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Common Mechanisms Underlying the Proconflict Effects of Corticotropin-Releasing Factor, A Benzodiazepine Inverse Agonist and Electric Foot-Shock[1]

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ABSTRACT

The effects of corticotropin-releasing factor (CRF), a benzodiazepine inverse agonist (methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate; DMCM) and electric foot-shock on rat conflict behavior were characterized and compared. Rats were trained to lever press under a multiple fixed-ratio schedule (FR 20) of food reinforcement in which responses during the first component were not punished, and the first response of each FR during the second component produced electric shock of an intensity sufficient to suppress responding by 10% to 15%. Intracerebroventricular injection of CRF (0.1–5.6 μg) caused a dose-dependent decrease in the rate of responding in both components of the schedule. However, CRF was more potent in decreasing rates of punished responding (proconflict effect). DMCM (10–100 μg; i.c.v.) also decreased rates of punished and nonpunished responding and was more potent during the punishment component. The suppression of punished and nonpunished responding by CRF and DMCM was mimicked by increasing the shock intensity (Δ = 0.1 to 0.6 mA) during the punishment component. To determine whether CRF, DMCM and electric shock shared common mechanisms for these effects, rats were pretreated with i.c.v. injections of either a CRF antagonist (α helical CRF_{9-41}, 50 μg), a benzodiazepine agonist (chlordiazepoxide, 10 μg) or a benzodiazepine antagonist (flumazenil, 10 μg) before the administration of equieffective doses of CRF or DMCM or an increase in shock intensity. Chlordiazepoxide attenuated the effects of all three stimuli. Flumazenil antagonized DMCM and CRF, but not shock, implicating a pharmacologic interaction between CRF and benzodiazepine systems. In contrast, α helical CRF_{9-41} antagonized CRF and shock, but not DMCM, suggesting that the effects of shock, but not of DMCM, may be due to endogeneous CRF release. Together, the present results indicate that the proconflict effects of CRF, DMCM and electric foot-shock share some common mechanisms and that the effects produced by CRF may require the release of an endogenous benzodiazepine inverse agonist.

The 41-amino acid neuropeptide, corticotropin-releasing factor, is the prime physiologic regulator of the hypothalamic-pituitary-adrenal axis during stress (Rivier *et al.*, 1982; Vale *et al.*, 1981, 1983). Apart from its neurohormonal role in the pituitary, CRF is also thought to serve as a neurotransmitter or neuromodulator in extrahypophyseal regions to mediate autonomic and behavioral components of stress responses (see Vale *et al.*, 1983; Valentino, 1988; Dunn and Berridge, 1990 for reviews). Many of the behavioral effects produced by central administration of CRF are consistent with a role of CRF in anxiety (Dunn and Berridge, 1990 for review). In support of this, abnormalities in CRF function have been associated with affective and anxiety-related disorders (for review see De Souza, 1991).

Substantial clinical and experimental evidence indicates that brain benzodiazepine/GABA-ergic systems are also intimately involved in the physiologic control of stress/anxiety responses. For example, it is well documented that anxiolytic benzodiazepine receptor agonists generally block or attenuate the physiologic, neuroendocrine and behavioral manifestations of stress (Hommer *et al.*, 1987; File *et al.*, 1988; De Boer *et al.*, in press). On the other hand, administration of benzodiazepine receptor inverse agonists such as the β-carbolines, FG 7142, β-CCE and DMCM, has been shown to evoke a profound stress/anxiety-like profile of electrophysiologic, neurochemical, endocrine/autonomic and behavioral responses qualitatively similar to those elicited by i.c.v. CRF injection (for reviews see: Hommer *et al.*, 1987; File and Baldwin, 1987; File *et al.*, 1988; Theibot *et al.*, 1988; De Boer *et al.*, in press). Further support for the role of brain benzodiazepine/GABA-ergic systems in stress and

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ABBREVIATIONS: CRF, corticotropin-releasing factor; GABA, γ-aminobutyric acid; β-CCE, β-carboline-3-carboxylic acid ethyl ester; DMCM, methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate; FR, fixed ratio; ANOVA, analysis of variance; DBI, diazepam binding inhibitor.

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anxiety comes from neurochemical studies showing rapid, stress-induced modifications of the number and biophysical properties of benzodiazepine/GABA-ergic receptors in discrete brain regions (Havoundjian *et al.*, 1987). Finally, clinical studies have shown that certain stress/anxiety-related disorders are associated with dysfunctions in the benzodiazepine/GABA-ergic receptor system (Nutt *et al.*, 1990; Roy-Byrne *et al.*, 1990).

The fact that brain CRF and benzodiazepine/GABA-ergic systems are both implicated in the (patho)physiologic expression of stress/anxiety responses suggests a possible functional interaction between the two. Indeed, it has been shown that anxiolytic benzodiazepine receptor agonists can reverse or antagonize several CRF-mediated behavioral effects that are thought to be related to anxiety, *e.g.*, locomotor activation in an open field (Lee *et al.*, 1987); decreases in social interaction (Dunn and File, 1987); enhancement of acoustic startle (Swerdlow *et al.*, 1986); increased defensive withdrawal (Yang *et al.*, 1990); and potentiation of punishment (proconflict effect) (Britton *et al.*, 1985, 1988; Zhang and Barrett, 1990). Accordingly, the anxiogenic benzodiazepine inverse agonist FG 7142; enhances the proconflict action of CRF (Britton *et al.*, 1988). A relationship between benzodiazepine and CRF neuronal systems is also supported at the neurochemical level. Thus, the anxiolytic triazolobenzodiazepine alprazolam exerts effects on CRF levels in locus ceruleus and hypothalamus/median eminence regions opposite to those observed after stress (Owens *et al.*, 1989, 1991). Additionally, *in vitro* studies showed that alprazolam and diazepam inhibited serotonin-induced CRF release from rat hypothalamic organ cultures, whereas the benzodiazepine inverse agonist β-carboline-3-carboxylic acid methylester stimulated CRF secretion in this preparation (Calogero *et al.*, 1988; Kalogeras *et al.*, 1990). Together, these findings suggest that the agonist (anxiolytic) and inverse agonist (anxiogenic) actions of benzodiazepine receptor ligands involve attenuation or enhancement of CRF neurohormonal/neurotransmitter function, respectively.

