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4-(2-Chloro-4-methoxy-5-methylphenyl)-*N*-[(1*S*)-2-cyclopropyl-1-(3-fluoro-4-methylphenyl)ethyl]5-methyl-*N*-(2-propynyl)-1,3-thiazol-2-amine Hydrochloride (SSR125543A): A Potent and Selective Corticotrophin-Releasing Factor$_1$ Receptor Antagonist. I. Biochemical and Pharmacological Characterization

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ABSTRACT

4-(2-Chloro-4-methoxy-5-methylphenyl)-*N*-[(1*S*)-2-cyclopropyl-1-(3-fluoro-4-methylphenyl)ethyl]5-methyl-*N*-(2-propynyl)-1,3-thiazol-2-amine hydrochloride (SSR125543A), a new 2-aminothiazole derivative, shows nanomolar affinity for human cloned or native corticotrophin-releasing factor (CRF)$_1$ receptors (pK_i values of 8.73 and 9.08, respectively), and a 1000-fold selectivity for CRF$_1$ versus CRF$_{2\alpha}$ receptor and CRF binding protein. SSR125543A antagonizes CRF-induced stimulation of cAMP synthesis in human retinoblastoma Y 79 cells (IC_{50} = 3.0 ± 0.4 nM) and adrenocorticotropin hormone (ACTH) secretion in mouse pituitary tumor AtT-20 cells. SSR125543A is devoid of agonist activity in these models. Its brain penetration was demonstrated in rats by using an ex vivo [^{125}I-Tyr^0] ovine CRF binding assay. SSR125543A displaced radioligand binding to the CRF$_1$ receptor in the brain with an ID_{50} of 6.5 mg/kg p.o. (duration of action >24 h). SSR125543A also inhibited the increase in plasma ACTH levels elicited in rats by i.v. CRF (4 μg/kg) injection (ID_{50} = 1, 5, or 5 mg/kg i.v., i.p., and p.o., respectively); this effect lasted for more than 6 h when the drug was given orally at a dose of 30 mg/kg. SSR125543A (10 mg/kg p.o.) reduced by 73% the increase in plasma ACTH levels elicited by a 15-min restraint stress in rats. Moreover, SSR125543A (20 mg/kg i.p.) also antagonized the increase of hippocampal acetylcholine release induced by i.c.v. injection of 1 μg of CRF in rats. Finally, SSR125543A reduced forepaw treading induced by i.c.v. injection of 1 μg of CRF in gerbils (ID_{50} = ~10 mg/kg p.o.). Altogether, these data indicate that SSR125543A is a potent, selective, and orally active CRF$_1$ receptor antagonist.

Corticotrophin-releasing factor (CRF) is the prime coordinator of the neuroendocrine and behavioral responses to stress (Owens and Nemeroff, 1991). This 41-amino acid peptide is the major hypothalamic factor responsible for the stimulation of corticotrophin (ACTH) secretion from the anterior pituitary, which in turn induces synthesis and release of glucocorticoids from the adrenal cortex (Vale et al., 1981). The highest density of CRF-containing cell bodies is found in the medial paraventricular nucleus of the hypothalamus, a brain region that projects to the median eminence (Sawchenko and Swanson, 1991). CRF-containing neurons are also found in extrahypothalamic areas, e.g., limbic structures (Gray and Bingaman, 1996), suggesting that CRF may also play a neurotransmitter role, mediating both stress response and affective behavior (Arborelius et al., 1999). Because CRF hypersecretion associated with overactivation of the hypothalamo-pituitary-adrenal (HPA) axis has been implicated in depression and anxiety, the discovery of nonpeptide molecules that selectively inhibit CRF activity is of major clinical interest (Holsboer, 1999).

ABBREVIATIONS: CRF, corticotropin-releasing factor; ACTH, adrenocorticotropin hormone; HPA, hypothalomo-pituitary-adrenal axis; CRF-BP, corticotropin-releasing factor-binding protein; DMSO, dimethyl sulfoxide; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; ANOVA, analysis of variance; ACh, acetylcholine; R-121919, 3-[6-(dimethylamino)-4-methyl-pyrid-3-yl]-2,5-dimethyl-*N*,*N*-dipropyl-pyrazolo[2,3-*a*]pyrimidin-7-amine; antalarmin, butylethyl-[2,5,6-trimethyl-7-(2,4,6-trimethylphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl]-amine.

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The functional effects of CRF are mediated via the activation of two receptor subtypes, CRF_1 and CRF_2, that are 70% homologous in their amino acid sequences but appear pharmacologically and anatomically distinct. Both receptor subtypes are members of the G protein-coupled receptor superfamily positively coupled to adenylate cyclase. CRF_1 is the predominant receptor within the pituitary, cerebellum, and neocortex. Two CRF_2 isoforms exist: the $CRF_{2\alpha}$, which is expressed in limbic regions, e.g., lateral septum and dorsal raphe nucleus; and the $CRF_{2\beta}$, more abundant in the periphery (Chalmers et al., 1995). Moreover, a CRF binding protein (CRF-BP) binds native rat/human CRF with higher affinity than CRF receptors (Behan et al., 1995). CRF-BP is expressed in the brain of numerous species, where it might regulate CRF-mediated neurotransmission.

