0013-7227/04/$15.00/0
Printed in U.S.A.

Endocrinology 145(12):5431–5438

doi: 10.1210/en.2004-0638

Endocannabinoid Signaling Negatively Modulates Stress-Induced Activation of the Hypothalamic-Pituitary-Adrenal Axis

SACHIN PATEL, CRAIG T. ROELKE, DAVID J. RADEMACHER, WILLIAM E. CULLINAN, AND CECILIA J. HILLARD

Department of Pharmacology and Toxicology, Medical College of Wisconsin (S.P., C.T.R., D.J.R., C.J.H.), Milwaukee, Wisconsin 53226; and Department of Biomedical Sciences, Marquette University (W.E.C.), Milwaukee, Wisconsin 53233

Activation of the hypothalamic-pituitary-adrenal (HPA) axis is critical for the adaptation and survival of animals upon exposure to stressful stimuli, and data suggest that endocannabinoid (eCB) signaling modulates neuroendocrine function. We have explored the role of eCB signaling in the modulation of stress-induced HPA axis activation. Administration of the CB_1 receptor antagonist/inverse agonist SR141716 (0.01, 0.1, 1, and 5 mg/kg, ip) to male mice produced a small, dose-dependent increase in the serum corticosterone (CORT) concentration. Despite this effect, the highest dose of SR141716 did not significantly increase neuronal activity within the paraventricular nucleus of the hypothalamus, as measured by the induction of Fos protein. Similarly, exposure of mice to 30 min of restraint increased serum CORT concentrations, but did not produce a consistent, statistically significant increase in Fos expression within the PVN. However, pretreatment of mice with SR141716 before restraint stress robustly potentiated restraint-induced CORT release and Fos expression within the PVN. Pretreatment of mice with either the CB_1 receptor agonist CP55940, the eCB transport inhibitor AM404, or the fatty acid amide hydrolase inhibitor URB597 significantly decreased or eliminated restraint-induced CORT release. Upon exposure to acute restraint, hypothalamic 2-arachidonylglycerol content was reduced compared with the control value; however, after 5 d of restraint exposure (which resulted in an attenuated CORT response), the hypothalamic 2-arachidonylglycerol content was increased compared with the control value. These data indicate that eCB signaling negatively modulates HPA axis function in a context-dependent manner and suggest that pharmacological augmentation of eCB signaling could serve as a novel approach to the treatment of anxiety-related disorders. (*Endocrinology* 145: 5431–5438, 2004)

CORTICOSTEROIDS PLAY AN important role in the regulation of behavioral, cognitive, and metabolic responses to stress. Upon exposure to stressful stimuli, activation of sensory and limbic brain systems ultimately results in the activation of hypothalamic paraventricular nucleus (PVN) neurons (1, 2). These neurons act as a final common neural substrate subserving activation of the hypothalamic-pituitary-adrenal (HPA) axis, which results in the release of corticosteroids from the adrenal cortex. In the short term, activation of the HPA axis is beneficial to the survival of an organism; however, long-term activation can have deleterious effects on metabolism, mood, and cognition and is associated with a variety of neuropsychiatric disorders, including anxiety and depression (see Refs. 3–5 for review). In this context, exploration of novel neurotransmitter/modulator systems involved in HPA axis regulation will probably enhance our understanding of the mechanisms regulating circulating glucocorticoid levels and thus could afford new opportunities for the treatment of stress-related neuropsychiatric disorders.

Abbreviations: AEA, Anandamide; 2-AG, 2-arachidonylglycerol; CORT, corticosterone; eCB, endocannabinoid; FAAH, fatty acid amide hydrolase; HPA, hypothalamic-pituitary-adrenal; PVN, paraventricular nucleus of the hypothalamus.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

Recent data suggest a role for endocannabinoid (eCB) signaling in the modulation of anxiety-related behaviors and HPA axis function. eCBs, including anandamide (AEA) and 2-arachidonylglycerol (2-AG), are synthesized from lipid precursors upon neuronal stimulation via phospholipase D- and phospholipase C-mediated enzymatic cascades and are degraded *in vivo* by fatty acid amide hydrolase (FAAH) and monoglyceride lipase, respectively (6). Both compounds are agonists of CB_1 cannabinoid receptors, and novel pharmacological tools have recently emerged to help elucidate the functional role of eCBs in the modulation of anxiety and HPA axis function. For example, compounds that augment AEA signaling through inhibition of FAAH activity reduce anxiety-like responses in the elevated zero maze and the isolation-induced ultrasonic vocalization paradigms (7). Similarly, low doses of Δ^9-tetrahydrocannabinol show anxiolytic properties in the light/dark box paradigm (8, 9). In line with these findings, some studies have shown that the CB_1 receptor antagonist SR141716 produces anxiety-like behaviors in animals (10, 11). However, the anxiogenic effects of SR141716 are not universally reported (12) and are probably context-dependent (13).

