatment	Blood sample	Blood sample	CRH treatment	Blood sampling
Time (min) and drug	**-60**	**-50**	**-40**	**-10**	**-2**	**0**	**Next 4–7 h**
Intracisternal pretreatment with astressin B							
I.v. antalarmin	Blood sample	Blood sample	Astressin B pretreatment	Blood sample	Blood sample	CRH treatment	Blood sampling

Dickenson and Company, Franklin Lakes, NJ) containing 0.04 ml of 7.5% EDTA and immediately placed on ice. After a blood sample was drawn, 1.5–3 ml of 30 U/ml heparin-saline solution was injected into the catheter. A maximum of 40 ml of blood was drawn over 8 h in each experiment, well within the 10 ml/kg guideline recommended by the University of Michigan Committee on Use and Care of Animals.

Blood samples were centrifuged at 5000 RPM for 5 min at 4°C and then the plasma (0.7 ml) was pipetted into 2 ml Cryovials (Corning Incorporated, Corning, NY) and stored at −80°C until assay. ACTH and cortisol levels were determined using commercially available radioimmunoassay kits (cortisol: Diagnostic Products Corporation, Los Angeles, CA; ACTH: Nichols Institute Diagnostics, San Juan Capistrano, CA). The limit of detection of the cortisol assay was 0.2 μg/dl, while the intra and interassay coefficients of variation were 5 and 6.5% respectively. The limit of detection for the ACTH assay was 0.5 pg/ml, with intra- and inter-assay coefficients of variation of 3 and 7% respectively.

Drugs

CRH (human/rat corticotropin releasing hormone) and astressin B were provided by JE Rivier (Salk Institute, La Jolla, CA). Astressin B and CRH were both water-soluble. Antalarmin (provided by KC Rice at NIDDK, Bethesda, MD) was solubilized immediately before use in a vehicle of one part ethanol, one part emulphor, and nine parts sterile water at a concentration of 20 mg/ml antalarmin. Methohexital was purchased from Ace Surgical Supplies (Brockton, MA) and diluted with sterile water.

Data Analysis

The raw cortisol and ACTH data, representing the HPA response to the CRH injection after pretreatment with astressin B (0.3, 0.03, and 0.003 mg/kg) or antalarmin, were averaged and plotted for presentation. ACTH and cortisol results were standardized for each subject prior to statistical analysis. Standardization involved averaging the baseline values and subtracting them from post-treatment samples to yield a difference score. This enabled direct comparison of the CRH-stimulated changes in HPA axis activity across subjects and treatment days. Data for the repeated antalarmin injection studies were transformed to area under curve (AUC) values. AUC provides an estimate of the total cortisol (μg min/dl) or ACTH (pg min/ml) release relative to basal levels during the 4 h sampling time following the CRH injection. AUC values were calculated using the trapezoidal rule (eg Tallarida and Murray, 1987) on a Microsoft Excel spreadsheet.

Statistics

All data are presented as mean ± standard error of the mean (SEM). Repeated measures analyses of variance (ANOVA), in which treatment and time were the within-subject variables, were conducted using standardized cortisol and ACTH data (see Data Analysis). Where appropriate, *post hoc* pairwise comparisons using the Tukey Honest Significant Difference test of significance ($p<0.05$) were carried out (Statistica v.5.0, Statsoft, Tulsa, OK).

RESULTS

CRH + Astressin B Pretreatment

Pretreatment with astressin B dose-dependently attenuated the rises in ACTH and cortisol levels that were stimulated by the i.v. administration of 10 μg/kg CRH (Figure 1). In the case of ACTH, CRH administration resulted in a rise of 222.4 ± 61.8 pg/ml from a baseline of 11.7 ± 2.5 pg/ml (Figure 1, upper panel). There was a significant effect of pretreatment with astressin B ($F_{5,10}=3.8$, $p=0.03$), with 0.3, 0.03, and 0.003 mg/kg astressin B significantly attenuating the ACTH response relative to CRH alone ($p<0.05$). There was also a significant sampling time effect ($F_{9,18}=5.23$, $p=0.001$) as well as an interaction between treatment and sampling time ($F_{45,90}=2.70$, $p<0.001$).

In the case of cortisol, CRH administration resulted in a rise of 21.3 ± 3.3 ng/ml from a baseline of 12.3 ± 1.9 ng/ml (Figure 1, lower panel). There was a significant effect of pretreatment with astressin B ($F_{5,10}=9.74$, $p=0.001$), with 0.3 and 0.03 mg/kg astressin B dose dependently attenuating the cortisol response relative to either CRH alone, or to CRH following pretreatment with 0.003 mg/kg astressin B ($p<0.05$). There was also a significant interaction between treatment and sampling time ($F_{45,90}=1.88$, $p=0.006$).