An alternative explanation for the complex CRF/benzodiazepine interactions is that the effects of CRF may be mediated through a direct or indirect modulation of benzodiazepine/GABA-receptor function. Consistent with this model, the specific benzodiazepine receptor antagonist, flumazenil, has been shown to reverse the proconflict effects of CRF (Britton *et al.*, 1988), indicating that either CRF itself, or an endogenous inverse agonist ligand released by CRF, interacts with the benzodiazepine receptor. In contrast, however, File and colleagues (1988) were not able to find a reversal by flumazenil of the CRF effects in the plus-maze or social interaction tests. Hence, this model of CRF/benzodiazepine receptor interaction is not clearly resolved yet.

The present study was designed to characterize the interaction between CRF and benzodiazepine systems in behaviors controlled by aversive stimuli and to determine whether: 1) the effects of benzodiazepine receptor inverse agonists are mediated *via* endogenous CRF release; 2) the effects of CRF are mediated through an interaction with benzodiazepine systems; and 3) the effects of electric shock are mediated through endogenous CRF and/or benzodiazepine systems. To test these hypotheses the Geller-Seifter conflict procedure (Geller and Seifter, 1960) was used. Previous studies using this procedure to investigate effects of CRF and benzodiazepine inverse agonists failed to show a selective proconflict effect of these compounds, *i.e.*, nonpunished and punished responding were equally suppressed (Prado de Carvalho *et al.*, 1983; Quintero *et al.*, 1985; Britton *et al.*, 1985, 1988; Koob *et al.*, 1986; Barrett *et al.*, 1989). The failure to find a selective proconflict effect may have been due to the shock intensity used, because shock intensity is a critical determinant for revealing proconflict effects of drugs acting on the benzodiazepine/$GABA_A$-receptor complex (Shekhar *et al.*, 1989; Giusti *et al.*, 1991; Takada *et al.*, 1992). Therefore, in the present study, a punishment procedure was used with a subthreshold intensity of shock that only minimally suppressed responding so that it would be possible to observe an increase in the effectiveness of electric shock after drug treatment. Using this procedure, the proconflict effects of CRF and the benzodiazepine inverse agonist, DMCM, were compared with those produced by increasing intensities of electric foot-shock, and the sensitivity of each stimulus to antagonism by the benzodiazepine agonist, chlordiazepoxide, the benzodiazepine antagonist, flumazenil, and the CRF antagonist, α helical CRF_{9-41}, was assessed.

Methods

Animals. Twenty adult male Sprague-Dawley rats (Taconic Farms, Inc., Germantown, NJ) weighing 276 to 300 g upon arrival in the laboratory were used as subjects in these studies. Animals were housed individually in polycarbonate cages with wood shavings and placed in a room with constant temperature (22 ± 0.5°C) and 12 hr light-12 hr dark lighting conditions (lights on from 7:00 A.M. to 7:00 P.M.). Tap water was freely available except during the experimental sessions. Rats were reduced to approximately 85% of their unrestricted-feeding body weights (347 ± 8 g) and then maintained on 12 to 15 g of standard laboratory chow (Purina, 5001) per day in addition to the food pellets obtained during the experimental sessions.

Apparatus. Three two-lever operant conditioning chambers (BRS/LVE, Laurel, MD), equipped with a houselight, three response lights above the levers (white, green and red) and a grid floor, were used. A food-pellet dispenser delivered 45-mg pellets (Bioserv, Inc., Frenchtown, NJ) to a tray placed between the levers. The bars of the grid floor were connected to a constant-current shock generator/scrambler (BRS/LVE). The operant chambers were enclosed individually within sound-attenuating boxes equipped with a ventilation fan, and all boxes were placed in a sound-attenuating room adjacent to a room containing the programming equipment. Experimental control and data collection were provided by a microcomputer operating MED-PC software and controlling a solid-state interface (MED Associates, East Fairfield, VT).

Surgery and infusions. To enable central administration of peptide, drug and vehicle solutions, rats were prepared with stainless-steel cannula guides aimed at the lateral ventricle. Rats were anesthetized with a mixture of halothane-in-air (1–2%) administered through a nose cone, then positioned in a Narishige stereotaxic instrument with non-injurious ear bars. The head was oriented at a 15° angle to the horizontal plane (nose down). Body temperature was maintained at 37°C with a small temperature-controlled heating pad. The skull was exposed with a scalpel blade, and xylocaine (4%) was applied to cut surfaces for local anesthesia. A hole (approximately 1.0 mm in diameter) was drilled 1.0 mm caudal to bregma and 1.5 mm lateral to the midline for placement of a sterilized 22-gauge stainless-steel cannula guide (Plastic Products, Roanoke, VA) 1 mm above the lateral ventricle, 4.6 mm ventral to the skull surface. Three additional holes were drilled for skull screws. The guide was positioned and cemented to the skull surface and screws with cranioplastic cement. A stylet was inserted into the guide to prevent exposure. The cut was then sutured, and 0.1 ml of an antibiotic (aqueous suspension of sterile benzathine penicillin G and penicillin G procaine, 300,000 U/ml) was administered s.c. All surgical tools and implanted materials (cannula guides and screws) were sterilized by soaking for at least 24 hours in Cidex. Surgery was done under aseptic conditions.