With regard to modulation of the HPA axis, SR141716 increases ACTH and corticosterone (CORT) release in rats (14). In addition, basal plasma ACTH concentrations are increased in $CB_1^{-/-}$ mice compared with those in wild-type animals, and a tendency toward an interaction between CB_1

receptor expression and environmental context in the elevation of ACTH has been reported (13). These studies suggest that eCB signaling negatively modulates HPA axis activity. In agreement with these findings and the widespread role of eCB signaling in the inhibition of neurotransmitter release, CB_1 receptor activation inhibits glutamatergic input onto PVN neurons (15).

Taken together, the available data suggest that environmental stress and CB_1 receptor activity interact in regulation of the HPA axis. In addition, these findings suggest that eCB signaling negatively modulates HPA axis activation and could thereby serve as an endogenous anxiolytic system. In the present study we tested the hypotheses that eCB signaling inhibits stress-induced HPA axis activation *in vivo* and that an interaction between environmental stress and CB_1 receptor activity exists in the control of HPA axis activation.

Materials and Methods

Drugs and animals

Male ICR mice (21–24 g) were used in all experiments (Harlan, Madison WI). All animals were housed (five per cage) on a 12-h light, 12-h dark cycle, with lights on at 0600 h. Animals had *ad libitum* access to food and water. All experiments were carried out in accordance with the NIH Guide for the Use and Care of Laboratory Animals.

AM404 was purchased from Tocris Cookson (Ellisville, MO). SR141716 was provided by the NIDA Drug Supply Program (Research Triangle Park, NC). CP55940 was a gift from Pfizer Central Research (Groton, CT). Cyclohexylcarbamic acid 3-carbamoyl-biphenyl-3-yl ester (URB597) was purchased from Cayman Chemical (Ann Arbor, MI). All drugs, except URB597, were dissolved in emulphor vehicle (18:1:1, saline/emulphor/ethanol) and delivered by ip injection in a volume of 10 ml/kg. URB597 was dissolved in dimethylsulfoxide and delivered in a volume of 50 μl by ip injection.

Restraint procedure

Animals were acclimated to the testing room 24 h before experimentation. Animals were marked for identification on the proximal region of the tail as needed. Experiments were conducted at the circadian nadir (0800–1000 h), when serum CORT levels are at their lowest. Mice were restrained for 30 min in modified, transparent 50-ml plastic conical tubes with numerous small air holes to increase ventilation; control animals were left undisturbed in their home cage. One end of the conical was closed using the screw top, the conical end was removed, and a tight gauze plug was inserted after the animals were inside. Animals were placed on the bench top for the restraint period. For the repeated restraint experiment, animals were restrained for 30 min/d for 5 consecutive days; group-housed control animals were left undisturbed in the home cage. Immediately after removal from the restraint apparatus, mice were decapitated. Either 2 ml trunk blood were collected or the hypothalamus was dissected and frozen on dry ice. For Fos experiments, animals were returned to their home cage for an additional 2 h after restraint exposure, then anesthetized and perfused.

CORT assays

Blood was allowed to coagulate at 4 C overnight. Samples were centrifuged at 1500 $\times$ *g* for 10 min; serum was removed and stored at −80 C until assayed. Serum CORT concentrations were determined by a commercially available ELISA kit according to the manufacturer's instructions (Assay Designs, Inc., Ann Arbor, MI). The sensitivity of the assay is 3.83 ng/ml, which is far less than the concentrations obtained from experimental animals in the present study. In our experiments, the intraassay coefficient of variation was 7.35%, and the interassay coefficient of variation was 3.62%; these values are very close to those provided by the manufacturer of the kit.

Fos immunohistochemistry

Fos immunohistochemistry was carried out as described previously (16). Briefly, animals were anesthetized with isoflurane and perfused transcardially with saline, followed by 4% formaldehyde. Brain were postfixed overnight, then incubated in 30% sucrose for 48 h. Sections (35 μm) through the hypothalamus were cut on a cryostat and stained for Fos using a rabbit anti-c-Fos antibody (Oncogen, Cambridge MA; 1:25,000 dilution). For FAAH staining, an antibody previously developed and characterized in our laboratory (17) was used (1:100 dilution). Sections were developed using the ABC kit (Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer's directions.

For quantitative analysis of Fos-like immunoreactive neurons, bright-field photomicrographs from matched sections (approximate bregma −0.8) through the PVN were obtained using a Eclipse E600 microscope (Nikon, Melville, NY) and Spot Advanced software (Amsterdam, The Netherlands). Images were opened in Image J and converted to 8-bit mono, and the region of the PVN was outlined using the freehand drawing tool. Bilateral cell counts were obtained using the threshold and particle analysis functions of Image J. Only cells that met OD (150/255 gray levels) and size requirements (50 pixels) were automatically counted as Fos-like immunoreactive neurons. Counts from each hemisphere were normalized to area and then averaged to obtain one data point per animal.

eCB quantification

Animals were decapitated immediately after removal from the restraint apparatus. The hypothalamus was dissected and frozen on dry ice 2 min after decapitation. Tissue was stored at −80 C until extraction. Extraction of AEA and 2-AG from tissue samples was carried out exactly as described previously (18). The amounts of AEA and 2-AG were determined by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS, Agilent 1100 LC-MSD, SL model). Samples (5 μl) were separated on a reverse phase C_{18} column (Kromasil; 250 $\times$ 2 mm, 5 μm diameter; Alltech, Deerfield, IL) using mobile phase A (deionized water, 1 mM ammonium acetate, and 0.005% acetic acid) and mobile phase B (methanol, 1 mM ammonium acetate, and 0.005% acetic acid) as described previously (18). eCB contents were normalized to wet tissue weight.

