Receptor Binding, Behavioral, and Electrophysiological Profiles of Nonpeptide Corticotropin-Releasing Factor Subtype 1 Receptor Antagonists CRA1000 and CRA1001

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ABSTRACT

Receptor binding, behavioral, and electrophysiological profiles of 2-[[N-(2-methylthio-4-isopropylphenyl)-N-ethylamino]-4-[4-(3-fluorophenyl)-1,2,3,6-tetrahydropyridin-1-yl]-6-methylpyrimidin-7-yl]-bis-(2-methoxy-ethyl)-amine; STL, step through latency; TH, tyrosine hydroxylase.

Corticotropin-releasing factor (CRF), a 41-amino acid peptide that regulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary (Vale et al., 1981), has been shown to mediate stress-induced changes in the autonomic nervous system, neuroendocrine functions, and behavior (Vale et al., 1981; Dunn and Berridge, 1990; Owens and Nemeroff, 1991). Intracerebroventricular (i.c.v.) administration of CRF to laboratory animals produces behavioral effects similar to those observed in both anxiety and depression, such as altered locomotor activity (Britton et al., 1986a,b), increased anxiety in an elevated plus-maze (Buwalda et al., 1997), cocaine withdrawal-induced anxiety (Sarnyai et al., 1995), social defeat-induced anxiety (Skutella et al., 1994), diminished food intake (Levine et al., 1983), decreased sexual behavior (Sirinathsinghji et al., 1983), and sleep disruption (Ehlers et al., 1983).

Clinical data indicate that patients with depression and post-traumatic stress disorder have significantly elevated concentrations of CRF in cerebrospinal fluid, as compared with normal controls (Nemeroff et al., 1984; Darnell et al., 1994). In addition, patients with depression, anxiety, anorexia nervosa, and post-traumatic stress disorders showed

ABBREVIATIONS: ACTH, adrenocorticotropic; CRA1000, 2-[[N-(2-methylthio-4-isopropylphenyl)-N-ethylamino]-4-[4-(3-fluorophenyl)-1,2,3,6-tetrahydropyridin-1-yl]-6-methylpyrimidine; CRA1001, 2-[[N-(2-bromo-4-isopropylphenyl)-N-ethylamino]-4-[4-(3-fluorophenyl)-1,2,3,6-tetrahydropyridin-1-yl]-6-methylpyrimidine (CRA1001), putative novel and selective CRF1 receptor antagonists for corticotropin-releasing factor1 (CRF1) receptor when examined using rat

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blunted ACTH responses to i.v. CRF (Holsboer et al., 1984; Taylor and Fishmann, 1988), indicating their CRF receptors may be down-regulated, possibly due to chronic hypersecretion of CRF (Nemeroff et al., 1988).

Receptors for CRF are distributed throughout the central and peripheral nervous systems (Owens and Nemeroff, 1991). In situ hybridization studies indicate that at least two CRF receptor subtypes, CRF₁ and CRF₂α, are expressed in the mammalian brain (Chalmers et al., 1995; Lovenberg et al., 1995a; Mansi et al., 1996). The heterogeneous anatomical distribution patterns of CRF₁ and CRF₂α mRNA expression suggests distinct functional roles for each receptor in CRF-related central nervous system circuit (Chalmers et al., 1995; Primus et al., 1997). Although CRF₁ receptor expression was abundant in the neocortical-, cerebellar-, and sensory-related structures, CRF₂α receptor expression was generally localized to specific subcortical structures, including the lateral septum and various hypothalamic nuclei (Chalmers et al., 1995). In addition, CRF₂β has been reported to be absent in the rat brain but abundant in the rat heart and skeletal muscle (Lovenberg et al., 1995b).

CRF₁ mRNA in the hypothalamic paraventricular nucleus (PVN) increased under various kind of stress, such as i.p. hypersonic saline injection (Luo et al., 1994), immune challenge (Rivest et al., 1995), and immobilization (Makino et al., 1995) and decreased with glucocorticoid treatment or adrenalectomy (Makino et al., 1995). Stress-induced increase in CRF₁ mRNA in the PVN corresponded to the increase in CRF binding (Luo et al., 1994). CRF₂α mRNA also showed a relatively high expression in the PVN even under unstressful conditions (Chalmers et al., 1995). However, CRF₂β mRNA levels in the PVN were not altered by corticosterone administration, adrenalectomy, or lipopolysaccharide injection (Makino et al., 1997). CRF₁ and CRF₂α receptor knockdown was achieved and confirmed autoradiographically within brain regions relevant to behavioral reactivity to stressors by chronic, central administration of antisense oligonucleotides (Heinrichs et al., 1997). CRF₁ but not CRF₂α knockdown produced a significant anxiolytic-like effect in the defensive withdrawal relative to vehicle-treated and two missense oligonucleotide negative control groups. In contrast, neither CRF₁ antisense treatment altered endocrine or behavioral reactivity to stressors (Chalmers et al., 1995; Primus et al., 1997). Although CRF₁ receptor expression was abundant in the neocortical-, cerebellar-, and sensory-related structures, CRF₂α receptor expression was generally localized to specific subcortical structures, including the lateral septum and various hypothalamic nuclei (Chalmers et al., 1995). In addition, CRF₂β has been reported to be absent in the rat brain but abundant in the rat heart and skeletal muscle (Lovenberg et al., 1995b).