A second CRF receptor endogenous agonist, urocortin, has been described (45% homology with CRF) and binds to CRF_2 receptors with a 10-fold higher affinity than CRF. Urocortin mRNA expression is prominent in the Edinger-Westphal nucleus, which does not contain CRF mRNA and is colocalized with the $CRF_{2\alpha}$ receptor mRNA in the rat lateral septum and dorsal raphe nucleus (Vaughan et al., 1995). Recently, urocortin II, which possesses only 26% homology with CRF, has been cloned and found to be a selective agonist at CRF_2 receptors (Reyes et al., 2001).

The hypothesis that CRF plays a role in the pathophysiology of affective disorders has been put forward on the basis of experimental behavioral data, and is consistent with the contribution of CRF system alterations to the etiology of psychiatric disorders exacerbated or precipitated by stress. Thus, high levels of cerebrospinal fluid CRF and an increased number of CRF immunoreactive neurons in the hypothalamic paraventricular nucleus have been measured in patients with depressive disorders (Nemeroff et al., 1984). After electroconvulsive therapy or antidepressant treatment, HPA axis and CRF function normalize, suggesting that CRF overactivity may be a marker for human depression (Nemeroff et al., 1991). Moreover, intra-amygdala injection of antisense oligonucleotides directed against the CRF_1 and CRF_2 receptor mRNA in the rat and knock out of the CRF_1 receptor gene in mice have been associated with reduced levels of anxiety and lower anxiogenic responses to i.v. CRF injections (Liebsch et al., 1995; Heinrichs et al., 1997; Smagin and Dunn, 2000). Furthermore, CRF_1 receptor antagonists have demonstrated anxiogenic effects in rodents (Gutman et al., 2000).

A number of synthetic CRF_1 receptor antagonists have been identified (Gutman et al., 2000), e.g., butylethyl-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl]-amine (CP-154,526), 5-chloro-*N*-cyclopropylmethyl-2-methyl-*N*-propyl-*N*′-(2,4,6-trichlorophenyl)-pyrimidin-4,6-diamine (NBI 27914), 4-(3-pentylamino)-2,7-dimethyl-8-(2-methyl-4-methoxyphenyl)-pyrazolo-[1,5-*a*]pyrimidine (DMP904), 2-[(*N*-(2-methylthio-4-isopropylphenyl)-*N*-ethylamino]-4-[4-(3-fluorophenyl)-1,2,3,6-tetrahydropyridin-1-yl)-6-methylpyrimidine (CRA 1000), and R-121919 (formerly NBI 30775). However, it is of interest to note that these molecules have close structural similarities, including pyrrolo-, pyrazolo-, and other substituted pyrimidine moieties. The more recent compounds offer better solubility and central nervous system penetration than their predecessors. For example, R-121919 (K_i value of 3 nM for the human CRF_1 receptor) has been shown to possess pharmacological activity in experimental models of anxiety after oral administration in the 3- to 30-mg/kg range (Gutman et al., 2000). Beneficial effects of this compound have been observed in an open clinical trial performed in depressed patients, supporting the view that CRF_1 receptor antagonism could be of therapeutic value in the treatment of depression. However R-121919's development has been stopped because of hepatic toxicity (Zobel et al., 2000). In the present study, we report on the characterization of a new CRF_1 receptor antagonist, 4-(2-chloro-4-methoxy-5-methylphenyl)-*N*-[(1*S*)-2-cyclopropyl-1-(3-fluoro-4-methylphenyl)ethyl]5-methyl-*N*-(2-propynyl)-1,3-thiazol-2-amine hydrochloride (SSR125543A), obtained by the optimization of a lead compound discovered by random screening of several thousand chemicals. This compound belongs to the novel 2-aminothiazole chemical family (Fig. 1).

Experimental Procedures

Animals

Male Sprague-Dawley CD rats and female OF1 mice purchased from Iffa Credo (L'Arbresele, France), and male Mongolian gerbils from Janvier (Le Genest St. Isle, France) were housed in a controlled temperature and light-dark environment with water and chow available ad libitum before the experiments. All experimental procedures were approved by the Animal Care and Use Committee of Sanofi-Synthelabo Recherche and were carried out in accordance with French legislation.