In order to assess duration of antagonist action, the effects of astressin B were evaluated when astressin B was given 15 min or 24 h prior to administration of 10 μg/kg CRH (Figure 2). When given 15 min before CRH, 0.1 mg/kg astressin B attenuated the cortisol (45–240, 360 min; $p<0.05$) and ACTH (15–180 min; $p<0.05$) response to CRH. This blockade was still present when measured 24 h after the administration of astressin B, but was significant at fewer sampling times.

The effectiveness of astressin B as a CRH receptor antagonist was compared following its administration by two different routes, i.v. and i.c. (Figure 3). The brief anesthesia with the short-acting barbiturate, methohexital (4 mg/kg with a 2 mg/kg booster), that was required for delivery of the i.c. injection, was evaluated and found to have no effect on the subsequent HPA response to i.v. CRH 40 min later (data not shown). I.v. (3.0 μg/kg) and i.c. astressin B (0.01 μg/kg) produced an overall attenuation of the ACTH ($F_{2,4}=6.52$, $p<0.05$) and cortisol ($F_{2,2}=21.88$, $p<0.05$) responses to CRH. Whereas both treatments attenuated ACTH and cortisol relative to when CRH was administered alone ($p<0.05$), the treatments did not differ from one another. There was an interaction between treatment and time for ACTH levels ($F_{16,32}=2.65$, $p<0.01$), whereby administration of astressin B by both routes attenuated the ACTH response to CRH at sampling times between 15 and 120 min ($p<0.05$; Figure 3, upper panel). These results indicate that astressin B may be up to 300-fold more potent when given by the i.c. (central) route than the i.v. (peripheral) route.

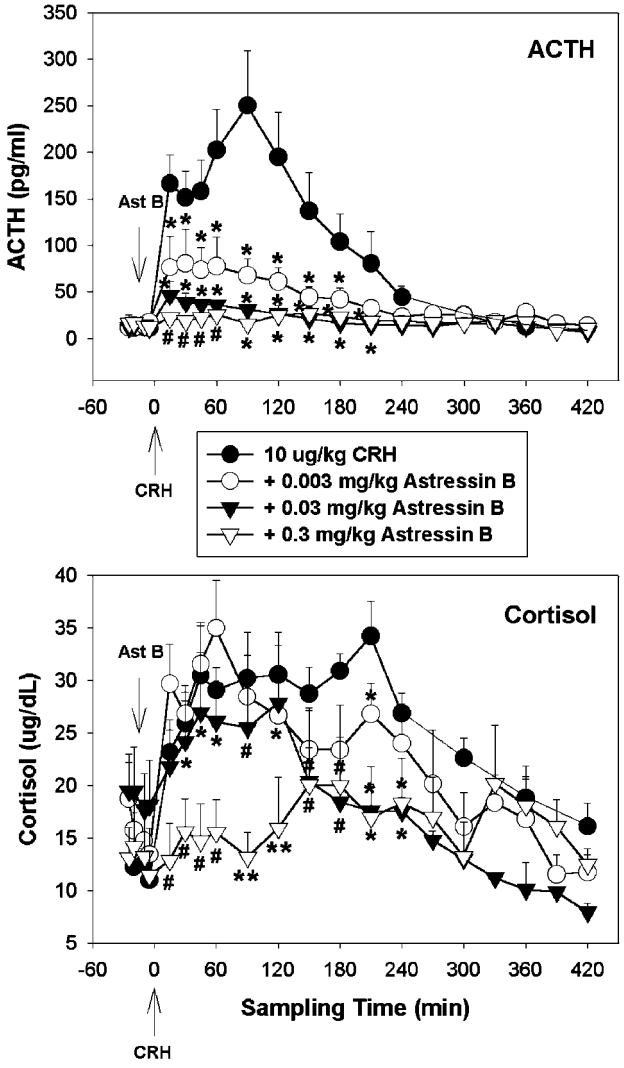

Figure 1 Averaged ACTH (upper panel) and cortisol (lower panel) levels in plasma following i.v. administration of 10 μg/kg CRH at time 0. Male rhesus monkeys ($n=4$) received CRH alone or 15 min after i.v. pretreatment with the CRH antagonist astressin B (0.3, 0.03, or 0.003 mg/kg). CRH-stimulated ACTH and cortisol levels were attenuated by prior treatment with astressin B (* *vs* CRH only; # *vs* CRH and 0.003 mg/kg astressin B; ** *vs* CRH, 0.03 and 0.003 mg/kg astressin B, $p<0.05$). Data for CRH plus the 0.1 and 0.001 mg/kg doses of astressin B are not plotted.