Statistical analysis

Data were analyzed by ANOVA, followed by *post hoc* Dunnett's or Bonferroni's multiple comparisons test as appropriate (see figure legends). Data are presented as the mean ± SEM. $P < 0.05$ was considered significant throughout.

Results

Blockade of CB_1 receptors increases CORT release

To determine whether eCB signaling modulates the basal activity of the HPA axis, we administered the CB_1 receptor antagonist/inverse agonist SR141716 and determined serum CORT concentrations 1 h later. SR141716 produced a dose-dependent increase in serum CORT concentrations (Fig. 1). One-way ANOVA revealed a significant effect of SR141716 administration on serum CORT concentrations [$F_{(4,42)} = 12.85$; $P < 0.0001$]. *Post hoc* analysis revealed a significant increase in serum CORT compared with vehicle treatment at the 1 and 5 mg/kg doses.

Blockade of CB_1 receptors potentiates restraint-induced CORT release

We explored the interactions between eCB signaling and 30 min of restraint stress in the regulation of HPA axis activity. The peak increase in serum CORT occurs approximately 30 min after the onset of restraint (19); therefore, in all subsequent experiments CORT concentrations were as-

sayed 30 min after the onset of restraint. SR141716 was administered 30 min before restraint (and, therefore, 1 h before death), so the interval between drug administration and death was the same as that in the previous experiment. To determine whether SR141716 treatment affects restraint-induced CORT release, mice were pretreated with SR141716 (1 and 5 mg/kg) before restraint, and serum CORT concentrations were determined.

Pretreatment of mice with SR141716 before restraint significantly potentiated the restraint-induced increase in serum CORT concentrations (Fig. 2). At both doses of SR141716, the increase in serum CORT was far greater in the presence of restraint than without restraint. For example, 5 mg/kg SR141716 increased serum CORT by 25 ng/ml compared with vehicle treatment under basal (nonstressed) conditions; in the presence of restraint, 5 mg/kg SR141716 increased serum CORT concentrations by 250 ng/ml compared with those in vehicle-treated, restrained mice. Two-way ANOVA with factors of restraint status (control or restrained) and drug treatment (vehicle or SR141716) revealed highly significant effects of drug treatment [$F_{(2,46)} = 49.8$; $P < 0.0001$] and restraint [$F_{(1,46)} = 596.7$; $P < 0.0001$] as well as an interaction between SR141716 treatment and restraint status [$F_{(2,46)} = 29.5$; $P < 0.0001$] on serum CORT concentrations. *Post hoc* analysis revealed that pretreatment of mice with SR141716 at both the 1 and 5 mg/kg doses before restraint significantly increased serum CORT concentrations compared with those in vehicle-treated, restrained mice.

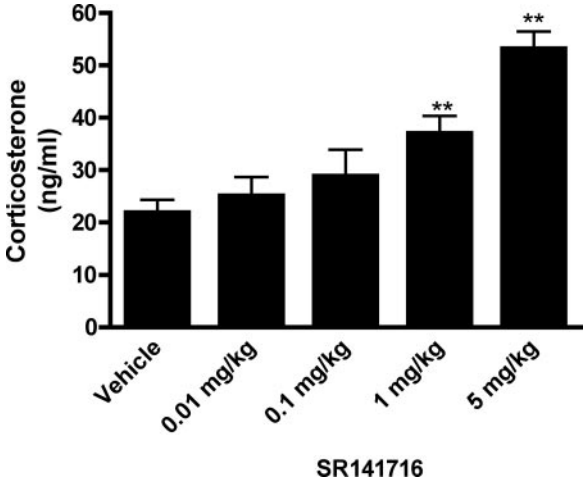

FIG. 1. Effects of SR141716 on serum CORT concentrations 1 h after drug administration (n = 8–10/group). **, $P < 0.01$, significantly different from vehicle treatment, by Dunnett's *t* test.