Materials

SSR125543A (Fig. 1) and antalarmin were synthesized by Sanofi-Synthelabo Recherche (Toulouse, France). Both compounds were solubilized in pure DMSO for the in vitro assays and in 5% DMSO and 5% Cremophor EL in saline when administered to mouse, rat, and gerbil. Rat/human CRF, ovine CRF, [D-Phe^{11},His^{12}]$Svg_{(11-40)}$ (antisauvagine-30), and rat/human $CRF_{(6-33)}$ from Neosystem (Strasbourg, France) were solubilized in 0.1% acetic acid solution containing 1 mg/ml serum bovine albumin. [^{125}I-Tyr^0] ovine CRF, [^{125}I-Tyr^0] rat/human CRF, and [^{125}I-Tyr^0] sauvagine were purchased from PerkinElmer Life Sciences (Boston, MA). Cell culture media, antibiotics, and fetal calf serum were obtained from Invitro-

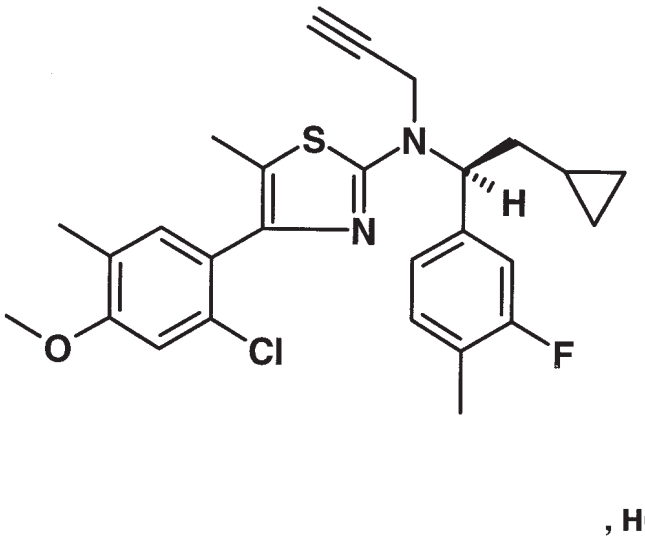

Fig. 1. Chemical structure of the selective CRF_1 receptor antagonist SSR125543A.

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gen (Cergy Pontoise, France). All other chemicals were from commercial sources.

Cell Cultures

CHO cells stably transfected with the human CRF_1 receptor ($hCRF_1$-CHO cells) or with the human $CRF_{2\alpha}$ receptor ($hCRF_{2\alpha}$-CHO cells) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated fetal calf serum, 300 μg/ml L-glutamine, nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.17 μg/ml amphothericine. Y 79 cells purchased from American Type Culture Collection (Rockville, MD) were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum and 300 μg/ml L-glutamine.

AtT-20 cells purchased from American Type Culture Collection were cultured in Dulbecco's modified Eagle's medium containing only in supplement 10% fetal calf serum, 300 μg/ml L-glutamine, HEPES, and sodium pyruvate. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 except AtT-20, which was incubated with 15% CO_2.

Preparation of Cell Membrane Homogenates

Cells were cultured to confluence and the flasks were washed with 10 ml of phosphate-buffered saline (PBS) medium and filled with an equal volume of PBS medium. Cells ($hCRF_1$-CHO, $hCRF_{2\alpha}$-CHO, and AtT-20) were detached from the flask with a cell scraper. Y 79 cells were cultured in suspension. After centrifugation at 800*g* for 5 min, the cell pellet was homogenized at 4°C by using a Polytron (setting 6, 2 × 20 s) in 50 mM Tris-HCl pH 7.4, 2 mM EDTA buffer for $hCRF_1$-CHO and $hCRF_{2\alpha}$-CHO cells. Homogenization was performed in 50 mM Tris-HCl pH 7.2, 10 mM $MgCl_2$, 2 mM EDTA, 0.1% serum bovine albumin, 8 mg/l aprotinin, and 0.5 mg/ml soybean trypsin inhibitor for Y 79 cells. After centrifugation at 40,000*g* for 20 min at 4°C, the pellet was homogenized at 4°C by using a Polytron in binding buffer (see below). Aliquots obtained from the membrane suspension were stored in liquid nitrogen.

Preparation of Brain Membrane Homogenates

Because the in vivo pharmacological profile of the compound was to be characterized in rodents, the inhibitory effects of SSR125543A on [^{125}I-Tyr^0] ovine CRF binding to rat, mouse, and gerbil brain were assessed.

Mouse, rat, and gerbil were sacrificed by decapitation and brains were rapidly removed and homogenized at 4°C by using a Polytron (setting 4, 30 s) in 50 mM Tris-HCl pH 7.4, 2 mM EDTA buffer. After centrifugation at 40,000*g* for 20 min at 4°C, the 0.5-mg/ml pellet was homogenized at 4°C by using a Polytron in binding buffer (see below). Aliquots obtained from the membrane suspension were stored were stored at −80°C.