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α-Helical CRF₉₋₄₁, a peptide antagonist, was initially characterized as a competitive inhibitor of CRF-induced ACTH secretion, in an in vitro pituitary cell culture system (Rivier et al., 1984). α-Helical CRF₉₋₄₁ has been used extensively in vivo to explore the physiological role of endogenous CRF systems in mediating various stress-induced hormonal and behavioral effects (Rivier et al., 1984; Heinrichs et al., 1992; Morimoto et al., 1993; Menzaghi et al., 1994). However, a peptide antagonist would not be expected to penetrate the blood-brain barrier, thereby limiting its clinical utility. Intensive research has focused on seeking nonpeptide CRF₁ antagonists. Data on several nonpeptide CRF₁ antagonists, butyl-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-ethylamine (CP-154,526) (Lundkvist et al., 1996; Schulz et al., 1996; Chen et al., 1997; Mansbach et al., 1997), [3-(2-bromo-4-isoproplyphenyl)-5-methyl-3H-[1,2,3]triazolo[4,5-d][pyrimidin-7-yl]-bis-(2-methoxy-ethyl)-amine (SC241) (Fitzgerald et al., 1996) and 5-chloro-N-(cyclopropyl)-2-methyl-N-propyl-N’-(2,4,6-trichlorophenyl)-4,6-pyrimidinediamine (NBI 27914) (Whitten et al., 1996; Baram et al., 1997) have been published.

We report here the receptor binding and neuropharmacological activities of the novel nonpeptide CRF₁ receptor antagonists, 2-(N-(2-methylthio-4-isoproplyphenyl)-N’-ethylamino-4-(3-fluorophenyl)-1,2,3,6-tetrahydropyridin-1-yl)-6-methylpyrimidine (CRA1000) and 2-(N-(2-bromo-4-isoproplyphenyl)-N’-ethylamino-4-(3-fluorophenyl)-1,2,3,6-tetrahydropyridin-1-yl)-6-methylpyrimidine (CRA1001) (Fig. 1).

Materials and Methods

Animals. Male ICR mice (20–30 g, Charles River, Yokohama, Japan) were housed 10/cage. Male Wistar rats (200–250 g, Japan SLC Inc., Japan) were housed 3/cage and used for receptor binding assay. Male Sprague-Dawley rats (220–240 g, Charles River, Yokohama, Japan) were used to assess stress-induced anxiogenic-like behavior. Male Wistar rats (300–400 g, Charles River, Yokohama, Japan) were used for electrophysiological experiments. Male Wistar rats (160–200 g, Charles River, Yokohama) were used to examine the hyperemotionality induced by destruction of olfactory bulbs. Rats were housed 3/cage. All the animals were maintained under a 12-h light/dark cycle (light on 7:00 A.M.) in a temperature- and humidity-controlled holding room. Food and tap water were available ad libium. All experiments were reviewed by The Taisho Pharmaceutical Co., Ltd. Animal Care Committee, and met The Japanese Experimental Animal Research Association Standards, as defined in 1987 Guidelines for Animal Experiments.

Membrane Preparations. The animals were decapitated and the frontal cortex, pituitary, and heart were rapidly dissected. Tissues were homogenized with 50 mM Tris-HCl buffer (pH 7.0) containing 10 mM MgCl₂ and 2 mM EDTA, and centrifuged at 48,000 g for 20 min at 4°C. The pellet was washed twice with the buffer, and the final pellet was suspended in the assay buffer (50 mM Tris-HCl buffer, pH 7.0, containing 10 mM MgCl₂, 2 mM EDTA, 0.1% BSA, and 100 Kallikrein unit (KU)/ml aprotinin), and used as crude membrane preparations for binding studies. Protein concentration was determined according to Bradford (1976).

Binding Studies. Binding assays for 125I-ovine CRF and 125I-sauvagine were done according to previously reported methods (De Souza, 1987; Grigoriadis et al., 1996) but with slight modification. The reaction was initiated by incubating 0.5 ml of membrane preparation with 0.2 nM 125I-ovine CRF or 0.2 nM 125I-sauvagine. The reaction mixture was incubated for 2 h at 25°C (for 125I-ovine CRF binding) or at 23°C (for 125I-sauvagine binding), and terminated by rapid filtration through Whatman GF/C glass fiber filters presoaked with 0.3% polyethyleneimine, after which the filters were washed three times with 3 ml of PBS containing 0.01% Triton X-100. The radioactivity was quantified in a gammacounter. Nonspecific binding was determined in the presence of unlabeled 1 μM ovine CRF (for 125I-ovine CRF binding) or 1 μM sauvagine (for 125I-sauvagine binding). Specific binding was determined by subtracting nonspecific

![Chemical structures of CRA1000 and CRA1001](image)
binding from total binding. In the competition binding assay, concentration of the test compound that caused 50% inhibition of specific radiolabeled ligand binding (IC_{50} values) was determined from each concentration-response curve.