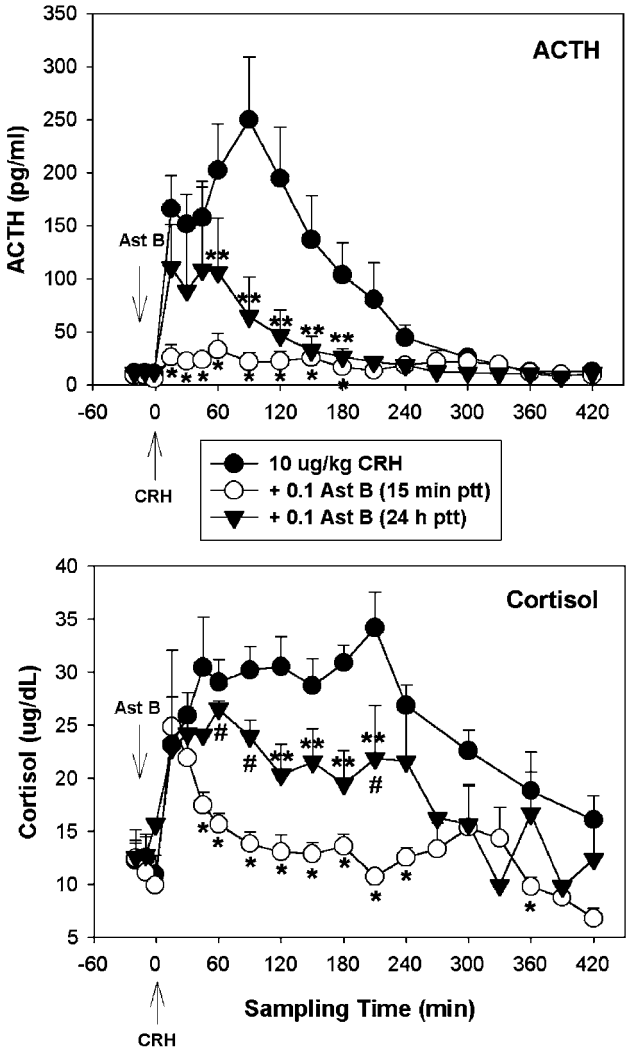

Figure 2 Plasma cortisol and ACTH levels following a single i.v. injection of 10 μg/kg CRH alone ($n=4$), or after 15 min ($n=4$) or 24 h pretreatment ($n=3$) with 0.1 mg/kg astressin B. At both 15 min and 24 h, astressin B pretreatment attenuated the cortisol and ACTH responses to CRH injection (*15 min ptt relative to CRH alone; **24 h ptt relative to CRH alone; # 15 min ptt *vs* 24 h ptt; $p<0.05$).

CRH + Antalarmin Pretreatment

There was a significant attenuation of CRH-stimulated ACTH levels following antalarmin pretreatment ($F_{3,6}=5.66$, $p<0.05$; Figure 4, upper panel). Administration of 1.0 and 3.2 mg/kg antalarmin attenuated the CRH-induced increase in ACTH relative to when CRH was administered alone ($p<0.05$). At a higher dose (10 mg/kg), antalarmin was a less effective antagonist of CRH-stimulated ACTH secretion than it was at lower doses. Despite the clear attenuation of ACTH following antalarmin pretreatment, antalarmin had no effect on CRH-stimulated cortisol levels (Figure 4, lower panel). Whereas there was no difference between the pre-antalarmin cortisol levels on the different treatment days, there was a dose-dependent elevation in cortisol levels 5 and 13 min after antalarmin pretreatment, before CRH was administered at time 0 ($F_{3,6}=9.59$, $p=0.01$). *Post hoc* analysis indicated that the 3.2 and 10 mg/kg antalarmin doses elevated cortisol levels significantly above those associated with 0 or 1.0 mg/kg antalarmin pretreatment ($p<0.05$), and that the 10 mg/kg antalarmin dose elevated cortisol significantly with respect to 0, 1.0, and 3.2 mg/kg antalarmin ($p<0.05$).