Blockade of CB_1 receptors potentiates restraint-induced Fos expression within the PVN

We determined whether the interaction between eCB signaling and restraint stress in the regulation of HPA axis activation involved increased activation of PVN neurons. SR141716 (5 mg/kg) was administered 30 min before restraint, and Fos protein expression was examined within the PVN. Our data indicate a significant interaction between SR141716 administration and restraint stress in the induction of Fos within the PVN (Fig. 3A). Two-way ANOVA with factors of SR141716 treatment and restraint status revealed a significant effect of SR141716 treatment [$F_{(1,15)} = 17.9$; $P = 0.0007$] and restraint [$F_{(1,15)} = 23.95$; $P = 0.0002$] and a significant interaction [$F_{(1,15)} = 18.17$; $P = 0.002$]. *Post hoc* analysis indicated that SR141716 pretreatment significantly increased PVN Fos expression compared with that in vehicle-treated, restrained mice. In the SR141716-pretreated group, Fos expression was increased within both the parvocellular and magnocellular divisions of the PVN (Fig. 3B).

Pharmacological enhancement of eCB signaling inhibits restraint-induced CORT release

Because our data suggest that eCB signaling serves to inhibit stress-induced HPA axis activation, we determined the ability of pharmacological activation of CB_1 receptors or eCB signaling to reduce restraint-induced CORT release. We administered the direct CB_1 agonist CP55940 (0.03 and 0.3 mg/kg), the eCB transport inhibitor AM404 (2 and 10 mg/kg), or the FAAH inhibitor URB597 (0.1 and 1 mg/kg) 30 min before restraint and assayed serum CORT concentrations 30 min after the onset of restraint.

Pretreatment of mice with two doses of the CB_1 receptor agonist CP55940 produced a significant biphasic effect on restraint-induced CORT release [by one-way ANOVA: $F_{(3,27)} = 23.42$; $P < 0.0001$]. *Post hoc* analysis revealed that the low dose of CP55940 (0.03 mg/kg) significantly decreased serum CORT concentrations compared with those in vehicle-treated, restrained mice, and the high dose (0.3 mg/kg) significantly increased serum CORT concentrations compared with those in vehicle-treated, restrained mice (Fig. 4A).

Pretreatment of mice with two doses of AM404 produced a significant effect on restraint-induced CORT release [by one-way ANOVA: $F_{(3,24)} = 23.98$; $P < 0.0001$]. *Post*

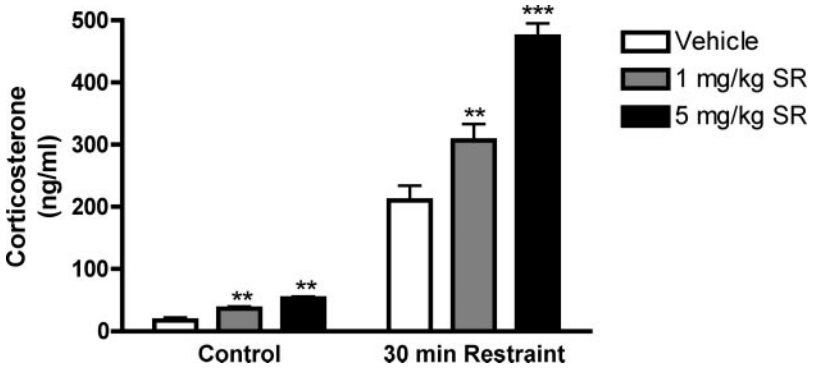

FIG. 2. Effects of SR141716 (SR; 1 and 5 mg/kg) on CORT release in control mice and mice restrained for 30 min (n = 8–10/group). ANOVA revealed a significant interaction between drug treatment and restraint status (see text). In both control and restrained subjects, CORT measurements were made 1 h after SR141716 administration. **, $P < 0.01$, ***, $P < 0.001$ (significantly different from corresponding vehicle group, by Dunnett's *t* test).