**Stress-Induced Anxiogenic-like Behavior in Mice.** The swim stress procedure consisted of placing mice in a 20-cm tall, 13-cm wide cylindrical plastic container containing 10 cm of water maintained at 25° ± 1°C. Duration of the swim stress was 10 min, and the light/dark exploration test was done 10 min after the swim stress. The light/dark exploration test was based on that validated for the mouse by Crawley and Goodwin (1980). The apparatus consisted of two polyvinylchloride boxes (20 × 20 × 14 cm) covered with Plexiglas; one of these boxes was darkened with cardboard. The light compartment was illuminated by a desk lamp (400 lux) placed 17 cm above the box, and the dark compartment provided the only room illumination. An opaque plastic tunnel (5 × 7 × 10 cm) separated the dark compartment from the light one. During observation, the experimenter always sat in the same place, next to the apparatus. The subjects were individually tested in 5-min sessions in the apparatus described above. Each mouse was placed in the center of light area to start the test session. The amount of time spent in the light area was recorded for 5 min after the first entry in the dark area. A mouse whose four paws were in the new box was considered as having changed boxes. Mice were naive to the apparatus. CRA1000 HCl (1, 3, and 10 mg/kg), CRA1001 HCl (1, 3, and 10 mg/kg), CP-154,526 (3, 10, and 30 mg/kg), and diazepam (0.1, 0.3, and 1 mg/kg) were administered orally 30 min before application of the swim stress. Ten to 16 mice for vehicle and for each of three dosages of drugs were used to generate dose-response reactions.

In nonstress conditions, the light/dark exploration test was performed 30 min after the oral administration of CRA1000 HCl (3 and 10 mg/kg), CRA1001 HCl (3 and 10 mg/kg), CP-154,526 (10 and 30 mg/kg), and diazepam (0.3, 1.3, and 10 mg/kg). Nine to 10 mice for vehicle and for each of two to four dosages of drugs were used to generate dose-response reactions.

**CRF-Induced Anxiogenic-like Behavior in Rats.** For experiments for i.c.v. infusion, rats were surgically equipped with a single cannula aimed above the lateral ventricle. Animals were anesthetized with sodium pentobarbital (40 mg/kg, i.p.), and placed in a stereotoxic apparatus (Narishige, Tokyo, Japan) where a 7-mm long, 23-gauge stainless steel guide cannula was lowered to within 1 mm of the ventricle and anchored to the skull with screws and dental cement. The implantation coordinates were 1.0 mm posterior to the bregma, 1.2 mm lateral to the midline, and 4.5 mm ventral to the cortical surface according to the rat brain atlas of Paxinos and Watson (1986). After a 7-day postsurgical recovery period, cannula patency was confirmed by gravity flow through an 8-mm, 30-gauge injector inserted through the guide to 1 mm beyond its tip. CRA1000 HCl (0.1, 0.3, and 1 mg/kg), CRA1001 HCl (0.3, 1, 3, and 10 mg/kg), CP-154,526 (0.3, 1, 3, and 10 mg/kg), and diazepam (0.3, 1, 3, and 10 mg/kg) were administered orally 30 min before i.c.v. infusion of CRF (1 μg/10 μl). Eight rats for vehicle and for each of three to four dosages of drugs were used to generate dose-response reactions.

The elevated plus-maze test was performed 60 min after CRF administration. The elevated plus-maze test was based on that validated for the rat by Guimaraes et al. (1991). The apparatus consisted of a plus-shaped maze elevated 50 cm from the floor and two opposite open arms, 50 × 10 cm, crossed at right angles by two arms of the same dimensions enclosed by 40 cm high walls with an open roof. In addition, a 1-cm high edge made of Plexiglas surrounded the open arms to avoid falls. Luminosity measured at the center of the maze was 80 lux. During observation, the experimenter always sat in the same place, next to the apparatus. The subjects were individually tested in 5-min sessions in the apparatus described above. Each rat was placed in the center of the plus-maze facing one enclosed arm. The amount of time spent in open arms of the maze was recorded. Rats were naive to the apparatus.

Lesioning of Olfactory Bulbs Induced Hyperemotivity in Rats. The animals were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and placed in a stereotoxic apparatus (Narishige). lesioning of olfactory bulbs was done by inserting a bipolar electrode 0.8 mm in diameter and made of insulated stainless steel wire, placed according to the rat brain atlas of Paxinos and Watson (1986) and applying a direct current of 3 mA for 40 s to both olfactory bulbs. The coordinate of the olfactory bulb was 6 mm anterior to the bregma, 1 mm lateral to the midline, and 6 mm ventral to the cortical surface. After lesioning of olfactory bulbs, the animal was immediately housed in an individual cage. Hyperemotivity of olfactory bulbectomized (OB) rats was measured by scoring the emotional responses to 1) air blowing on the dorsum, 2) a rod presented in the front of the snout, 3) resistance upon capturing and handling, and 4) reaction upon tail pinch. These responses were graded as follows: 0, no reaction; 1, slight; 2, moderate; 3, marked; or 4, extreme response (Yamamoto et al., 1982; Shibata et al., 1984). In each emotional response, vocalization during the test was also scored and graded as follows, 0, no vocalization; 1, occasional vocalization; or 2, marked vocalization. After the 7- to 21-day postsurgical period, the emotional responses were measured. Only rats that exhibited hyperemotivity (score: >14) were selected for study.

In the case of the acute administration study, CRA1000 HCl (0.3, 1, 3, and 10 mg/kg), CRA1001 HCl (0.3, 1, 3, and 10 mg/kg), and CP-154,526 (0.3, 1, 3, and 10 mg/kg) were administered orally 2 h before measurement of emotional responses. Six to 20 rats for vehicle and for each of four dosages of drugs were used to generate dose-response reactions. In the case of the chronic administration, CRA1000 HCl (3 and 10 mg/kg), CRA1001 HCl (3 and 10 mg/kg), and CP-154,526 (3 and 10 mg/kg) were administered orally once daily for 7 days, and emotional responses were measured 2 h after the final administration. Six to 12 rats for vehicle and for each of two dosages of drugs were used to generate dose-response reactions.