CRF_1 Receptor Binding Assay

[^{125}I-Tyr^0] ovine CRF binding was performed with $hCRF_1$-CHO cell membranes, Y 79 cell membranes, or rodent brain membrane homogenates in the presence of 25 pM radiolabeled CRF in 50 mM Tris-HCl pH 7.2, 10 mM $MgCl_2$, 2 mM EDTA, 0.1% serum bovine albumin, 8 mg/l aprotinin, and 0.5 mg/ml soybean trypsin inhibitor under a final volume of 400 μl. Nonspecific binding was determined in the presence of 1 μM rat/human CRF. Agonists and antagonists were added in 1% DMSO (final concentration). After incubation at 20°C for 2 h, the incubation mixture was filtered on Whatman GF/B filters presoaked in 0.5% bovine serum albumin solution for 2 h. The filters were washed twice with ice-cold Tris-HCl pH 7.2 buffer and the radioactivity was determined with a gamma scintillation counter (LKB 1261 multi gamma; EG G Instruments, Evry, France). Specific binding was determined as the difference between total and nonspecific binding. IC_{50} values were determined using a nonlinear least-square regression analysis (Munson and Rodbard, 1980) with RS/1 (BBN Software Product Corporation, Cambridge, MA) and an internal computerized interactive procedure.

CRF_2 Receptor Binding Assay

[^{125}I-Tyr^0] sauvagine binding was performed using a similar protocol as with [^{125}I-Tyr^0] ovine CRF binding. In this case, $hCRF_{2\alpha}$-CHO cell membranes were used at the concentration of 2.5 μg of protein/tube in presence of 20 pM radiolabeled sauvagine, under a final volume of 250 μl. Nonspecific binding was determined in presence of 1 μM unlabeled sauvagine.

CRF-BP Binding Assay

Displacement of CRF from CRF-BP was measured by a detergent phase separation assay. Recombinant human CRF-BP was incubated at 20°C for 2 h with 30 pM [^{125}I-Tyr^0] rat/human CRF in 0.02% Nonidet-40 phosphate-buffered saline, pH 7.4. Bound and free CRF were then separated by the addition of Triton X-114 (octylphenoxypolyethoxyethanol) buffer stirring and incubation 20 min at 37°C. Free CRF segregates to the detergent phase at the bottom of the tube, and the CRF/CRF-BP complex remains in the aqueous phase. The amount of radioactivity in an aliquot of the aqueous phase was determined with a gamma scintillation counter (LKB 1261 multi gamma; EG G Instruments). Values were expressed as the mean ± S.E.M. of at least three determinations performed in triplicate. Specific binding was determined as in CRF_1 binding assays.

Measurement of Intracellular cAMP Synthesis in Y 79 Cells

CRF-induced cAMP synthesis in human retinoblastoma Y 79 cells was assessed as described by Hauger et al. (1997). In the present article, two types of experiments were performed on Y 79 cells. In the first experiment, Y 79 cells were incubated for 15 min at 37°C under stirring in presence of 10 nM rat/human CRF with increasing concentrations of SSR125543A in 1 mM isobutylmethyl xanthine supplemented RPMI buffer, pH 7.2. The intracellular cAMP content was measured after lysing the cells by 0.5% ice-cold Triton X-100 by using a cAMP ^{125}I scintillation proximity assay kit (Amersham Biosciences plc, Little Chalfont, Buckinghamshire, UK). IC_{50} values were determined using a nonlinear least-square regression analysis (Munson and Rodbard, 1980) with RS/1 (BBN Software Product Corporation) and an internal computerized interactive procedure.

In the second experiment, Y 79 cells were incubated for 15 min at 37°C under stirring with increasing concentrations of rat/human CRF alone or in presence of three concentrations of SSR125543A. Intracellular cAMP synthesis was expressed as the percentage of maximal release after subtraction of basal release. Values were expressed as the mean of at least three determinations performed in duplicate.

Measurement of ACTH Secretion by AtT-20 Cells

CRF-induced ACTH secretion in mouse pituitary AtT-20 cells was previously described by Litvin et al. (1984). A subclone of AtT-20/D16v cells was used in this study. Cells were seeded in 12-well plates and cultured overnight in their growth medium. They were incubated for 120 min at 37°C with 2 ml of basal medium, alone or with increasing concentrations of rat/human CRF in the presence or absence of three concentrations of SSR125543A. ACTH release was measured on supernatant samples by using a radioimmunoassay (Diasorin, Stillwater, MN). Values were expressed as mean values of three determinations performed in triplicate.

Ex Vivo Binding Assay in Rats

SSR125543A or the corresponding vehicle was administered p.o. or i.v. to rats (three per group) at various doses (dose-effect studies) and times (time course studies) before rat decapitation and organ (brain and pituitary) removal. Tissues were homogenized in 10 ml of incubation buffer by using a Polytron (speed 21,500 rpm × 17 s) then

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diluted (1/20) with the same incubation buffer and submitted to a [^{125}I-Tyr^0] ovine CRF binding assay procedure as previously described. To determine the relative population of CRF_1 binding sites present in crude homogenates from rat brain and pituitary, binding studies were performed in vitro, on naïve brain and pituitary tissue. Competition curves were determined for ovine CRF, antisauvagine-30, and rat/human $CRF_{(6-33)}$, two peptides selective for CRF_2 receptor and CRF-BP, respectively (Behan et al., 1995; Ruhmann et al., 1998). In the ex vivo binding assay, nonspecific binding was defined with 100 nM antalarmin. Values were expressed as the mean percentage of specific binding ± S.E.M. Statistical differences between drug- and vehicle-treated groups were assessed by a Student's *t* test.