HPA Response Following Repeated Treatment with Antalarmin

Experiment 1. Antalarmin (3.2 mg/kg) was administered on several occasions, either alone or prior to 10 μg/kg CRH administration (Figure 5). CRH alone significantly

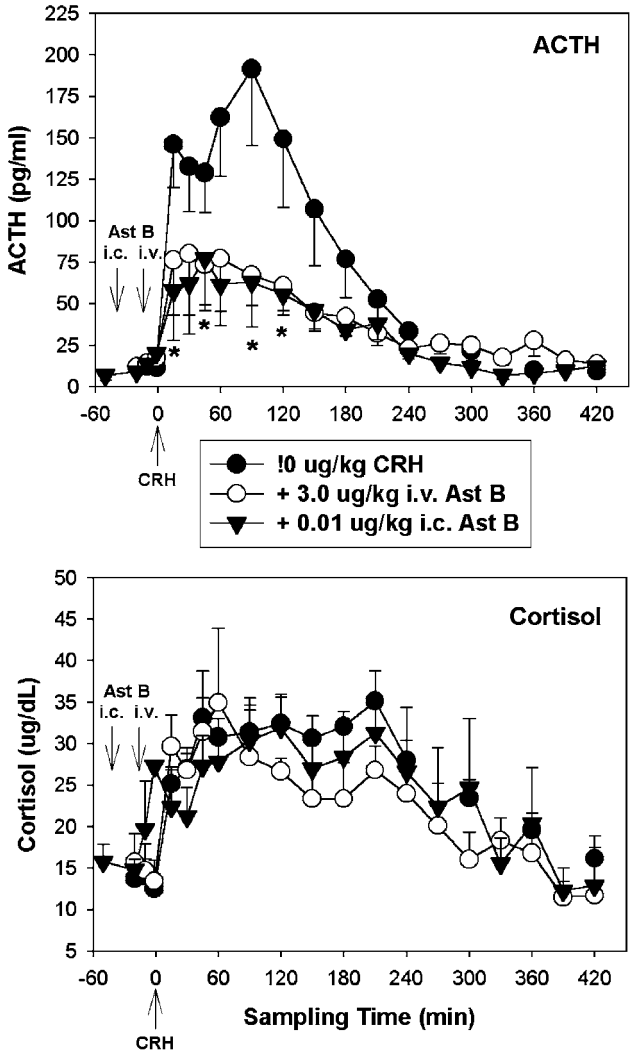

Figure 3 Comparison of the effects of i.v. or i.c. injections of astressin B on CRH-stimulated cortisol and ACTH release ($n=3$). Astressin B (3.0 μg/kg i.v.) was administered 15 min prior to CRH administration, whereas 0.01 μg/kg i.c. astressin B was administered under methohexital anesthesia 40 min prior to CRH. Whereas both pretreatments produced an attenuation of the ACTH ($F_{2,4}=6.52$, $p<0.05$*) and cortisol ($F_{2,2}=21.88$, $p<0.05$) responses to CRH relative to when CRH was administered alone, there was no difference between the treatments in their effects on CRH-stimulated cortisol and ACTH. The i.c. route of administration was approximately 300-fold more potent than the i.v. route in producing a blockade of the CRH-stimulated release of ACTH.