**Electrophysiological Experiments.** Animals were anesthetized with urethane (1.5 g/kg, i.p.), and fixed in a stereotoxic apparatus. Body temperature was maintained at 37° ± 1°C with a heating pad (KN-474, Natsume, Tokyo, Japan). Extracellular single unit recordings of the locus coeruleus (LC) neurons were made as previously described (Nakamura, 1977; Arakawa et al., 1995). Stimulating electrodes consisting of two insulated stainless wires with an exposed tip of 0.5 mm were implanted into the dorsal noradrenergic bundle. The same type of electrode was implanted into the frontal cortex to record an electroencephalogram. Coordinates of the dorsal noradrenergic bundle were 1.5 mm anterior to the lambda, 0.8 mm lateral to the midline, and 5.7 to 6.0 mm ventral to the cortical surface, and those for the frontal cortex were 2.0 mm anterior to the bregma, 2.0 mm lateral to the midline, and 1.0 mm ventral to the cortical surface. Stimuli applied to the dorsal noradrenergic bundle were single square pulses of 0.5 to 1 ms with currents ranging from 0.1 to 0.5 mA. The frequency of stimulation was 1 Hz in all experiments. The single-unit activity of LC neurons was recorded extracellularly by means of a glass micropipette filled with 2 M NaCl. Location of the LC was determined by appearance of field responses evoked by dorsal noradrenergic bundle stimulation (Fig. 2A). When the tip of a recording electrode was localized correctly in the LC, the single-unit activity of LC neurons was recorded and was superimposed upon the field response (Nakamura, 1977; Arakawa et al., 1995).

The i.c.v. cannula for CRF infusion was placed. CRA1000 HCl (0.5, 1, and 5 mg/kg), CRA1001 HCl (1 and 5 mg/kg), and CP-154,526 (5 mg/kg) were administered via an i.c.v. catheter implanted in the tail vein 5 to 10 min before i.c.v. infusion of CRF (1 μg/2 μl). Five to 9 rats for vehicle and for each of dosage of drugs were used. A CRF-induced change in neuronal activity was plotted as percentage of increase from baseline firing rate, as calculated over a 10-min period. The percentage of increase of each group was calculated.

**Potentiation of Hexobarbital-Induced Anesthesia in Mice.** Hexobarbital-induced anesthesia of mice was estimated based on the
CRA1000 H₂SO₄ (5 mg/kg i.v.) on CRF-induced increase in firing rate of LC neurons.

The animal was then placed in the dark compartment, the guillotine door was closed and an animal from escaping, was run at 10 rpm. All animals were given control trials before the test. A mouse was placed on the roller, and duration of righting reflex loss. Hexobarbital (70 mg/kg, i.p.) was administered 30 min after the oral administration of CRA1000 HCl (1, 10, and 100 mg/kg), CRA1001 HCl (1, 10, and 100 mg/kg), CP-154,526 (1, 10, and 100 mg/kg), and diazepam (0.1, 0.3, 1, and 3 mg/kg). Eight to 12 mice for each of the treatments were used to generate dose-response reactions. The sleep duration of treated groups was defined as 100% for the vehicle-treated control group. The percentage of potentiation of each treatment group was calculated and ED₅₀ values were determined.

### Rotarod Test in Mice.
The rotarod (Campden Instruments, Loughborough, UK), consisting of a gritted plastic roller (3.2 mm diameter, 30 cm height), and locomotor activity was recorded every 5 min for 30 min, using a SCANET apparatus (Neuroscience Inc., Tokyo, Japan) placed in a soundproof box. Spontaneous locomotor activity was measured 30 min after the oral administration of CRA1000 HCl (1, 10, and 100 mg/kg), CRA1001 HCl (1, 10, and 100 mg/kg), CP-154,526 (1, 10, and 100 mg/kg), and diazepam (3, 10, and 30 mg/kg). Eight to 12 mice for each of the treatments were used to generate dose-response reactions. The total count for the vehicle-treated control group was defined as 100%; percentage of inhibition of each treatment group was calculated and ED₅₀ values were determined.

### Passive Avoidance Task in Rats.
The apparatus consisted of two compartments: one light compartment (12 cm long, 12 cm wide, and 25 cm high) and one dark compartment (30 cm long, 12 cm wide, and 25 cm high) connected by a guillotine door. Once the rat had entered the dark compartment, the guillotine door was closed and an electrical shock (0.3–0.35 mA for 3 s) was delivered to the animal via the foot from a floor grid (acquisition trial). The animal was then put back into the home cage until the retention trial. The retention trial was carried out 24 h after the acquisition trial. At that time, the animals were returned to the light compartment and the time taken to enter the dark compartment was recorded (step through latency or STL). A maximum latency of 300 s was used. CRA1000 HCl (10 and 100 mg/kg), CRA1001 HCl (10 and 100 mg/kg), CP-154,526 (10 and 100 mg/kg), and diazepam (3, 10, and 30 mg/kg) were administered orally 30 min before the acquisition trial. Ten rats for each of the treatments were used to generate dose-response reactions.

### Statistical Analysis.
In binding assays, IC₅₀ values were determined for each compound to bind to the CRF₁ receptor. A series of novel 4-(4-aryl-1,2,3,6-tetrahydropyridin-1-yl)pyrimidine derivatives were found to have affinity for the CRF₁ receptor, and CRA1000 and CRA1001 were shown to have the highest affinity, with IC₅₀ values of 20.6 and 22.3 nM, respectively. The brain concentrations of CRA1000 H₂SO₄ and CRA1001 H₂SO₄ were dissolved in 50% polyethylene glycol 400 and saline. CP-154,526 was dissolved in 0.1 N HCl and saline. Recombinant human CRF (rCRF) (Peptide Institute Inc. Osaka, Japan) was dissolved 0.1% acetic acid and saline with the addition of 0.1% BSA.