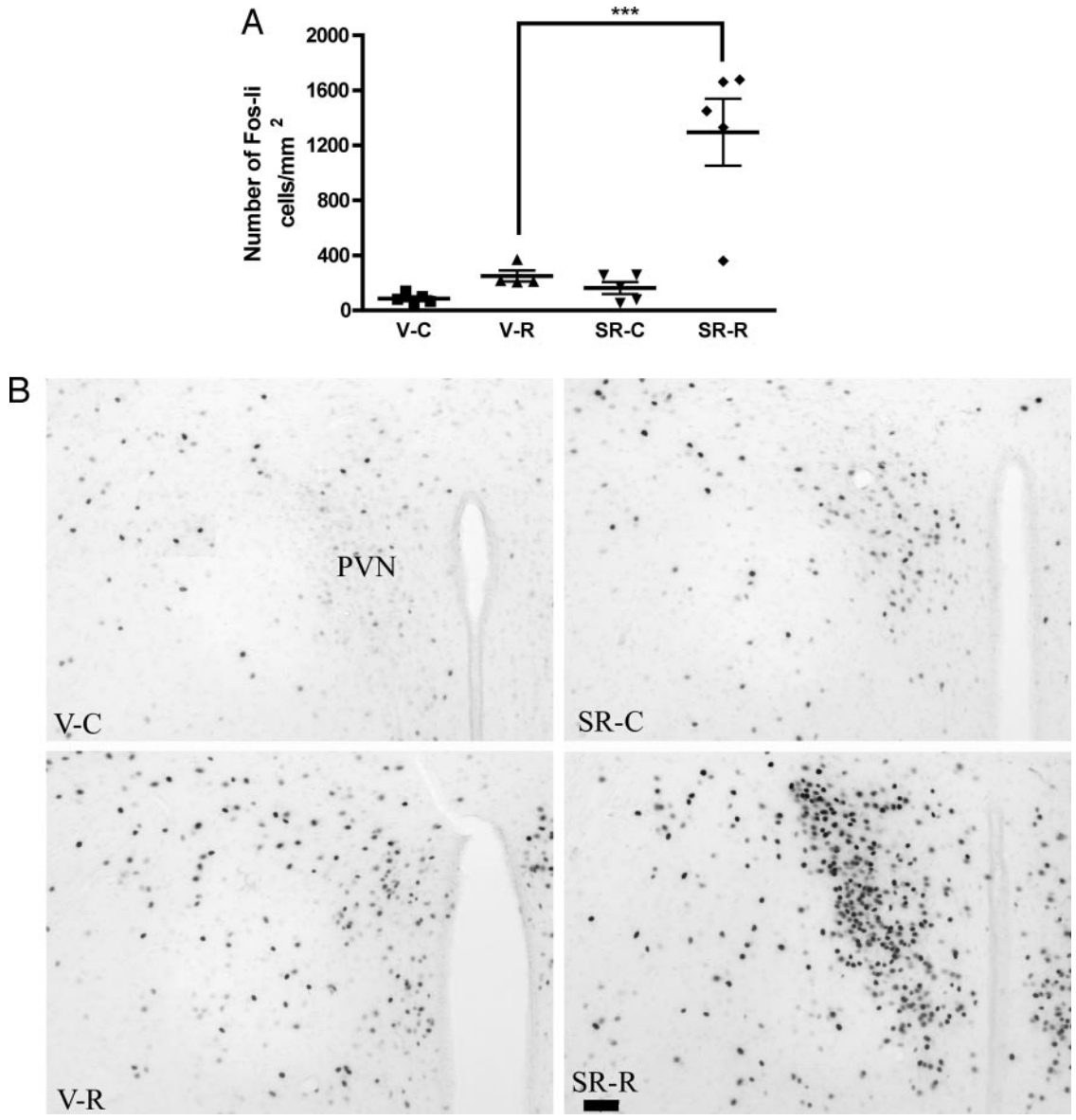

FIG. 3. A, Effects of SR141716 (SR; 5 mg/kg) on restraint-induced Fos expression within the PVN (n = 5/group). ***, $P < 0.001$, significantly different from the vehicle-restraint group, by Bonferonni's multiple comparisons test. B, Brightfield photomicrographs depicting the interactions between restraint stress and SR141716 in the expression of Fos protein within the PVN. Note the robust Fos expression in the SR-R group. Treatment groups: vehicle control (V-C), vehicle-restraint (V-R), SR141716-control (SR-C), and SR141716-restraint (SR-R). *Bar* in SR-R figure, 50 μm.

hoc analysis revealed that the low dose of AM404 (2 mg/kg) significantly decreased serum CORT concentrations compared with those in vehicle-treated, restrained mice (Fig. 4B). Pretreatment with the high dose of AM404 (10 mg/kg) did not significantly affect serum CORT concentrations compared with those in vehicle-treated, restrained mice (Fig. 4B).

Pretreatment of mice with the FAAH inhibitor URB597 dose-dependently inhibited restraint-induced CORT release [by one-way ANOVA: $F_{(3,32)} = 27.79$; $P < 0.0001$]. *Post hoc* analysis revealed that both the low (0.1 mg/kg) and high (1 mg/kg) doses of URB597 significantly decreased restraint-induced CORT release compared with that in vehicle-treated, restrained mice (Fig. 4C). In a separate experiment, mice were pretreated with either SR141716 (1 mg/kg) or an equivalent amount of vehicle 1 h before the administration of URB597 (0.1 mg/kg). In this study restraint alone produced an increase in CORT of 8.4 ± 0.8-fold over that in unrestrained mice. Plasma CORT was increased 2.1 ± 0.3-fold in restrained mice pretreated with URB597; this was significantly less than the effect of restraint in vehicle-treated mice ($P < 0.001$). In the mice pretreated with SR141716 and URB597, the fold increase in CORT (6.5 ± 1.0) was not significantly different from that in vehicle-treated, restrained mice. These data indicate that the effect of URB597 is dependent upon CB_1 receptor activation.