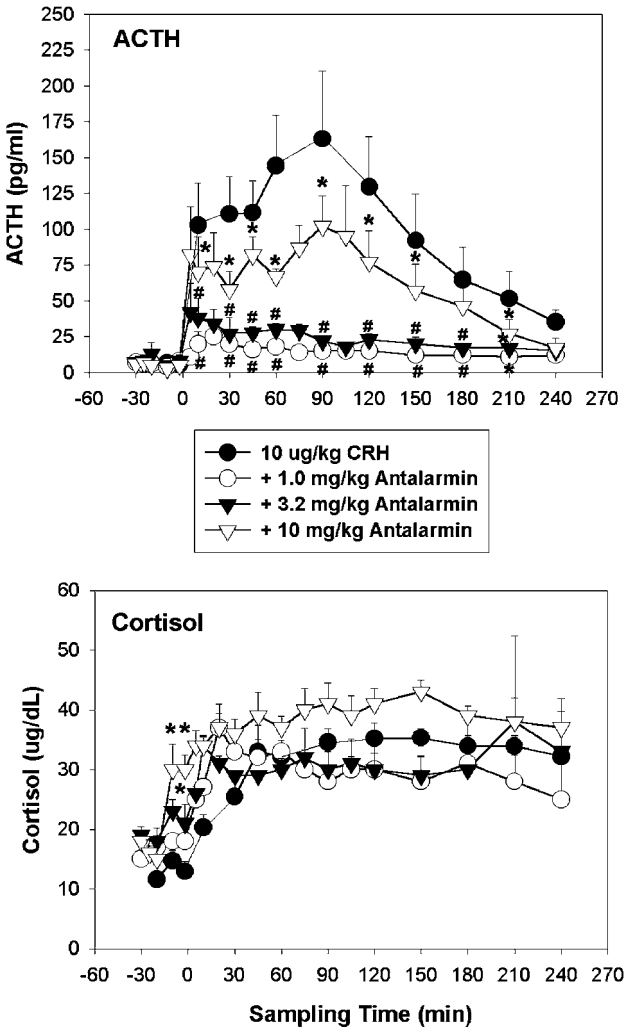

Figure 4 Averaged ACTH (upper panel) and cortisol (lower panel) levels in blood following i.v. administration of 10 μg/kg CRH at time 0. Male rhesus monkeys ($n=3$) received CRH alone or after 15 min i.v. pretreatment with the CRH antagonist antalarmin (1.0, 3.2, or 10 mg/kg). CRH-stimulated ACTH levels were attenuated by prior treatment with antalarmin (upper panel: * *vs* CRH only; # *vs* CRH and 10 mg/kg antalarmin, $p<0.05$). The CRH-stimulated release of cortisol was not affected by prior treatment with antalarmin (lower panel). However, there was a significant, dose-related elevation of cortisol levels measured in samples taken following antalarmin pretreatment, but prior to CRH administration (* *vs* no antalarmin pretreatment; ** *vs* 0, 1.0, and 3.2 mg/kg antalarmin pretreatment, $p<0.05$).

increased both ACTH ($F_{2,6}=12.60$, $p<0.01$) and cortisol release ($F_{2,6}=42.38$, $p<0.001$) when given on days 0 and 21. When given alone on days 1, 4, 7, and 12, antalarmin (3.2 mg/kg) produced consistent, nonsignificant decreases in ACTH and cortisol. When given prior to administration of 10 μg/kg CRH on days 2, 9, and 14, antalarmin (3.2 mg/kg) produced significant decreases in ACTH release ($p<0.05$), but no change in cortisol levels. Antalarmin was also evaluated in three subjects using the same experimental protocol but with a lower dose of CRH (1 μg/kg). This dose of CRH produced a mean cortisol increase of 15.4 ± 3.3 ng/ml, and increased ACTH levels by 35.3 ± 14.8 pg/ml. Two doses of antalarmin (1.0 and 3.2 mg/kg) were evaluated. Neither dose of antalarmin significantly attenuated the cortisol or ACTH responses following the administration of 1 μg/kg CRH (data not shown).

Experiment 2. The effects of saline, antalarmin vehicle, and 10 mg/kg antalarmin on ACTH and cortisol were examined on several occasions (Figure 6). Neither saline nor vehicle injection differed in its effects on ACTH and cortisol release. However, i.v. administration of 10 mg/kg antalarmin resulted in a stimulation of the HPA axis. This effect appeared to peak on the second or third occasion that antalarmin was tested, and then diminished until it resembled a saline/vehicle-like response on the sixth and final test day. The increase in ACTH reached statistical significance the third time that antalarmin was given,

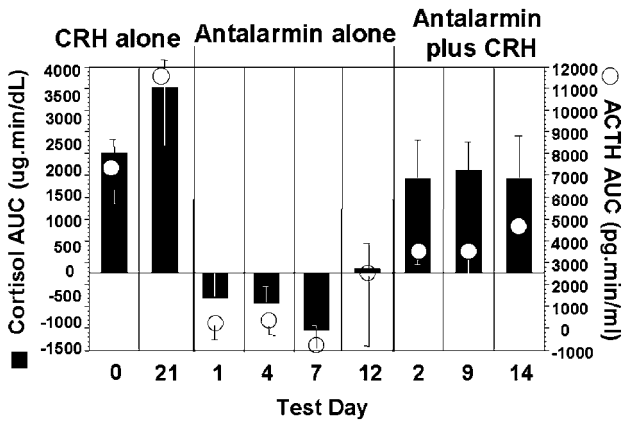

Figure 5 Comparison of the effects of repeated administration of 10 μg/kg CRH alone, 3.2 mg/kg antalarmin alone, and antalarmin given as a pretreatment to CRH, on ACTH and cortisol release when tested over a 21-day period ($n=4$). Antalarmin when given alone did not differ from saline in its effects on cortisol and ACTH release. CRH when given alone stimulated cortisol and ACTH release. When antalarmin was administered prior to CRH, antalarmin attenuated the ACTH response (**$p<0.001$), but not the cortisol response following CRH administration. The acute effects of CRH, antalarmin, and CRH + antalarmin on ACTH and cortisol secretion were maintained when retested across multiple test days.