### Results
Affinities of CRA1000 and CRA1001 for CRF Receptors. Both CRA1000 and CRA1001 were identified from screening in which ¹²⁵I-ovine CRF binding to membranes of the rat frontal cortex was used to determine the potential of the compounds to bind to the CRF₁ receptor. A series of novel 4-(4-aryl-1,2,3,6-tetrahydropyridin-1-yl)pyrimidine derivatives were found to have affinity for the CRF₁ receptor, and CRA1000 and CRA1001 were shown to have the highest affinity, with IC₅₀ values of 20.6 and 22.3 nM, respectively (Table 1). CRA1000 and CRA1001 inhibited ¹²⁵I-ovine CRF

### Compounds.
CRA1000, CRA1001, CP-154,526, and SC241 were synthesized in the laboratories of Taisho Pharmaceutical Co., Ltd. (Saitama, Japan). ¹²⁵I-ovine CRF (specific radioactivity: 81.4 TBq/mmol) and ¹²⁵I-sauvagine (specific radioactivity: 81.4 TBq/mmol) were purchased from NEN Life Sciences (Boston, MA).

CRA1000 HCl, CRA1001 H₂SO₄, CRA1001 HCl, CRA1001 H₂SO₄, and CRA1001 H₂SO₄ have good pharmacokinetics with good oral bioavailability in rodents (bioavailability [area under the concentration curve (AUC)₀–∞, p.o./i.v.]: 12, 14, and 18%, C₅₀ = 398 ± 164, 452 ± 68, and 1129 ± 306 ng/ml, T₅₀ = 2.7 ± 0.7, 0.8 ± 0.2, and 0.8 ± 0.2 h, respectively, at 10 mg/kg p.o. in rats). The brain concentrations of CRA1000 H₂SO₄ and CRA1001 H₂SO₄ were 38 ± 8 and 21 ± 3 ng/g tissue, respectively, at 10 mg/kg p.o. in rats. In the present study, the hydrochloride and sulfate salts of two compounds were used. All the sulfate salt turned into hydrochloride in the stomach. Therefore, it can be concluded that there is probably no difference between the two types of salts in vivo.

### Statistical Analysis.
In binding assays, IC₅₀ values were determined using the Marquardt-Levenberg nonlinear least-squares curve-fitting procedure of the Microcal ORIGIN program (MicroCal, Northampton, MA) running on Microsoft Windows 3.1. Data from the light/dark exploration task, the elevated plus-maze, hexobarbital-induced anesthesia, spontaneous locomotor activity, rotarod test, and electrophysiological experiments were analyzed by one-way ANOVA, and significant differences between groups were determined by Dunnett's test. Data from lesioning of olfactory bulb-induced hyperemotionality were analyzed by one-way ANOVA and significant differences between groups were determined by Tukey-Kramer's nonparametric comparison test. Data from the passive avoidance task were expressed in terms of medians and interquartile ranges, and were analyzed using the Kruskal-Wallis test, with significant differences between groups determined by Dunnett's test. The ED₅₀ values were calculated from the dose response of each compound, using nonlinear least-squares regression analysis.

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binding in CRF<sub>1</sub> membrane sources used in this study, including the rat frontal cortex, pituitary (Table 1). Competition curves of CRA1000 and CRA1001 for all preparations were monophasic, and Hill slopes were approximately 1.0 (Fig. 3). The affinity of CRA compounds for the CRF<sub>1</sub> receptor were monophasic, and Hill slopes were approximately 1.0. 

**TABLE 1**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>CRF&lt;sub&gt;1&lt;/sub&gt; receptor</th>
<th>CRF&lt;sub&gt;2&lt;/sub&gt; receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frontal cortex</td>
<td>Pituitary</td>
</tr>
<tr>
<td>CRA1000</td>
<td>20.6 ± 5.4</td>
<td>15.7 ± 4.0</td>
</tr>
<tr>
<td>CRA1001</td>
<td>22.3 ± 5.7</td>
<td>18.6 ± 2.6</td>
</tr>
<tr>
<td>CP-154,526</td>
<td>1.8 ± 0.1</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td>SC241</td>
<td>14.8 ± 0.8</td>
<td>20.7 ± 2.9</td>
</tr>
</tbody>
</table>

Data represent mean ± S.E.M. from three to four determinations, each done in duplicate.

In nonstress conditions, CRA1000 HCl (3 and 10 mg/kg, p.o.), CRA1001 HCl (3 and 10 mg/kg, p.o.), and CP-154,526 (10 and 30 mg/kg, p.o.) did not attenuate the time spent in the light area in the light/dark exploration task in mice. In contrast, administration of diazepam (0.3–10 mg/kg, p.o.) resulted in a dose-related increase in the time spent in the light area, with significant increases with 10 and 30 mg/kg, p.o. (Fig. 5).

**Fig. 4.** Effects of CRA1000 HCl (A), CRA1001 HCl (B), CP-154,526 (C), and diazepam (D) on stress-induced reduction of time spent in light area in light/dark exploration test in mice. Data are represented as mean ± S.E. (n = 10 to 16). *P< .01 versus normal (nonstress group) (Dunnett’s test). **P< .01 versus control (vehicle-treated swim-stress group) (Dunnett’s test).