FAAH immunoreactivity was highly enriched within neurons of the PVN (Fig. 5, A and B), indicating the molecular

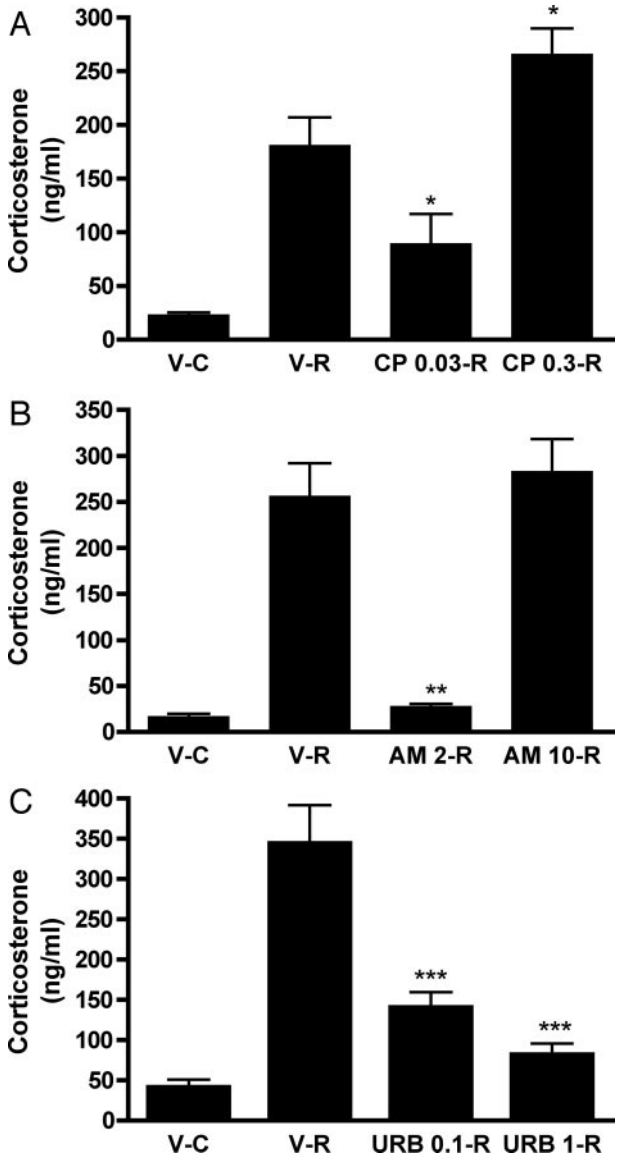

FIG. 4. A, Effects of CP55940 (CP; 0.03 and 0.3 mg/kg) on restraint-induced CORT release. B, Effects of AM404 (AM; 2 and 10 mg/kg) on restraint-induced CORT release. C, Effects of URB597 (URB; 0.1 and 1 mg/kg) on restraint-induced CORT release (n = 8–10/group). Treatment groups: vehicle control (V-C), vehicle-restraint (V-R), drug-control (CP, AM, URB, -C), and drug-restraint (CP, AM, URB, -R). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (significantly different from V-R group, by Bonferonni's multiple comparisons test).

target of URB597, and a key component of AEA signaling is present within the PVN.

Effects of acute and repeated restraint on hypothalamic eCB content

We examined the effect of acute and repeated restraint episodes on serum CORT and hypothalamic eCB content (Table 1). A single 30-min restraint exposure increased serum CORT, and exposure to 30 min of restraint for 5 consecutive days resulted in a habituated CORT response. In response to a single episode of restraint, the hypothalamic content of the eCB 2-AG was significantly reduced compared with control.

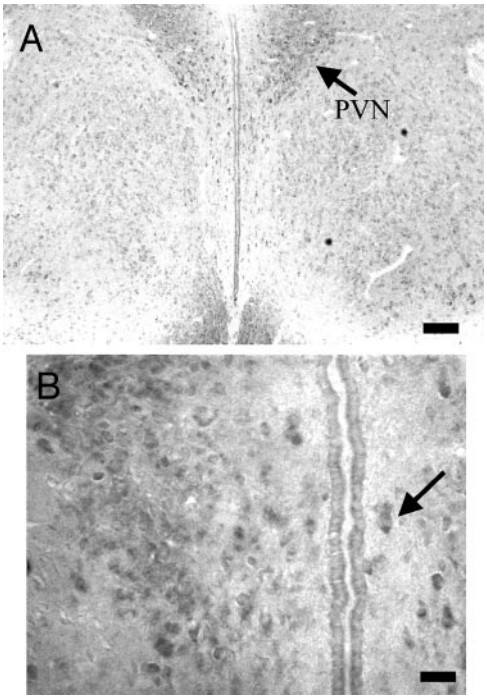

FIG. 5. A, Localization of FAAH within the PVN region (*black arrow*). B, Note the predominantly cytoplasmic expression of FAAH within PVN neurons (*black arrow*, for example). *Scale bar*, 100 μm in A and 25 μm in B.

TABLE 1. Effects of acute and repeated restraint (5 d) on serum CORT concentrations and hypothalamic eCB content

	Control	Acute restraint	Repeated restraint
Serum CORT (ng/ml)	27.4 ± 4.7	78.5 ± 9.2[a]	46.1 ± 3.2[b]
AEA content (pmol/g)	9.2 ± 0.7	7.1 ± 0.8	8.7 ± 0.9
2-AG content (nmol/g)	23.8 ± 1.7	16.3 ± 1.9[c]	31.3 ± 2.5[b,c]

Because the control values for the acute and repeated restraint experiments were not different, they were combined into one column.