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For i.c.v. injections, the stylet was removed and a 26-gauge cannula (Plastic Products, Roanoke, VA) which was connected by PE tubing to a 10-μl Hamilton syringe was inserted into the cannula guide. The length of the cannula exceeded the guide by 1.0 mm so that its tip was in the lateral ventricle. The tubing was filled with the solution to be tested. The cannula was left in place for at least 30 sec following the injection to prevent efflux of the injected solutions. After removal of the cannula, the stylet was replaced. To verify patency of the i.c.v. cannula during the course of the experiment, angiotensin (50 μg in 3 μl) was administered through the i.c.v. cannula and the onset (<60 sec) and duration (>30 sec) of drinking were recorded.

Drugs. All solutions were prepared freshly on the day they were used. CRF and the CRF antagonist, α helical CRF_{9-41} (generously supplied by Dr. Jean Rivier of The Peptide Biology Laboratory, The Salk Institute, San Diego, CA), as well as chlordiazepoxide HCl (Hoffmann-La Roche Inc., Nutley, NJ) were dissolved in sterile 0.9% saline solution. Flumazenil (kindly supplied by Hoffmann-La Roche Inc.) was suspended in a vehicle consisting of sterile distilled water to which Tween 80 (2 drops/10 ml) was added. DMCM (R.B.I., Natick, MA) was dissolved in an acidified (HCl) saline solution. All drugs were administered i.c.v. in a volume of 3 μl (CRF, DMCM) or 5 μl (α helical CRF_{9-41}, chlordiazepoxide and flumazenil).

Behavioral training. Rats were trained to press the right lever with each response producing food reinforcement (FR 1 schedule). The experimental session was initiated by the illumination of a white houselight. Initially, the white and green lights above the right lever were on; 3 min later, these were turned off and a red light signaling the initiation of the second component of the schedule was turned on. These two schedule components alternated every 3 min, and the session lasted 30 min. With continued training the FR response requirement was progressively increased until 20 lever responses were required for each food pellet (FR 20). After about 25 sessions, response rates were stable and comparable in both schedule components and ranged from 0.94 to 1.88 responses/sec. At this time, electric shock was introduced into the second component of the schedule such that the first response of each FR 20 resulted in the delivery of a 0.5-msec constant current scrambled shock to the grid floor of the chamber. The intensity of the shock used varied between individual rats (range: 0.05–0.4 mA) and was chosen on the basis of its ability to decrease the rate of responding in the second (punished) component by 10% to 15% (mean: 13.8 ± 2.9%), *i.e.*, 85% to 90% of nonpunishment response rates. Shock levels were never adjusted during periods of drug administration. The experimental sessions were run once daily, 6 days a week (except on Sunday) between 10:00 A.M. and 2:00 P.M.

Behavioral testing. When response rates appeared to be stable (*i.e.*, about 10 sessions after the introduction of electric shock), as judged by their variation of less than 10% over three consecutive sessions, rats were prepared with i.c.v. cannulas for peptide and drug administration. After a recovery period of 1 week, rats were retrained in the punishment procedure. When stable base lines of responding were obtained, similar to those obtained before surgery (*i.e.*, after about 16 sessions), behavioral testing began. Either CRF (0.1, 0.3, 1.0, 3.0, 5.6 μg), DMCM (3, 10, 30, 56, 100 μg) or saline (3 μl) was administered i.c.v. over a 30-sec period, 5 min before the start of the session. The efficacy of various shock intensities as punishment was determined in sessions in which the shock intensity used in the second component of the schedule was increased (*i.e.*, changes cf 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mA) and varied between 0.15 and 1.0 mA. Only one intensity of shock was used in a single session. Rats were usually tested twice a week and retrained on the other 4 days. Drug doses and shock intensity increases were studied in a mixed sequence that differed between subjects.

For pharmacologic antagonism experiments, either chlordiazepoxide (10 μg), flumazenil (10 μg), α helical CRF_{9-41} (50 μg) or vehicle (5 μl) was administered 5 min before CRF or DMCM. In sessions designed to investigate the effects of these antagonists on the punishing efficacy of different shock intensities, the antagonists were administered 5 min before the start of the session.

Histology. At the end of the experiment, neutral red dye (5 μl) was injected through the i.c.v. cannula and the rats were sacrificed by i.p. injection of 150 mg/kg pentobarbital. The brains were removed and sectioned for visualization of the dye in the ventricular system.

Data analysis. Data were obtained as the mean number of lever responses per second (response rate) during the nonpunishment and punishment components of the schedule. Drug and shock effects on response rates were expressed as the percentage of the rates determined during the non-drug training sessions conducted on the days before the test sessions (% control response rate). In addition, individual suppression ratio values were calculated for each drug dose or shock intensity. Suppression ratio was defined as the ratio of response rate in the punishment component and the sum of the response rates in both components. This ratio typically takes values between 0, indicating a selective and complete suppression of punished responding compared with nonpunished responding, and 0.5, indicating that the nonpunished and punished responding are equally affected.

The dose- or intensity-effect curves for the % control response rates were analyzed by a two-way ANOVA, with schedule component as within-subject factor 1 (two levels: nonpunished and punished responding) and drug dose or shock intensity as within-subject factor 2 (six or seven levels, respectively). The dose- or intensity-effect curves for the suppression ratios were analyzed by one-way ANOVA, with drug dose or shock intensity as within-subject factor (six or seven levels, respectively). The pharmacologic antagonism effects on nonpunished responding and punished responding were analyzed separately by a two-way ANOVA, with pretreatment as within-subject factor 1 (four levels) and treatment as within-subject factor 2 (four levels). Further analyses were made by Duncan's new multiple range test to determine the source of detected significance in the ANOVAs. The criterion of significance was set at $P < .05$.