In OB rats, emotional responses were significantly reduced by all three antagonists only at the highest doses, with CRA1000 H<sub>2</sub>SO<sub>4</sub> (1 mg/kg, p.o.), CRA1001 H<sub>2</sub>SO<sub>4</sub> (3 and 10 mg/kg, p.o.), and diazepam (3 mg/kg, p.o.) (Fig. 6). The order of potency of compounds was: diazepam > CRA1000 H<sub>2</sub>SO<sub>4</sub> > CRA1001 H<sub>2</sub>SO<sub>4</sub> = CP-154,526.

**Lesioning of Olfactory Bulbs Induced Hyperemotionality in Rats.** In the acute administration study, the score of emotional responses was significantly (P < 0.01) increased in OB rats compared with sham-operated rats. Emotional responses of OB rats were significantly reduced by CRA1000 H<sub>2</sub>SO<sub>4</sub> (1 mg/kg, p.o.), CRA1001 H<sub>2</sub>SO<sub>4</sub> (3 and 10 mg/kg, p.o.), and diazepam (3 mg/kg, p.o.) (Fig. 7). Figure 7 shows only a partial effect of all three antagonists only at the highest doses, with CRA1000 H<sub>2</sub>SO<sub>4</sub> much more potent than CRA1001 H<sub>2</sub>SO<sub>4</sub> and CP-154,526.

Emotional responses of OB rats were significantly reduced...
by chronic administration of CRA1000 H₂SO₄ (3 and 10 mg/kg, p.o.), CRA1001 H₂SO₄ (3 and 10 mg/kg, p.o.), and CP-154,526 (10 mg/kg, p.o.), with CRA1000 H₂SO₄ much more potent than CRA1001 H₂SO₄ and CP-154,526. The inhibition of emotional responses by CRA1000 H₂SO₄, CRA1001 H₂SO₄, and CP-154,526 was augmented with chronic administration compared with findings in case of acute administration (Fig. 8).

Effects on CRF-Induced Excitation of LC Neuron Firing. The firing rate of the LC neurons was significantly (P < 0.01) increased among the CRF (1 μg/10 μl, i.c.v.)-treated rats compared with findings in the vehicle-treated rats (Fig. 2C and Table 2). This excitation of LC neurons was significantly blocked by the pretreatment with CRA1000 H₂SO₄ (1 and 5 mg/kg, i.v.), CRA1001 H₂SO₄ (5 mg/kg, i.v.), and CP-154,526 (5 mg/kg, i.v.) (Table 1). CRA1001 H₂SO₄ was much less potent than CRA1000 H₂SO₄, yet CRA1001 H₂SO₄ is equipotent with CP-154,526.

Effects on Spontaneous Locomotor Activity, Potentiation of Hexobarbital-Induced Anesthesia and Rotarod in Mice and Passive Avoidance Task in Rats. Diazepam dose dependently inhibited spontaneous locomotor activity (ED₅₀ = 11.9 mg/kg, p.o.), potentiated hexobarbital-induced anesthesia (ED₅₀ = 0.4 mg/kg, p.o.), and reduced the time mice spent on the rotarod (ED₅₀ = 4.1 mg/kg, p.o.). In contrast, CRA1000 HCl, CRA1001 HCl, and CP-154,526 at doses up to 100 mg/kg, p.o. had no effect on the spontaneous locomotor activity, hexobarbital-induced anesthesia, and rotarod test in mice (Table 3).

In the passive avoidance task, STL (retention time) was significantly decreased by diazepam (10 and 30 mg/kg, p.o.). In contrast, CRA1000 HCl, CRA1001 HCl, and CP-154,526 at doses up to 100 mg/kg, p.o. had no effect on the STL in a passive avoidance task (Fig. 9).

Discussion

We obtained evidence that CRA1000 and CRA1001 are potent and selective antagonists for the CRF₁ receptor. The CRF receptor has been divided into two major subtypes, CRF₁ and CRF₂, based on molecular cloning techniques (Chen et al., 1993; Lovenberg et al., 1995a). Moreover, CRF₂ has two isoforms, CRF₂α and CRF₂β (Perrin et al., 1995) in which the sequences of the N-terminus differ. The distributions and pharmacological properties of these subtypes are distinct. Among CRF and CRF-related peptides such as sauvagine, urotensin I, and urocortin, CRF₁ has a higher affinity for CRF itself than for CRF-related peptides, whereas CRF₂ has a higher affinity for CRF-related peptides than for CRF itself (Chen et al., 1993; Lovenberg et al., 1995a; Perrin et al., 1995). Thus, we made use of radioligand binding to the rat frontal cortex and pituitary to determine the affinity for CRF₁ and 125I-sauvagine binding to the rat heart to determine the affinity for CRF₂β.

CRA1000 and CRA1001 inhibited 125I-CRF binding to membranes of the rat frontal cortex and pituitary, whereas these compounds had no effect on 125I-sauvagine binding to membranes of the rat heart. CRA1000 and CRA1001 were without effect on 125I-sauvagine binding to membranes of COS-7 cells expressing the CRF₂α receptor (Chaki et al., 1998). CRA1000 and CRA1001 had weak or negligible affinities for 84 other receptors, including neurotransmitters and peptides, and ion channels. These results indicate that both...
CRA1000 and CRA1001 are specific ligands for the CRF1 receptor and that these compounds have no affinity for both CRF2α and CRF2β.