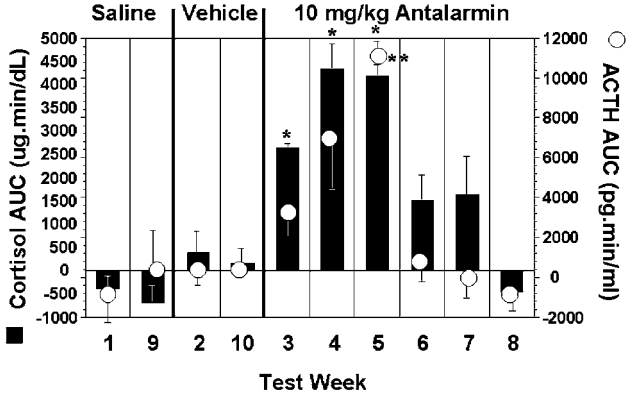

Figure 6 Comparison of the effects of repeated administration of saline, vehicle and 10 mg/kg antalarmin on ACTH and cortisol secretion when tested at weekly intervals ($n=3$). Neither saline nor vehicle had any effect on ACTH and cortisol release. I.v. administration of 10 mg/kg antalarmin resulted in an acute stimulation of ACTH (**$p<0.05$; week 3) and cortisol (*$p<0.05$; weeks 1, 2, and 3) that diminished with repeated testing. The stimulation of stress hormone secretion was accompanied by behavioral sedation and lack of responsiveness to environmental stimuli, the extent and duration of which also diminished upon repeated exposure to antalarmin.

whereas the increase in cortisol levels attained significance during the first, second, and third antalarmin tests ($p<0.05$).

Behavioral Effects of Antalarmin

The highest dose of antalarmin that was administered (10 mg/kg) also produced profound changes in behavior for a period of 25–45 min following injection. Animals appeared sedated and either lay down in their cages or leaned on the cage interior. They exhibited drooling and were unresponsive to sound or motion. Gently touching the brow resulted in an eye blink with little or no movement of the head. Monkeys recovered normal function by 60–90 min following injection. The extent and duration of these behavioral changes diminished with repeated administration of 10 mg/kg antalarmin, following a similar time course to the changes in HPA response shown in Figure 6.

DISCUSSION

The prediction that antalarmin, a nonpeptidic CRH-R_1-selective antagonist, and astressin B, a peptidic CRH $R_{1\&2}$-selective antagonist, would differ in their effects on pituitary CRH receptors following i.v. administration was supported by the results of this study. Astressin B produced an effective blockade of pituitary CRH receptors, attenuating the HPA response to exogenously administered CRH, and decreasing both ACTH and cortisol levels in a dose-dependent manner. However, pretreatment with antalarmin only attenuated the CRH-stimulated increase in ACTH release but had no effect on the CRH-stimulated release of cortisol. The inhibitory effect of antalarmin on ACTH release diminished when the dose of antalarmin was increased.

CRH + Antalarmin Pretreatment

In the present study there was no evidence that antalarmin decreased CRH-stimulated cortisol levels at any time during the 4 h sampling period following the CRH injection. This lack of effect of antalarmin on CRH-stimulated cortisol levels may have been due simply to an incomplete blockade of CRH receptors by antalarmin, with the subsequent release of ACTH still being sufficient to stimulate cortisol release following a single injection of CRH. It appears more likely though that i.v. antalarmin pretreatment may actually have stimulated cortisol secretion, since plasma cortisol levels were already elevated prior to CRH injection in antalarmin-pretreated subjects. This paradoxical increase in cortisol secretion may have offset the inhibitory effect of the reduced ACTH levels on cortisol, with both effects occurring as a result of antalarmin pretreatment. There was no corresponding increase in ACTH levels, suggesting either that antalarmin has a direct effect on the adrenal cortex, or that antalarmin's effect on ACTH is more difficult to detect. An interesting corollary to this is the observation that repeated administration of 10 mg/kg antalarmin to monkeys that were initially antalarmin-naïve resulted in a significant elevation in cortisol on the first three occasions on which antalarmin was administered, but the increase in ACTH reached significance only after the third administration. There are similarities between the results described here and those reported by Habib *et al* (2000). In their study, antalarmin was administered orally (p.o.) to rhesus monkeys prior to presentation of an 'intruder paradigm', a 30-min exposure to another male monkey in which the two were separated only by a transparent barrier. Blood and CSF were taken under ketamine anesthesia 30 min later. As in the present study, only one (20 mg/kg p.o.) of the four doses of antalarmin that were tested significantly attenuated the