Results

Proconflict effects of CRF, DMCM and shock. Control rates of nonpunished responding and punished responding ranged from 0.95 to 2.16 (mean: 1.58 ± 0.12) and from 0.88 to 1.80 (mean: 1.33 ± 0.08) responses per second, respectively. Figure 1 shows representative data from sequential sessions for one subject. Rates of responding in the two components of the schedule varied little from one session to the next. With i.c.v. administration of either CRF or DMCM, or with an increase in the intensity of electric shock, rates of responding were decreased, with those decreases generally greater in the punishment component (proconflict effect). At low to intermediate doses the decreases were selective to the punishment component (*e.g.*, 0.1 and 0.3 μg CRF; 3.0 and 10.0 μg DMCM; 0.2 mA shock). Performances recovered to base line during sessions immediately following those in which the effects of drugs or changes in intensity of shock were assessed. Neither repeated administration of the drugs (no more than twice weekly) nor the order of treatments appeared to alter the reliability of results obtained under this schedule.

The selective aspects of the effects of these drugs and changes in shock intensity can be better observed in the dose-effect, or intensity-effect functions (figs. 2 and 3). CRF, delivered i.c.v., produced a dose-related decrease in rates of both nonpunished and punished responding. However, rates of punished responding were affected at doses lower than those necessary to decrease nonpunished responding (fig. 2A, compare open and filled squares). In particular, the 0.1- to 1.0-μg doses produced selective proconflict effects, whereas higher doses (3.0, 5.6 μg) decreased both punished and nonpunished responding more similarly. Thus, the CRF dose-effect curve for effects on punished responding was shifted to the left of that for effects on nonpunished responding ($F_{1,11} = 68$; $P < .001$). The selective

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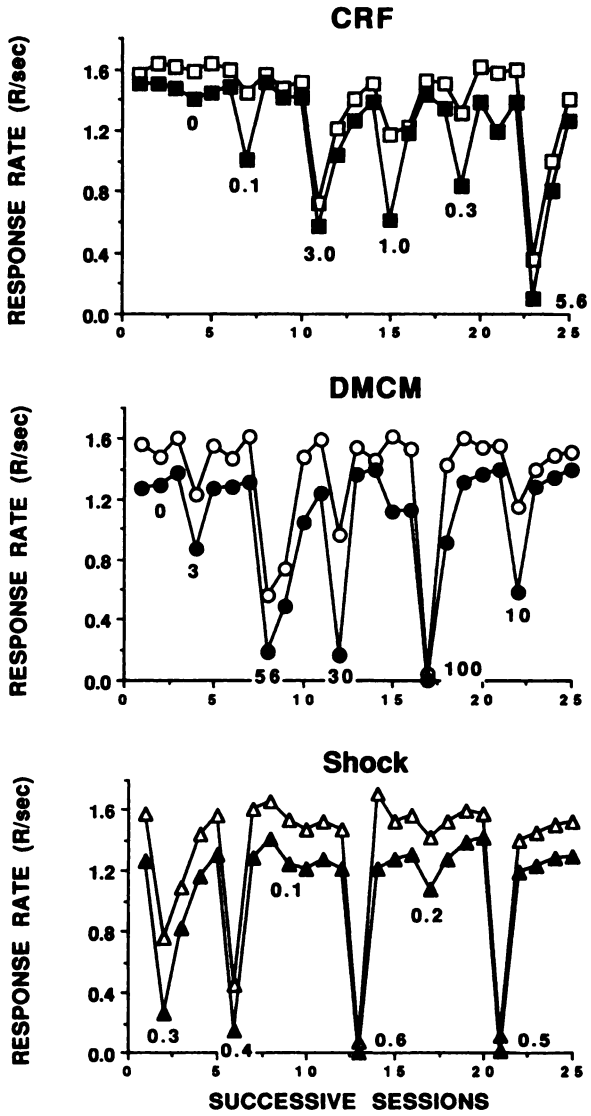

Fig. 1. Effects of CRF, DMCM and shock on rates of nonpunished (open symbols) and punished (filled symbols) responding in a representative subject. The abscissae indicate the number of successive sessions. The ordinates indicate mean response rates expressed as number of lever presses per second. Each point represents the response rate determined in each component of the multiple schedule during that particular session. The numbers under certain points indicate a drug dose (micrograms, i.c.v.) or increase in shock intensity (milliamperes) that was used in the test session. Note selective suppression of punished responding elicited by CRF (0.1–1.0 μg) and DMCM (3–30 μg).