Stress- or CRF-induced anxiogenic-like behavior has been used as an animal model of anxiety. Activation of CRF secretion by stress has been extensively documented. Portal-vesSEL cannulation studies indicate pronounced release of CRF in anesthetized preparations (Plotsky et al., 1989). CRF, when administered to animals i.c.v. caused anxiogenic-like behavior (Britton et al., 1986a,b; Dunn and Berridge, 1990; Skutella et al., 1994; Sarnyai et al., 1995; Buwalda et al., 1997). Similar findings have been noted in animals in which reduced time was spent in contact with novel stimuli and resembled behavior observed following a period of restraint stress (Berridge and Dunn, 1986, 1987, 1989a,b). CRA1000 and CRA1001, CP-154,526, and diazepam dose dependently reversed i.c.v. administration of CRF-induced reduction of the time spent in the open arms in the elevated plus-maze task in rats. Reduction in the time spent in the open arms in case of i.c.v. administration of CRF may reflect increased anxiety, because administration of the anxiolytic diazepam blocks the effect of CRF.

CRA1000 and CRA1001 as well as CP-154,526 and diazepam improved behavioral deficits caused by swim stress. In nonstress conditions, CRA1000, CRA1001, and CP-154,526 had no effect on mice in the light area in the light/dark exploration task. CP-154,526 exhibited signs of anxiolytic-like activity in the elevated plus-maze task in rats, whereas these effects were not dose dependent because higher doses did not induce significant effects (Lundkvist et al., 1996). CRA1000 and CRA1001 as well as CP-154,526 had no effect on anxiogenic-like behavior in nonstress conditions. We have no adequate explanation for the lack of anxiolytic effects of CRA1000, CRA1001, and CP-154,526 in nonstress conditions; the stress-induced anxiogenic-like behavior may be mainly mediated by CRF1 receptors.

Administration of CRF or exposure to stressful situations leads to activation of the hypothalamic-pituitary-adrenal axis, and increase in plasma ACTH and corticosterone (File, 1991). The CRF-induced increase in plasma ACTH was blocked by both α-helical CRF9–41 and CP-154,526 (Schulz et al., 1996). Although CRF-induced cAMP accumulation and ACTH elevations were inhibited by CRA1000, CRA1001, CP-154,526, and SC241 in AtT-20 cells (Chaki et al., 1998), CRA1000 HCl (1, 10, and 100 mg/kg, p.o.), CRA1001 HCl (1, 10, and 100 mg/kg, p.o.), CP-154,526 (10 and 30 mg/kg, s.c.), and SC241 (10 mg/kg, p.o.) had no effects on swim stress (2 min)- and/or CRF (4 μg/kg, i.v.)-induced increase in ACTH and corticosterone in rat serum (data not shown). We have no precise explanation for this difference. Because many of the behavioral effects induced by CRF were found to be indepen-
dent of hypothalamic-pituitary-adrenal activation (Britton et al., 1986a; Berridge and Dunn, 1989b; Adamec and McKay, 1993), involvement of extrahypothalamic CRF receptors was suggested. Indeed, studies using in situ hybridization histochemistry and autoradiography revealed the existence of CRF1 receptor mRNA and CRF receptors not only in the hypothalamus, but also in the cerebral cortex and in the limbic structures such as the amygdaloid complex (De Souza et al., 1985, 1987; Potter et al., 1994; Chalmers et al., 1995; Lovenberg et al., 1995a; Mansi et al., 1996). Evidence has accumulated that the central nucleus of the amygdala with its CRF-containing cells and fibers, and its direct anatomical connections to the neocortex, hypothalamus, and brainstem appear to be an important center in pathways underlying emotional integration (Davis, 1992; Liebsch et al., 1995). CRF receptors in this nucleus may participate in mediating anxiety-related behavior (Heinrichs et al., 1992; Rassnick et al., 1993; Swiergiel et al., 1993). Thus, the anxiolytic-like effects of CRA1000 and CRA1001 may be related to blockade of CRF1 receptors in the cerebral cortex and in the limbic structures.

A characteristic pattern of hyperemotionality is induced in rats by bilateral OB. This hyperemotionality of OB rats is useful for evaluating antidepressant activity (Leonard and Tuite, 1981; Shibata et al., 1984; van Riezen and Leonard, 1991). The acute administration of CRA1000, CRA1001, and CP-154,526 inhibited hyperemotionality in OB rats. The hyperemotionality of OB rats was markedly reduced by acute administration of anxiolytics such as diazepam and chlordiazepoxide concomitant with sedation, ataxia, and muscle relaxation (Nurimoto et al., 1974; Shibata et al., 1984). In contrast, acute administration of antidepressants such as desipramine, imipramine and amitriptyline caused a slight inhibition of the hyperemotionality of OB rats, even at larger doses (Nurimoto et al., 1974; Shibata et al., 1984). Although hyperemotionality was suppressed by acute administration of CRA1000, CRA1001, and CP-154,526, the OB rats did not show sedation, ataxia, or muscle relaxation. Therefore, CRA1000 and CRA1001 as well as CP-154,526 have antihyperemotional actions in OB rats.