[a] $P < 0.001$ *vs.* control.

[b] $P < 0.01$ *vs.* acute restraint condition by Bonferroni's multiple comparisons test.

[c] $P < 0.05$ *vs.* control.

After five repeated episodes of restraint, 2-AG content was significantly increased compared with the control value. Neither acute nor repeated restraint significantly affected hypothalamic AEA content.

Discussion

The main findings of the present study are 1) the CB_1 receptor antagonist SR141716 alone produced a small, but significant, activation of the HPA axis and potentiated HPA axis activation by restraint stress; 2) pharmacological augmentation of eCB signaling significantly reduces restraint-induced CORT release; and 3) acute restraint-induced activation of the HPA axis is associated with a decrease in hypothalamic 2-AG content, and the attenuated CORT response observed during the habituated state is associated with an increase in hypothalamic 2-AG content compared with those in control and acute restraint conditions. These data indicate that eCB signaling negatively modulates activation of the HPA axis and that there is a strong interaction

between CB_1 receptor blockade and environmental stress in the regulation of HPA axis activity. In addition, modulation of PVN activity appears to contribute to this interaction. These data suggest the eCB system is an important regulator of the central stress response and thus could be a novel target for the development of therapeutic interventions for anxiety disorders.

These data are consistent with the finding that intracerebroventricular injection of SR141716 increases plasma CORT concentrations in rats (14). In addition, context-dependent effects of CB_1 receptor gene deletion on anxiety-like behaviors and ACTH release in mice have been demonstrated (13). In particular, the anxiogenic effects of CB_1 receptor gene deletion were only apparent under high light conditions, an anxiogenic context. Similarly, ACTH release in response to novelty stress was greater in $CB_1^{-/-}$ than WT mice (13). When considered together with our data that SR141716-induced increases in serum CORT and PVN neuron activity are robustly potentiated by restraint stress, these findings indicate that eCB signaling negatively modulates the activity of the HPA axis. Our findings that CP55940, AM404, and URB597 reduce or eliminate restraint-induced CORT release provide additional evidence to support this hypothesis. Our finding that the hypothalamic content of the endocannabinoid 2-AG is affected by restraint stress (discussed more fully below) is also consistent with the hypothesis that eCB signaling is an important physiological regulator of HPA axis activity.

A recent electrophysiological study by Di *et al.* (15) provides a cellular model of eCB signaling within the PVN that is consistent with the present findings. These investigators found that excitatory input onto PVN neurons is inhibited by CB_1 receptor activation (15). Our physiological data are consistent with this model because activation of the CB_1 receptor by exogenous administration of a low dose of the CB_1 receptor agonist CP55940 inhibits stress-induced CORT release. In addition, we found that blockade of CB_1 receptors during restraint potentiated restraint-induced CORT release and Fos expression within the PVN, suggesting that functional eCB signaling inhibits stress-induced activation of PVN neurons, either directly or via modulation of afferent circuits. Interestingly, SR141716 pretreatment potentiated restraint-induced Fos expression within both magnocellular and parvocellular divisions of the PVN, suggesting a more widespread role for eCB signaling in the inhibition of many neuroendocrine pathways in addition to the glucocorticoid system. This conclusion was also reached by Di *et al.* (15), who found that CB_1 receptor activation inhibits the glutamatergic activation of many different cell types within the PVN. Indeed, we demonstrated many years ago that Δ^9-tetrahydrocannabinol nearly abolished circulating TSH via a mechanism that was consistent with inhibition of TRH release (20).

Restraint stress affects eCB content in the hypothalamus. Animals killed immediately after 30 min of restraint exhibited a significant decrease in the hypothalamic content of 2-AG. In contrast, mice exposed to the same restraint for 5 consecutive days exhibited significantly elevated 2-AG content compared with that under control and acute restraint conditions. When these data are compared with the CORT response of similarly treated mice, an interesting relationship is observed. Specifically, 2-AG is reduced, and CORT is elevated after a single restraint, whereas in restraint-habituated mice, 2-AG is elevated (compared with both control and acute restraint conditions), and the CORT response is attenuated compared with that under the acute restraint condition. Taken together with the effects of pharmacological manipulations of the CB_1 receptor on restraint-induced CORT release, we hypothesize that the 2-AG content within the hypothalamus is high during nonstressful situations and functions to dampen HPA axis activity. Stress induces a reduction in hypothalamic 2-AG content, leading to reduced activation of the CB_1 receptor and facilitation of stress-induced glutamatergic afferent activation of the corticotropin-releasing factor-releasing neurons. This mechanism is consistent with our findings that CB_1 receptor antagonist treatment increases CORT release in nonstressed animals and profoundly potentiates release in stressed animals, whereas pharmacological elevation (or maintenance) of hypothalamic eCB concentrations during stress reduces HPA axis activation. We also hypothesize that the lack of a reduction, rather an elevation, in hypothalamic 2-AG content in response to repeated restraint contributes to the attenuated CORT response observed in restraint-habituated mice.