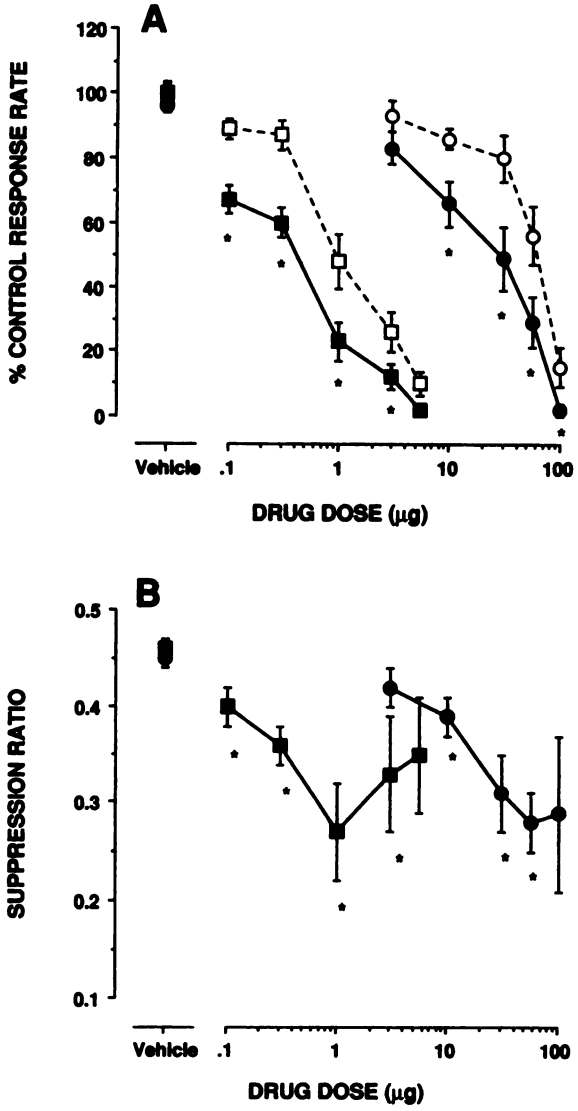

Fig. 2. Dose-response curves for the proconflict effects of CRF and DMCM on punished behavior. The abscissae indicate the dose (micrograms, i.c.v.; log scale). Panel A: Shown are dose-response curves for effects of CRF (squares) and DMCM (circles) on nonpunished (open symbols) and punished (filled symbols) responding. The ordinates indicate the response rates expressed as a percentage of the rates determined during the nondrug control session 1 day before the test sessions. Each point is the mean of 10 to 12 rats. Vertical lines indicate ± 1 S.E.M. Points at vehicle represent sessions when vehicle was administered. Two-way ANOVA on the values in panel A revealed significant main effects of schedule component (CRF: $F_{1,11} = 68.3$, $P < .001$; DMCM: $F_{1,9} = 39.1$, $P < .001$) and drug dose (CRF: $F_{5,55} = 9.7$, $P < .001$; DMCM: $F_{5,45} = 59.4$, $P < .001$) as well as a significant schedule component × drug dose interaction effect (CRF: $F_{5,55} = 9.7$, $P < .001$; DMCM: $F_{5,45} = 7.2$; $P < .001$). Asterisks indicate that values for punished responding are significantly (at least $P < .05$; Duncan's) lower than corresponding values for nonpunished responding. Panel B: Shown are dose-response curves for CRF and DMCM on the suppression ratio. The ordinate indicates the suppression ratio, which provides a measure of suppression in response rate during the punished component relative to the nonpunished responding. One-way ANOVA on these values yielded a significant effect of drug dose (CRF: $F_{5,55} = 4.72$, $P < .01$; DMCM: $F_{5,45} = 6.7$, $P < .001$). Asterisks indicate a significant (at least $P < .05$; Duncan's) difference from the vehicle value. Note that both drugs are more potent in decreasing punished responding and that CRF is about 50 times more potent than DMCM.

aspects of the effects can also be seen in the suppression-ratio values (fig. 2B, squares) which show a dose-related decrease up to a dose of 1 μg, with a reversal of this trend at the higher doses.

DMCM also decreased response rates in both components of the schedule (fig. 2A, circles). Rates of responding in the punishment component were selectively decreased (fig. 2A, compare open and filled circles), particularly at the lower doses (10–30 μg). Similar to CRF, the DMCM dose-effect curve for effects on punished responding was shifted to the left of that for effects on nonpunished responding ($F_{1,9} = 39$; $P < .001$). The selective aspects of the effects of DMCM are also exhibited as a decrease in the suppression-ratio values (fig. 2B, circles). These values decrease at doses of 3 to 30 μg, with a plateau at the highest doses.

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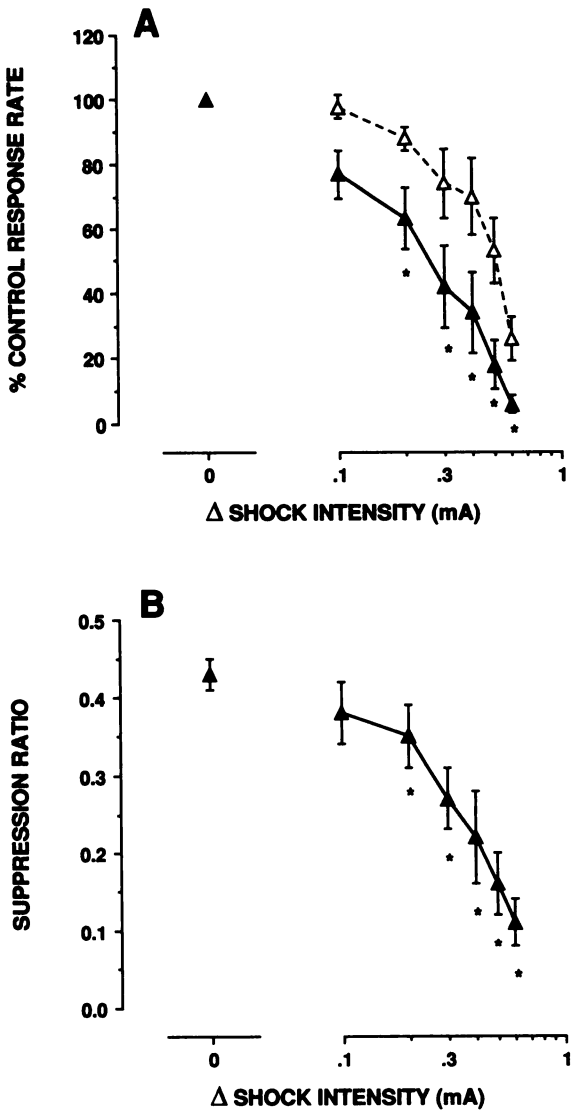