CRF-Induced ACTH Secretion in Rats

Animals were habituated to the experimental procedure 1 day before the experiment. SSR125543A or its vehicle was administered p.o. or i.v. to rats (3–7/group) at various doses (dose-effect studies) and times (time course studies) before intravenous injection of 4 μg/kg rat/human CRF. Thirty minutes later, animals were sacrificed by decapitation and trunk blood samples were collected in a 1 mg/ml EDTA solution for the determination of ACTH plasma levels by radioimmunoassay (Diasorin). Results were expressed as the mean values ± S.E.M. Statistical differences between drug- and vehicle-treated groups were assessed by a Student's *t* test. The median inhibitory doses (ID_{50}) with 95% confidence limits were determined by fitting of the dose-response curve to the four-parameter logistic model according to Ratkowsky and Reedy (1986). The adjustment was performed by nonlinear regression by using the Levenberg-Marquardt algorithm in the RS/1 software.

Restraint Stress-Induced ACTH Secretion in Rats

One hour after oral administration of SSR125543A or its vehicle, rats were placed into hemicylindrical Plexiglas enclosures (6 cm in width and 4 cm in height) for 15 min. After this stress period, the animals were placed back in their cages, carried to an adjacent room and immediately sacrificed. Nonstressed control animals remained in their cage for 15 min before sacrifice. Blood was collected in a 1 mg/ml EDTA solution for the determination of ACTH plasma levels by radioimmunoassay (Diasorin). Values were expressed as the mean ACTH levels ± S.E.M. Statistical differences between drug- and vehicle-treated groups were assessed by a single factor ANOVA or by the nonparametric Kruskall-Wallis test followed by Dunnett's *t* test or by the Mann-Whitney *U* test with α adjustment of Holm on RS1/software, respectively.

CRF-Induced Hippocampal Acetylcholine Release

Surgery and Microdialysis. Rats were anesthetized with urethane (1.4 g/kg i.p.) and then placed in a stereotaxic frame. A microdialysis probe (CMA-12, length 2 or 3 mm and outer diameter 0.5 mm; Carnegie Medicine AB, Stockholm, Sweden) was stereotaxically implanted in the dorsal hippocampus. The coordinates were 3.5 mm posterior to bregma, 2 mm lateral to the midline, and 3.8 mm down from the dural surface for the hippocampus (Paxinos and Watson, 1986). For i.c.v. injection of CRF, ejection pipettes were implanted into the left lateral ventricle at the following coordinates: 0.8 mm posterior to bregma, 1.5 mm lateral to the midline, and 3.4 mm down from the dural surface. The ejection of CRF (1 μg/2 μl/90 s) was performed by applying air pressure with a 1-ml syringe connected to the nontapered side of the pipette by Tygon tubing. The probes were perfused with a gassed Ringer's solution containing 125 mM NaCl, 3 mM KCl, 1.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, 23 mM $NaHCO_3$, and 1.5 mM KH_2PO_4, pH 7.4, at a rate of 2 μl/min by using a microinjection pump (CMA-100; Carnegie Medicine AB). To reduce acetylcholine degradation in the dialysate, 1 μM neostigmine was added to the Ringer's solution perfused in the hippocampal probe. Microdialysis sampling started 90 min after the probe was placed in the hippocampus. Serial samples were collected at 30-min intervals. SSR125543A, antalarmin, and vehicle were given intraperitoneally (5 ml/kg of body weight) 180 and 30 min before peptide application.

The time course of the CRF effects was analyzed by ANOVA with repeated measures and Dunnett's *t* test was used for individual time comparisons. The antagonism of the CRF effect was evaluated by comparing the area under the curve during the 120 min after peptide injection. A statistical analysis was carried out by using the Student's *t* test.

Assay of Acetylcholine (ACh). ACh levels were measured in 30-min dialysate samples (50 μl) by using a high-performance liquid chromatography system (Waters, Milford, MA) as previously described by Steinberg et al. (1995) except for the electrochemical detection system (Coulochem II; ESA, Chelmsford, MA). Briefly, the analytical system for ACh included a trapping precolumn and immobilized enzyme reactor (BAS.MF-6151). The mobile phase, 35 mM phosphate buffer, pH 8.5, supplemented with the antibacterial reagent Kathon (5 ml/l; BAS DF-2150), was pumped at a flow rate of 0.8 ml/min and replaced with a fresh preparation every 3 days. The enzyme postcolumn reactor converted ACh to hydrogen peroxide that was electrochemically detected using a platinum electrode (ESA P/N 55-0183) set at 0.3 V. The chromatographic column and enzyme reactor were kept at 35°C. The detection sensitivity was 0.2 pmol/50 μl.