ACTH response to the social stressor and this effect was not dose-dependent, since a higher dose (40 mg/kg) did not produce a significant inhibition of ACTH secretion. The lack of an inhibitory effect of acute antalarmin administration on cortisol in the present study also concurs with earlier reports that chronic administration of antalarmin was necessary in order to detect reductions in basal corticosterone levels in rats (Bornstein *et al*, 1998; Wong *et al*, 1999).

Behavioral Effects of Antalarmin and Astressin B

In the few studies in which ACTH and/or corticosterone have been measured following antalarmin treatment, little or no effect of antalarmin on HPA measures was found (Deak *et al*, 1999; Wong *et al*, 1999). Yet anxiolytic activity of antalarmin has been reported in rats (Deak *et al*, 1999) and rhesus monkeys (Habib *et al*, 2000). Given the unusual HPA axis profile of antalarmin in the present study, and the lack of clear evidence of pituitary CRH-R1 receptor blockade in earlier studies, it seems reasonable to question whether antalarmin is producing it effects solely via CRH-R1 receptors, or whether another mechanism is being recruited. This question has particular salience given the HPA and behavioral effects seen in association with antalarmin administration. When 10 mg/kg i.v. antalarmin was first administered to antalarmin-naïve rhesus monkeys, it had a clear stimulatory effect on cortisol. This effect was coupled with a period of profound sedation and behavioral unresponsiveness lasting 30–45 min. Both the HPA and behavioral changes that accompanied the administration of 10 mg/kg antalarmin diminished with repeated administration. I.v. antalarmin has also been shown to have transient reinforcing effects in rhesus monkeys across a range of doses, with some drug intakes resulting in visible intoxication (Broadbear *et al*, 2002). While there have been no prior reports associating antalarmin treatment with behavioral changes, it was noted by the authors that behavioral suppression by CP-154,526 may have been responsible for some of its effects on free exploration in mice (Griebel *et al*, 1998). In addition to the behavioral testing carried out in rhesus monkeys following oral antalarmin administration, antalarmin was also administered i.v. in the study by Habib *et al*, (2000). A single i.v. injection of 20 mg/kg antalarmin, which is double the maximum dose used in the present study, was administered in order to study antalarmin's pharmacokinetics. However, since this part of their study was carried out under ketamine anesthesia, behavioral observations were not possible. I.v. antalarmin clearly had acute sedative and HPA stimulatory effects in monkeys in the present study.

In contrast to antalarmin, astressin B produced neither behavioral nor HPA stimulatory effects at any dose tested. This may be because unlike antalarmin, astressin B is unselective with respect to CRH receptor subtypes, and is unlikely to have ready access to the CNS following i.v. administration. Perhaps the most direct way to assess whether antalarmin's sedative and HPA stimulatory effects are mediated by central CRH-R1 receptors will be to similarly evaluate other nonpeptidic CRH-R1 antagonists as they become available.

CRH + Astressin B Pretreatment

As well as effectively blocking the ACTH and cortisol responses when administered 15 min prior to an injection of CRH, astressin B (0.1 mg/kg) was also active 24 h after administration. This concurs with results from adrenalectomized (ADX) rats, in which subcutaneous (s.c.) treatment with astressin B produced a dose-dependent suppression of ACTH release lasting in excess of 24 h (Rivier *et al*, 1999). Since astressin B is a bulky peptide, it is unlikely to have ready access to the CNS following systemic administration. A study carried out with astressin, another peptide from the same chemical series, showed that i.c. astressin (10 μg), but not i.v. astressin (10 μg), was effective at attenuating the delay in gastric emptying in rats following i.c. CRH (0.6 μg) administration (Martinez *et al*, 1999). The effects of higher doses of i.v. astressin were not reported.