Fig. 3. Intensity-effect curves for the suppressant effects of electric footshock on punished behavior. The abscissae indicate the change in electric shock intensity in milliamperes (log scale). Panel A: Shown are intensity-response curves for effects on nonpunished (open symbols) and punished (filled symbols) response rate. The ordinate indicates response rate expressed as a percentage of the rates determined during the training session 1 day before the test session. Each point is the mean of 8 rats. Vertical lines represent ± 1 S.E.M. Two-way ANOVA on these values revealed significant effects of the main factors, schedule component ($F_{1,7} = 54.9$, $P < .001$) and shock intensity ($F_{6,42} = 32.8$, $P < .001$), as well as a significant interaction effect ($F_{6,42} = 5.3$, $P < .001$). Asterisks indicate that values for punished responding are significantly (at least $P < .05$; Duncan's) lower than corresponding values for nonpunished responding. Panel B: Intensity-response curve for the effects of shock on the suppression ratios. One-way ANOVA on these values yielded a significant effect of shock intensity ($F_{6,42} = 21.3$, $P < .001$). Asterisks indicate a significant (at least $P < .05$; Duncan's) difference from the point zero value.

Increases for a single session in the intensity of shock delivered in the punishment component produced decreases in rates of responding in the two components (fig. 3A). Changes in intensity as small as 0.1 mA were effective in decreasing rates of responding. These decreases in rates of responding were greater in the punishment component, but also occurred in the alternate component in which shocks were never delivered. Smaller increases in shock intensity were effective in decreasing rates of responding in the punishment component compared with the alternate component, resulting in a statistically significant shift to the left in the intensity-effect curve for effects on punished responding compared with that for effects on nonpunished responding ($F_{1,7} = 55$, $P < .001$). The suppression ratio values (fig. 3B) show a monotonic decrease with increasing values for the change in the intensity of electric shock.

Antagonism of CRF, DMCM and shock. Figure 4 shows the effects of various pretreatments on the behavioral effects produced by CRF, DMCM and shock-intensity changes. Pretreatment with two i.c.v. vehicle injections (left-most open bar) did not appreciably affect rates of responding in either the nonpunishment component (fig. 4A) or the punishment component (fig. 4B). The anxiolytic benzodiazepine, chlordiazepoxide (10 μg), when administered alone i.c.v. (left-most bar with horizontal stripes), did not alter either rates of nonpunished (fig. 4A) or punished responding (fig. 4B). Pretreatments with either the benzodiazepine antagonist, flumazenil (10 μg; left-most filled bar), or the CRF antagonist, α helical CRF_{9-41} (50 μg; left-most bar with vertical stripes), were similarly inactive.

The 10-μg dose of chlordiazepoxide significantly attenuated the response rate suppressing effects of equally effective doses of both CRF and DMCM (bars with horizontal stripes above appropriate drugs). This antagonism was obtained in both components of the schedule. While the effects of these drugs were attenuated, rates of responding were not restored completely to control levels. Chlordiazepoxide pretreatment similarly diminished the effects of a change in shock intensity that was as effective as CRF and DMCM (fig. 4, right-most bar with horizontal stripes).

Pretreatment with the CRF antagonist, α helical CRF_{9-41} (50 μg), almost completely prevented the effects of CRF on responding in both components of the schedule (fig. 4, filled bar over CRF). Similarly, the suppression of responding by electric shock was significantly attenuated by the CRF antagonist, although to a lesser extent than was suppression by CRF (fig. 4, right-most filled bar). In contrast, the suppressant effects of DMCM were not significantly attenuated by pretreatment with α helical CRF_{9-41} (fig. 4, filled bar over DMCM).

The benzodiazepine antagonist, flumazenil (10 μg), produced virtually a complete antagonism of the effects of CRF and DMCM on rates of nonpunished and punished responding (fig. 4, bars with vertical stripes). In contrast, the effects of a change in the intensity of electric shock were unaffected by flumazenil pretreatment.

Discussion

The results demonstrate that i.c.v. administration of CRF and DMCM have identical effects on food-reinforced responding generated by the multiple schedule used in the present study; *i.e.*, CRF and DMCM decreased nonpunished responding and responding that was punished by a relatively low-intensity shock. The proconflict effect of each of these drugs was indicated by the finding that punished responding was more sensitive to the effects of certain doses of the drugs than was nonpunished responding. Increasing the shock intensity in the punishment component mimicked the drug effects, further indicating a proconflict effect of these drugs. These results are consistent with other studies that demonstrate suppressant effects of CRF and benzodiazepine inverse agonists on food-reinforced responding (Prado de Carvalho *et al.*, 1983; Quintero

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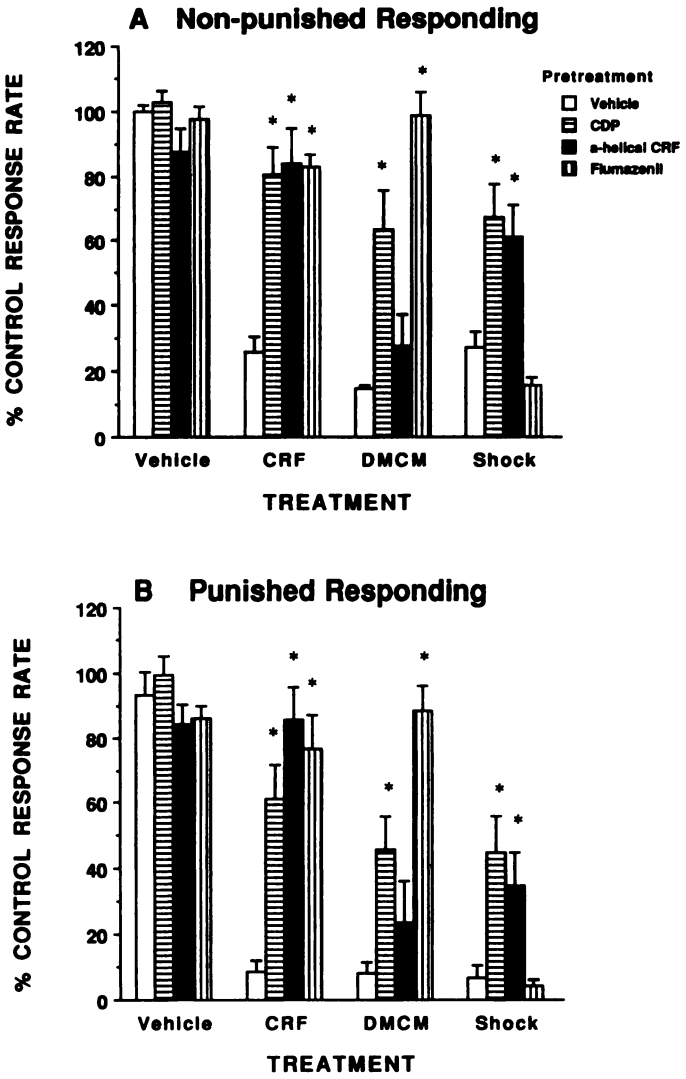