CRF-Induced Forepaw Treading in Gerbils

This test was based on the observation that i.c.v. injection of CRF (1 μg/2 μl) produces forepaw treading ("piano playing") in gerbils, an effect that is prevented by treatment with the CRF_1 receptor antagonist R-121919 (Owens and Nemeroff, 1999). Gerbils were placed individually in small transparent plastic cages for 30 min. They were then pretreated with SSR125543A p.o. or antalarmin i.p. CRF (1 μg) was injected i.c.v. (free-hand method; Jung et al., 1996) 15 min (antalarmin) or 60 min (SSR125543A) later. In each experiment, a control group was injected i.c.v. with the vehicle. Forepaw treading was measured by an observer unaware of the drug treatment, for 1 min every 15 min over a 2-h period (8 min in total cumulative times). The cumulative forepaw treading time was calculated for each gerbil and then expressed as the mean and S.E.M. Comparisons between control and treated groups were performed using the Kruskall-Wallis test, followed by Mann-Whitney *U* test with α adjustment of Holm.

Results

Affinity of SSR125543A for CRF_1 Receptors

SSR125543A inhibited the specific binding of [^{125}I-Tyr^0] ovine CRF to human CRF_1 receptors expressed in CHO cells

TABLE 1
Affinity of SSR125543A for CRF receptor subtypes and for CRF_1 receptors of various species
Values of pK_i and Hill coefficients are the means ± S.E.M. of three experiments performed in triplicate.

Binding Assay	Results	
CRF_1 binding: [^{125}I-Tyr^0]ovine CRF ligand	pK_i	*n*
hCRF_1-CHO cells	8.73 ± 0.15	1.25 ± 0.10
Y 79 cells	9.08 ± 0.20	0.97 ± 0.17
Rat brain	8.77 ± 0.23	0.89 ± 0.12
Mouse brain	8.90 ± 0.10	1.16 ± 0.13
Gerbil brain	9.00 ± 0.00	0.75 ± 0.11
$CRF_{2\alpha}$ binding: [^{125}I-Tyr^0] ovine sauvagine ligand	Inhibition at 10 μM	
h$CRF_{2\alpha}$-CHO cells	0%	
CRF-BP binding: [^{125}I-Tyr^0] rat/human CRF ligand	Inhibition at 10 μM	
hCRF-BP	0%	
rCRF-BP	0%	

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with a pK_i value of 8.73 ± 0.15 (mean ± S.E.M.; Table 1), which was comparable to that of antalarmin and higher than that of the natural ligand rat/human CRF (pK_i = 8.70 and 8.22, respectively). It also recognized with high affinity the native CRF_1 receptors present on the human retinoblastoma cell line Y 79 (pK_i = 9.08 ± 0.20). At 10 μM, SSR125543A did not interact with the human $CRF_{2\alpha}$ receptor expressed in CHO cells, or human and rat recombinant CRF-BP (Table 1). Binding studies performed with [^{125}I-Tyr^0] ovine CRF on membrane preparations obtained from rodent brains (rat, mouse, and gerbil) did not reveal species differences in affinity because the respective pK_i values of 8.77 ± 0.23, 8.90 ± 0.10, and 9.00 ± 0.00 were very close to the pK_i for the human CRF_1 receptor (Table 1). The high selectivity of SSR125543A for the CRF_1 receptor was demonstrated by its lack of activity (inhibition lower than 50%) at 1 or 10 μM in 125 assays performed by Panlabs and Cerep (receptors, transporters, enzymes, and ion channels) (Table 2).

CRF_1 Receptor Antagonism by SSR125543A: In Vitro Studies

When rat/human CRF was applied to Y 79 cells, which express constitutively CRF_1 receptors, the intracellular cAMP production was increased by ~7-fold over basal levels, with an EC_{50} value of 4.0 ± 0.9 nM (mean ± S.E.M). SSR125543A did not modify the basal level of cAMP but fully blocked the CRF (10 nM) response with an IC_{50} value of 3.0 ± 0.4 nM (mean ± S.E.M., n = 3; Fig. 2A). Under similar experimental conditions, the IC_{50} for antalarmin was 0.8 ± 0.1 nM (mean ± S.E.M., n = 3; data not shown). Increasing concentrations of SSR125543A produced a rightward shift of the rat/human CRF concentration-response curve (Fig. 2B), without modifying the maximal cAMP production obtained with rat/human CRF alone. EC_{50} values for CRF were 2.5 nM (2.1–2.9), 8.3 (5.5–12.4), 55.6 (42.5–73.0), and 92.2 (55.7–144.9) (means and confidence limits) in the presence or absence of 3, 30, and 100 nM SSR125543A, respectively.