Table 2
Effects of CRA1000 HSO₄, CRA1001 HSO₄, and CP-154,526 on CRF-induced excitation of firing of LC neurons

<table>
<thead>
<tr>
<th>Treatments</th>
<th>n</th>
<th>Percentage of Increase from Baseline Firing Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (i.c.v.)</td>
<td>5</td>
<td>1.8 ± 3.9</td>
</tr>
<tr>
<td>+ Vehicle (i.v.)</td>
<td>7</td>
<td>53.8 ± 6.9*</td>
</tr>
<tr>
<td>+ CRA1000 HSO₄ (0.5 mg/kg, i.v.)</td>
<td>6</td>
<td>32.6 ± 8.1</td>
</tr>
<tr>
<td>+ CRA1000 HSO₄ (1 mg/kg, i.v.)</td>
<td>6</td>
<td>2.9 ± 6.4*</td>
</tr>
<tr>
<td>+ CRA1000 HSO₄ (5 mg/kg, i.v.)</td>
<td>7</td>
<td>1.0 ± 8.1*</td>
</tr>
<tr>
<td>+ CRA1001 HSO₄ (1 mg/kg, i.v.)</td>
<td>5</td>
<td>46.6 ± 5.3</td>
</tr>
<tr>
<td>+ CRA1001 HSO₄ (5 mg/kg, i.v.)</td>
<td>9</td>
<td>19.0 ± 10.3*</td>
</tr>
<tr>
<td>Vehicle (i.c.v.)</td>
<td>5</td>
<td>1.8 ± 3.9</td>
</tr>
<tr>
<td>+ Vehicle (i.v.)</td>
<td>5</td>
<td>61.9 ± 6.4*</td>
</tr>
<tr>
<td>+ CP-154,526 (5 mg/kg, i.v.)</td>
<td>6</td>
<td>19.2 ± 6.6*</td>
</tr>
</tbody>
</table>

n, Number of animals. Results are shown as mean ± S.E.  
* P < .01 versus Vehicle (i.c.v.) + Vehicle (i.v.-treated group (Dunnett’s test).  
* P < .01 versus Vehicle (i.v.-treated group treated group (Dunnett’s test).  

Table 3
Behavioral profiles of CRA1000 HCl and CRA1001 HCl: comparison with CP-154,526 and diazepam

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ED₅₀ (mg/kg, p.o.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRA1000 HCl</td>
<td>&gt;100</td>
</tr>
<tr>
<td>CRA1001 HCl</td>
<td>&gt;100</td>
</tr>
<tr>
<td>CP-154,526</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Diazepam</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Fig. 9. Effects of CRA1000 HCl (A), CRA1001 HCl (B), CP-154,526 (C), and diazepam (D) on STL (retention time) in a passive avoidance task in rats. Data are represented as terms of medians and interquartile ranges (n = 10). *P < .05 and **P < .01 versus control (vehicle-treated group) (Dunnett’s test).
was reduced with chronic administration, whereas that by desipramine and amitriptyline was augmented with chronic administration (Shibata et al., 1984, van Riezen and Leonard, 1991). In the present study, the inhibition of hyperemotionality by the chronic administration of CRA1000, CRA1001, and CP164,526 was stronger than that seen in cases of the acute administration. Thus, CRA1000, CRA1001, and CP-154,526 is effective in an animal model shown to be predictive of antidepressant activity in humans. In addition, Mansbach et al. (1997) reported antidepressant-like effects of CP-154,526 in the learned helplessness procedure, a putative model of depression with documented sensitivity to diverse classes of antidepressant drugs.

With respect to stress systems, the LC has been shown to be a key mediator of neurogenic responses to stress (Brady, 1994; Weiss et al., 1994). The LC is rich in CRF immunoreactivity (Swanson et al., 1983). Infusion of CRF into LC increases certain stress-related behavior (Butler et al., 1990). Intracerebroventricular administered CRF increases tyrosine hydroxylase protein in the LC (Melia and Duman 1991). a-Helical CRF9-41 infusion immediately lateral to the LC blocks chronic stress-induced activation of tyrosine hydroxylase protein in the LC (Melia and Duman, 1991). Stress- or i.c.v. administered CRF-induced increases in the LC neuronal firing are blocked by CRF receptor antagonists (Swanson et al., 1983; Valentino et al., 1983; Valentino and Wehby, 1988; Schulz et al., 1996). Systemic administration of CP-154,526 antagonizes the stimulatory effects of exogenous CRF on LC neuronal firing (Schulz et al., 1996). In the present study, the firing rate of LC neurons was increased by i.c.v. administered CRF. This excitation of LC neurons was significantly blocked by the pretreatment with i.v. administration of CRA1000 and CRA1001 as well as CP-154,526. Thus, stress- or CRF induced behavioral deficits observed in animal models could be due to a conditioned increase in activity of the LC-noradrenergic system, which could be acutely terminated by CRA1000 and CRA1001.

Diazepam, but not CRA1000, CRA1001, and CP-154,526 significantly inhibited the spontaneous locomotor activity and the number of mice who stayed on the rotarod, and potentiated the hexobarbital-induced anesthesia in mice. Additionally, although memory for the task was not altered by CRA1000, CRA1001, and CP-154,526, a significant disruption of retention was noted for diazepam-treated animals. These data suggest that CRA1000 and CRA1001 may not produce central nervous system depression in humans.

In conclusion, CRF is a key regulator of the overall response to stress. Clinical data have implicated CRF in the etiology and pathophysiology of endocrine, psychiatric, neurologic, and inflammatory illnesses (Owens and Nemeroff, 1991; Dieterich et al., 1997). Hypersecretion of CRF in the brain may contribute to the symptomatology seen in neuropsychiatric disorders such as depression and anxiety-related disorders (Owens and Nemeroff, 1991; Dieterich et al., 1997). CRA1000 and CRA1001, nonpeptide CRF, receptor antagonists, warrant further study for possible treatment of anxiety and depression as well as other diseases in which excessive stimulation of CRF receptors contributes to the pathology.

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