Fig. 4. Antagonism of the effects of CRF, DMCM and shock. The ordinate represents the response rate expressed as a percentage of the rate determined on training sessions 1 day before the test. The abscissae indicate vehicle (3 μl), CRF (1.0 μg), DMCM (56 μg) or shock (0.4 mA increase) treatment during the test session. Rats were pretreated (10 min before test session) with either vehicle (5 μl; open bars), chlordiazepoxide (CDP; 10 μg; horizontal striped bars), α helical CRF_{9-41} (50 μg; closed bars) or flumazenil (10 μg; vertical striped bars). Each bar is the mean of 6 rats and vertical lines indicate ± 1 S.E.M. Panels A and B show effects on nonpunished and punished responding, respectively. Two-way ANOVA on the values in panels A and B revealed significant treatment (A: $F_{3,15} = 36.5$, $P < .001$; B: $F_{3,15} = 60.5$, $P < .001$) and pretreatment (A: $F_{3,15} = 58.3$, $P < .001$; B: $F_{3,15} = 23.2$, $P < .001$) main effects, as well as a significant treatment × pretreatment interaction (A: $F_{9,45} = 17.0$, $P < .001$; B: $F_{9,45} = 11.8$, $P < .001$). Asterisks indicate a significant (at least $P < .05$; Duncan's) difference from the corresponding vehicle pretreatment values.

et al., 1985; Britton *et al.*, 1985, 1988; Britton and Koob, 1986; Koob *et al.*, 1986; Barrett *et al.*, 1989; Zhang and Barrett, 1990) and extend these studies by demonstrating that selective effects of CRF and DMCM on punished responding (proconflict effects) are determined by the intensity of the punishing stimulus. The finding that both a benzodiazepine agonist and antagonist prevent the effects of DMCM and CRF implies a pharmacologic interaction between CRF and endogenous benzodiazepine receptor systems. Furthermore, the CRF antagonist, α helical CRF_{9-41}, did not antagonize the behavioral effects of DMCM, indicating that the effects of the benzodiazepine inverse agonist DMCM are not mediated through endogenous CRF release. Finally, the finding that the CRF antagonist attenuates the effects of electric shock suggests that the shock-mediated punishment process may be, at least in part, mediated by CRF release.

Previous studies of the effects of CRF on punished behavior failed to show selective proconflict effects of any dose of CRF (Britton *et al.*, 1985, 1988; Britton and Koob, 1986; Barrett *et al.*, 1989). In these studies the CRF dose-response curves for suppression of punished responding and suppression of nonpunished responding were almost superimposable. This general reduction in operant responding was interpreted to be related to the ability of CRF to suppress appetitive motivation (Levine *et al.*, 1983). The subjects of these studies were either rats (Britton *et al.*, 1985, 1988; Britton and Koob, 1986) or pigeons (Barrett *et al.*, 1989), and either random-interval (Britton *et al.*, 1985, 1988; Britton and Koob, 1986) or fixed-ratio (Barrett *et al.*, 1989) schedules were used. One major difference between the present and previous studies involves the intensity of the punishing stimulus. In the previous studies, shock intensities were used that decreased the rate of responding to 3% to 15% of the rate of nonpunished responding. This is in contrast to the present study, which used a shock intensity that decreased the rate of responding to only 85% to 90% of the nonpunishment rate. Like CRF, the benzodiazepine inverse agonists, β-CCE and noreleagnine, have been shown to have selective proconflict effects that are determined by the intensity of the shock used as a punisher (Shekhar *et al.*, 1989; Takada *et al.*, 1992). Taken together, these studies suggest that there is an optimal range of shock intensities that can be used to demonstrate selective proconflict effects. It is likely that if shock intensity is too low, selective proconflict effects will not be observed. Alternatively, if shock intensity is too high, a ceiling effect may be reached whereby drug pretreatment cannot decrease responding further. The proconflict effects of both CRF and DMCM are also dependent on drug dose such that only intermediate doses have selective effects. This was also observed with β-CCE (Takada *et al.*, 1992). Although it is possible that the differences in dose- or intensity-effect curves for effects on punished and nonpunished responding observed in the present study were due to differences in response rates in the two components, this is unlikely because the response rates were selected to be relatively similar in both components. Additionally, the predicted rate-dependent effect would be opposite to the results obtained, *i.e.*, higher rates of responding in the nonpunished component would be more sensitive to suppression.