Our conclusions differ from those of Di *et al.* (15), who postulated that stress induces an increase in hypothalamic eCB content, possibly mediated by CORT activation of nonnuclear receptors. These researchers suggested that the function of eCBs within the hypothalamus is to mediate rapid feedback inhibition of the CORT response by reducing excitatory input to PVN CRF neurons. Several technical and theoretical possibilities exist that could explain the difference in the conclusions reached. First, it is possible that highly localized increases in eCB content occur within the PVN during stress that are overwhelmed or diluted by decreases in other hypothalamic regions. Second, it is possible that increases in eCB content occur at different time points than the one assayed. If an eCB-mediated feedback mechanism is operative, then it may occur minutes to hours after termination of the stressor, not during the stress (as assayed in the present study). In fact, stress itself could antagonize or counteract an eCB-mediated feedback mechanism. The optimization of eCB detection techniques with higher spatial and temporal resolution will help clarify these issues.

Consistent with previous data that direct CB_1 agonists have biphasic effects on HPA axis activation and anxiety-like behaviors, we found that only the low dose of CP55940 (0.03 mg/kg) suppressed stress-induced CORT release, whereas the high dose (0.3 mg/kg) facilitated CORT release in response to restraint. This finding is in agreement with other studies in which high doses of CB_1 agonists were found to increase HPA axis activation (14, 21). It is quite likely that cannabinoid effects on brain regions, either upstream or downstream of the PVN, result in opposing effects on CORT. In fact, deafferentation of the hypothalamus reduces Δ^9-tetrahydrocannabinol-induced CORT release, evidence that the cannabinoids can increase HPA axis activity via actions within brain regions upstream of the hypothalamus (22). In particular, recent studies suggest that eCB signaling within

the amygdala and/or anterior pituitary could contribute to the activation of HPA axis by CB_1 receptor agonists (23–25).

AM404, which inhibits the reuptake of AEA and 2-AG (26, 27) as well as FAAH activity (28), blocked restraint-induced CORT release at a low dose only. However, in contrast to CP55940, the high dose of AM404 (10 mg/kg) did not potentiate restraint-induced CORT release. It is possible that higher doses of AM404 would have potentiated restraint-induced CORT release; however, the use of doses higher than 10 mg/kg have not been reported in the literature.

Interestingly, URB597, a compound that selectively inhibits FAAH activity and increases brain fatty acid ethanolamide content (including AEA) (7), blocked restraint-induced CORT release at both a low and a high dose. Although inhibition of FAAH would be expected to increase the brain content of the entire family of *N*-acylethanolamines, its effect on CORT was reversed by the CB_1 receptor antagonist SR141716, suggesting that AEA is the FAAH substrate responsible for this effect. It is unlikely that higher doses of URB597 would potentiate restraint-induced CORT release because the high dose used in this study (1 mg/kg) is several-fold higher than that which produces maximal and irreversible FAAH inhibition (~95% inhibition for several hours). This finding is consistent with data showing that URB597 produces monophasic, dose-dependent, anxiolytic actions in animals (7). We found that FAAH protein is abundant within PVN neurons, so URB597 could elevate the fatty acyl ethanolamides, including AEA, within the PVN, resulting in increased CB_1 receptor activation locally. Alternatively, increased CB_1 receptor activity in other brain regions could be responsible for the anxiolytic effects of URB597. Regardless of the site of action, the present data combined with data from a previous study (7) indicate that selective inhibitors of FAAH, such as URB597, are attractive candidates for eCB-based treatments for anxiety-related disorders because they produce monophasic anxiolytic effects. This approach is less likely to precipitate the adverse panic and anxiety reactions commonly observed during marijuana intoxication (29, 30).

In conclusion, the present study demonstrates a robust relationship between environmental stress and eCB signaling in the regulation of HPA axis activation and indicates that the role of eCB signaling is to inhibit activation of this pathway. Our eCB data lead us to the working hypothesis that endogenous CB_1 receptor activation is high during nonstressed periods and is reduced during stress. The reduction in CB_1 receptor activation could result in increased synaptic activity at glutamatergic afferents to the PVN, thus allowing for stressful stimuli to activate the HPA axis. This hypothesis fits with the well documented role of the CB_1 receptor as an inhibitor of synaptic activity (6). Because URB597 completely blocked stress-induced CORT release without exhibiting the biphasic properties of direct CB_1 agonists, we suggest that inhibition of FAAH activity represents a novel approach to the development of antianxiety therapies. These data underscore the importance of eCBs in the regulation of neuroendocrine function and anxiety.

Acknowledgments

Received May 19, 2004. Accepted August 20, 2004.

Address all correspondence and requests for reprints to: Dr. Cecilia J. Hillard, Department of Pharmacology and Toxicology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, Wisconsin 53226. E-mail: chillard@mcw.edu.

This work was supported by National Institutes of Health Grants R01-DA-016967 and F30-DA-15575 (to S.P.).

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