When rat/human CRF was applied to mouse pituitary AtT-20 cells, which express CRF_1 receptors, ACTH secretion was stimulated by ~3-fold over basal levels. SSR125543A did not modify basal secretion of ACTH but antagonized the ACTH secretion induced by increasing concentrations of rat/human CRF. Increasing concentrations of SSR125543A also produced a rightward shift of the rat/human CRF dose-response curve and a concentration-dependent inhibition of the maximal ACTH secretion elicited by rat/human CRF alone (Fig. 3). In the course of three experiments, EC_{50} values for CRF were 1.6 (1.4–1.9), 11.9 (10.0–14.1), 49.8 (34.2–74.3), and 128.1 (104.6–160.0) (means and confidence limits) in the presence or absence of 3, 30, and 100 nM SSR125543A, respectively.

Ex Vivo Binding Assay

The nonspecific binding obtained in the presence of 1 μM rat/human CRF in the ex vivo [^{125}I-Tyr^0] ovine CRF binding assay averaged 20%. To determine the real proportion of CRF_1 binding sites in this model, competition studies were performed with ovine CRF, antalarmin, SSR125543A, antisauvagine-30, and rat/human $CFR_{(6-33)}$ on crude brain and pituitary homogenates prepared from untreated rats. As shown in Fig. 4A and Table 3, the binding of radiolabeled ovine CRF could be competed by ovine CRF with a biphasic curve, suggesting two populations of 50 and 30% of the total binding sites and respective pK_i values of 8.35 and 6.23. Antalarmin and SSR125543A displaced only the first population of sites in a monophasic manner and similar pK_i values of 8.80 and 8.89. Antisauvagine-30 competed weakly with iodinated ovine CRF with a shallow monophasic curve (pK_i = 6.77, n_H = 0.67). In contrast, the selective CRF-BP ligand rat/human $CRF_{(6-33)}$ inhibited only 30% of the total binding that represents the non-CRF_1 component. Its low affinity for ovine CRF compared with rat/human CRF explains the weak pK_i (6.43) measured in this study. Unlike crude brain membranes, the specific binding of [^{125}I-Tyr^0] ovine CRF to crude rat pituitary homogenates represented 90% of the total binding and was completely displaced by 100 nM antalarmin. Taking into account the high selectivity of antalarmin, CRF_1 specific binding to crude tissue homogenates was considered as the maximal displacement measured in the presence of 100 nM antalarmin.

The blockade of brain and pituitary CRF_1 receptors was evaluated in binding studies performed on crude tissue homogenates prepared from rats treated with SSR125543A (ex vivo binding assay). No specific CRF_1 binding could be measured after a 2-h oral treatment at the dose of 30 mg/kg, whereas at 4 h postadministration, the binding was still reduced by 76 ± 2% (Fig. 4C). The presence of SSR125543A at the pituitary level was also demonstrated in the same experiment, by a decrease of 67 ± 1% in binding 1 h after SSR125543A oral administration that reached 78 ± 1% at 2 and 4 h (Fig. 4C). Another experiment performed under similar conditions demonstrated that ligand binding inhibition was still present 24 h after SSR125543A treatment, with 62 ± 14% inhibition in the brain and 80 ± 2% in the pituitary (mean and S.E.M., n = 3). Dose-effect study performed 2 h after oral treatment revealed a dose-dependent inhibition of brain CRF_1 receptor binding with an ID_{50} of 6.5 (3.2–11.8) mg/kg (mean and confidence limits; Fig. 4B). SSR125543A also reached both brain and pituitary after i.p. injection, ID_{50} determined 2 h post-treatment being 11.7 (6.0–23.0) mg/kg, slightly higher than after oral administration (data not shown).

CRF_1 Receptor Antagonism by SSR125543A: In Vivo Studies

CRF-Induced ACTH Secretion in Rats. In conscious rats, the plasma level of ACTH determined by radioimmunoassay was 34 ± 4 pg/ml (mean ± S.E.M., n = 12). Oral administration of 30 mg/kg SSR125543A, 2 h before blood sampling significantly diminished the ACTH level (18 ± 2 pg/ml, $p < 0.01$, n = 5). CRF (4 μg/kg i.v.) injection, 30 min before blood sampling, induced a more than 10-fold stimulation (269 ± 20 pg/ml) of the ACTH secretion. When administered orally at the dose of 30 mg/kg, SSR125543A inhibited the increase in ACTH secretion induced by CRF injection with significant effects from 1 to 6 h (Fig. 5A). Dose-effect studies performed after oral administration of SSR125543A, 2 h before the CRF injection, yielded ID_{50} values of 4.9 (3.0–8.6) mg/kg (Fig. 5B) (means and confident limits). After i.v. injection of 3 mg/kg SSR125543A, the maximal inhibition of CRF-induced ACTH secretion was observed at 5 min postinjection. The dose-effect study performed at the same time yielded an ID_{50} of 1.3 (1.2–1.4) mg/kg i.v. (Fig. 5C).