In spite of the finding that CRF exhibited more selectivity in this study than in previous studies using other schedules or higher shock intensities, it may be surprising that a greater separation between dose-response curves for effects on punished and nonpunished responding was not observed. The small degree of selectivity was not specific to CRF but was also observed with the benzodiazepine inverse agonists, DMCM (present study) and β-CCE (Takada *et al.*, 1992), and may be a function of the multiple schedule used to assess proconflict effects. When rats were tested at a higher shock intensity in the present study, response rates in both components of the multiple schedule decreased and the degree of selectivity of suppression was comparable with that observed with CRF or DMCM pretreatment. In contrast to the present study, other

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studies of intensity of the punishing stimulus have found a greater selective suppression of response rates by shock as intensity was increased (Dinsmoor, 1952; Brethower and Reynolds, 1962; Katz and Goldberg, 1986). One explanation for this discrepency is that the repeated exposure to the high shock intensity that occurs during behavioral training can result in enhanced discriminative control by the stimuli associated with the schedule components. The high shock intensities used in the present study were relatively novel, because they were not presented on a daily basis. It has been demonstrated previously that high-intensity or novel aversive stimuli have generalized behavioral suppressant effects (Azrin and Holz, 1966).

The effects of CRF on food-reinforced responding were likely mediated by an interaction with specific CRF binding sites because the doses of CRF that were effective were comparable with those that elicit adrenocorticotropin hormone release (Vale *et al.*, 1981) or mimic autonomic responses to stress (Brown *et al.*, 1982; Fisher *et al.*, 1982). More importantly however, these behavioral effects of CRF are prevented by pretreatment with a dose of a CRF antagonist that has been demonstrated to prevent other effects of CRF (Rivier *et al.*, 1982; Kalin *et al.*, 1988; Lenz *et al.*, 1988) or stress-elicited effects (Brown *et al.*, 1986; Lenz *et al.*, 1988; Valentino and Wehby, 1988). This finding confirms previous studies (Britton *et al.*, 1986; Barrett *et al.*, 1989). Interestingly, the DMCM-induced effects on nonpunished and punished responding were not blocked by the CRF antagonist. Although higher doses of α helical CRF_{9-41} may be necessary to antagonize DMCM, the dose used was sufficient to almost completely antagonize an equally effective dose of CRF. This suggests that the effects of benzodiazepine inverse agonists on punished responding are not mediated *via* endogenous CRF release. Furthermore, while the CRF antagonist did not alter the suppressive effects of low-intensity shock on response rate, it did attenuate the behavioral suppression elicited by the higher intensity shock, suggesting that part of the punishing effects of shock are due to endogenous CRF release. In two earlier studies (Britton *et al.*, 1986; Barrett *et al.*, 1989) α helical CRF_{9-41} had no effect on response suppression elicited by high-intensity shock in a conflict procedure. However, in contrast to previous studies, the high shock intensity used in the present study was novel. This additional characteristic of shock, *i.e.*, novel *vs.* nonnovel, may be an important determinant in sensitivity to a CRF antagonist and suggests that endogenous CRF systems are in effect in behavioral responses to novel aversive stimuli.

As expected, both the benzodiazepine receptor agonist, chlordiazepoxide, and the benzodiazepine receptor antagonist, flumazenil, attenuated the suppressive effects of the benzodiazepine inverse agonist, DMCM, indicating a central benzodiazepine receptor-mediated mechanism of action for this DMCM effect. As previously reported in a variety of animal tests of anxiety, including punishment procedures (Britton *et al.*, 1985, 1988; Lee *et al.*, 1987; Dunn and File, 1987; Yang *et al.*, 1990; Zhang and Barrett, 1990), the anxiolytic chlordiazepoxide also attenuated the suppressive effects of CRF and high-intensity shock. It is likely that higher i.c.v. doses of chlordiazepoxide are needed to completely reverse the suppressant effects of these stimuli. The reversal of CRF by chlordiazepoxide has been interpreted as a physiologic antagonism, *i.e.*, an anxiolytic opposing the effects of an anxiogenic, rather than a pharmacologic interaction at the benzodiazepine/GABA receptor complex (Dunn and File, 1987; File, 1990). However, this explanation does not seem to be valid, since 1) the clinically effective anxiolytic buspirone, which does not interact with the benzodiazepine/GABA receptor complex, failed to attenuate the effects of CRF (Zhang and Barrett, 1990; Lazosky and Britton, 1991), and 2) the benzodiazepine receptor antagonist, flumazenil, was an effective antagonist of CRF effects in punishment procedures (Britton *et al.*, 1988; this study). The latter finding clearly points to a pharmacologic antagonism of the CRF effects, which may occur either at the CRF-receptor level (*i.e.*, flumazenil serves as a CRF-receptor antagonist) or at the level of the benzodiazepine receptor (*i.e.*, CRF itself or an endogenous ligand released by CRF acting as a benzodiazepine inverse agonist). Thus far, there is no direct evidence available either from ligand-binding studies that CRF has affinity for benzodiazepine receptors or that benzodiazepine ligands bind to CRF receptors, or from neurochemical studies that exogenously administered CRF induces the release of endogenous benzodiazepine inverse-agonist ligands.

Recently, a brain peptide, DBI, was isolated, purified, sequenced, measured and cloned, which seems to fulfill many of the requirements for an endogenous inverse-agonist ligand for the benzodiazepine receptor (see for review: Costa and Guidotti, 1991). When given i.c.v., DBI and one of its natural processing products, *i.e.*, octadecaneuropeptide, were found to elicit proconflict effects that were antagonized by flumazenil pretreatment (Costa and Guidotti, 1991). Therefore, it is tempting to speculate that exogenous CRF may produce its "anxiogenic" effects by releasing an endogenous inverse-agonist ligand for benzodiazepine receptors (*i.e.*, DBI or its fragment, octadecaneuropeptide), which in turn can be blocked by flumazenil.

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