In the present study, using doses that produced equivalent reductions in CRH-stimulated ACTH release (i.v.: 3.0 μg/kg, i.c.: 0.01 μg/kg) in the same subjects, we also determined that astressin B was approximately 300 times more potent following i.c. administration. This finding differs from those published previously, in which astressin (not astressin B), given via both i.c. and i.v. routes, was found to be equipotent in blocking the i.v. (not i.c., as discussed above) CRH-induced delay in gastric emptying in rats (Martinez *et al*, 1999). In another study, rats were shown to have a reduced ACTH response to i.v. CRH 4 h after treatment with i.c.v. sheep anti-CRH (Turnbull and Rivier, 1998). At 4 h, the plasma concentration of anti-CRH administered i.c.v. was 68–76% of the anti-CRH levels measured 4 h after i.v. administration, sufficient to produce a peripheral (pituitary) blockade of exogenous CRH. Turnbull and Rivier did not report whether shorter pretreatment times, when the plasma levels of anti-CRH following i.c.v. administration were much lower, were effective in blocking exogenous CRH-induced ACTH release. The potency difference measured in the present study implies that astressin B may be concentrated in the hypophysial portal circulation, as the i.c. injection of 0.00001 mg/kg astressin B that attenuated the effect of i.v. CRH on ACTH would have been ineffective if given via the i.v. route. A number of antisera (Turnbull and Rivier, 1998), cytokines (Chen *et al*, 1997; Chen and Reichlin, 1999) and large molecules such as albumin (Reed and Woodbury, 1963) have been injected centrally and had their passage to the periphery traced. All are detectable in plasma within 5–30 min following injection. Clearly, these large molecules 'leak' out of the CNS to appear in the general circulation via a route that does not appear to differentiate between them. However, it has been shown that CRH is actively transported out of the CSF into blood (Martins *et al*, 1996). Astressin B, being a modified fragment of CRH, may also be a substrate for this mechanism, thus becoming concentrated in the cerebral vasculature, including the hypophysial portal system that supplies the anterior pituitary, soon after administration. This may explain the greater potency of astressin B following administration via the i.c. route 35 min prior to i.v. CRH administration. However, a simpler explanation could be that despite there being no measurable effect of anesthesization with methohexital on the subsequent CRH-stimulation of ACTH and

cortisol, subthreshold effects of both methohexital and low-dose astressin B may have combined to increase the apparent potency of astressin B given via the i.c. route.

Drug Self-Administration History

There is also a question of whether having a history of cocaine or methohexital self-administration may have influenced the results of our study. We have published several studies showing that self-administered cocaine dose-dependently stimulates the HPA axis, and about half of the monkeys in the present study have contributed data to these earlier papers. We have seen no evidence of changes in either baseline ACTH and cortisol activity, or changing trends in the monkeys' responses to self-administered cocaine and exogenous CRH injection (eg Broadbear *et al*, 1999a, b). Although studies in both humans (Buydens-Branchey *et al*, 2002) and rats (Zhou *et al*, 2003) have indicated that chronic cocaine exposure may change the responsiveness of the HPA axis (with higher cortisol levels being associated with high-dose cocaine use in humans; tolerance to the HPA-stimulating effects of cocaine reported in rats), the amount of cocaine administered in these published studies was probably or certainly much larger than that experienced by the monkeys described in the current study. In the present study, the available doses were small, access was limited to short periods of the day, and saline was substituted in at least 50% of the self-administration opportunities. The monkeys ate well, behaved normally, and appeared normal throughout this and the prior studies. In any event, it is not clear that a history of cocaine exposure would account for the differences observed between the effects of antalarmin and astressin B on the HPA axis response to CRH administration. Indeed, knowledge of the effects of CRH antagonists in subjects that have a history of substance use may be directly relevant to the investigation of CRH antagonists for the treatment of substance abuse disorders.

In summary, the data presented in this study show that the unselective, peptidic CRH antagonist, astressin B, effectively attenuated the CRH-stimulated ACTH and cortisol responses. In contrast, antalarmin, despite its apparent selectivity and affinity for CRH-R1 receptors *in vitro*, appears to have a self-limiting effect at pituitary CRH-R1 receptors *in vivo* following acute administration. Paradoxically, antalarmin itself produced a stimulation of the HPA axis and profound behavioral sedation when administered at a high dose. Why should these two CRH-antagonists show such different profiles in the same experimental paradigm? Since astressin B is water-soluble and antalarmin is administered as an emulsion, it is possible that antalarmin is poorly distributed and at higher doses has nonselective effects on other physiological systems. Alternatively, the behavioral and HPA effects of antalarmin could be a consequence of its simultaneous blockade of both central and peripheral CRH-R1 receptors, revealing a novel profile of activity for this nonpeptidic R1-selective antagonist.

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Animals used in these studies were maintained in accordance with the University of Michigan Committee on Animal Care and Guidelines of the Committee on the Care and Use of Laboratory Animal Resources, National Health Council (Department of Health, Education and Welfare, ISBN 0-309-05377-3, revised 1996).

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