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TABLE 2

Screening of the selectivity of SSR125543A

Targets and tissue/cell sources for binding, transporter, ion channel, and enzyme assays (Panlabs and Cerep screening). (1), (2), and (3) in the target column refer to the respective number in the tissue/cell source column.

Target	Tissue/Cell Source
Receptors	
Adenosine A_1, A_{2A}, A_{2B}	Human recombinant
Adrenergic α_{1A}(1), α_{1B}(2), α_{1D}, α_{2A}, α_{2B}, α_{2C}, β_1, β_2, β_3	Rat submaxillary gland(1), liver(2); Human recombinant
Angiotensin AT_1, AT_2	Human recombinant
Atrial natriuretic factor	Guinea pig adrenal gland
Bombesin	Rat brain
Bradykinin B_2	Human recombinant
Calcitonin	Human T-47D cells
Calcitonin gene-related peptide	Human SK-N-MC cells
Cannabinoid CB_1, CB_2	Human recombinant
Chemokine CCR_5, $CXCR_1$, $CXCR_2$	Human recombinant
Cholecystokinin CCK_1, CCK_2	Human recombinant
Dopamine D_1, D_{2L}, D_3, $D_{4\text{-}2}$, D_5	Human recombinant
Endothelin ET_A, ET_B	Human recombinant
Epidermal growth factor	Human A431 cells
Estrogen ERa, ERb	Human recombinant
Galanin	Human Bowes cells
Glucocorticoid	Human HeLa S3 cells
Glutamate kainate(1), NMDA agonism(2)	Rat brain(1) and cerebral cortex(2)
Glycine strychnine-sensitive	Rat spinal cord
Histamine H_1 central(1), H_2(2) and H_3(3)	Guinea pig brain(1) and striatum(2); Rat brain(3)
Imidazoline I_2 central	Rat cerebellar cortex
Inositol triphosphate IP_3	Rat cerebellum
Insulin	Rat liver
Interleukin IL-1α	Mouse 3T3-SWISS cells
Leukotriene B_4(1), D_4(2)	Human U937 cells(1); Guinea pig lung(2)
Melanocortin MC_4(1), ML_1(2)	Human recombinant(1); Chicken brain(2)
Muscarinic M_1,M_2,M_3,M_4 and M_5	Human recombinant
N neuronal, α-BGTX-insensitive(1); muscle-type(2)	Rat cerebral cortex(1); Human BC3H-1 cells(2)
Neuropeptide Y_1(1), Y_2(2)	Human recombinant(1); Human KAN-TS cells(2)
Neurotensin	Mouse brain
Nicotinic acetylcholine, central	Rat brain
Opiate δ, K, μ	Human recombinant
Orphanin	Human recombinant
PCP	Rat cerebral cortex
Platelet-activating factor	Rabbit platelets
Progesterone	Calf uterus
Purinergic P_{2X}	Rabbit urinary bladder
Rolipram	Rat brain (minus cerebellum)
Serotonin 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}	Human recombinant
Serotonin 5-HT_2(1), 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}	Rat brain(1); human recombinant
Serotonin 5-HT_3, 5-HT_4(1), 5-HT_{5A}, 5-HT_6, 5-HT_7	Guinea pig striatum(1); Human recombinant
Sigma σ_1, σ_2	Human recombinant
Sst_4	Human recombinant
Tachykinin NK_1, NK_2, NK_3	Human recombinant
Testosterone	Rat ventral prostate
Thromboxane A_2 (TXA_2)	Rabbit platelets
Thyrotropin-releasing hormone (TRH)	Rat brain
Tumor necrosis factor, Nonselective	Human U937 cells
Vascular endothelial growth factor (VEGF)	HUVEC
Vasoactive instestinal peptide VIP_1	Human HT29 cells
Vasopressin V_{1A}	Human recombinant
Transporters	
Adenosine	Guinea pig cerebral cortex
Adrenergic, norepinephrine	Human recombinant
Choline	Rat striatum
Dopamine	Human recombinant
Serotonin 5-HT	Human recombinant
Ion channels	
Calcium type N	Rat brain frontal lobe
Chloride	Rat cerebral cortex
$GABA_A$ antagonist site(1), benzodiazepine central(2), chloride TBOB(3)	Rat brain(1,2) and cerebral cortex(3)
Sodium site 1 and site 2	Rat cerebral cortex
Potassium K^+_{ATP}, K^+_V, SK^+Ca	Rat cerebral cortex
Enzymes	
ACE	HUVEC cells
Acetylcholinesterase	Human recombinant
ATPase (Na^+/K^+)	Dog kidney
COX_1(1), COX_2(2)	Human platelets(1), HUVEC cells(2)
ECE	HUVEC cells
MAO-A(1) and MAO-B(2)	Human placenta(1), rat brain(2)
NOS inducible	RAW 264-7 cells
Phosphodiesterase III(1) and IV(2)	Guinea pig heart(1), human U-937 cells(2)
Protein kinase C	Rat brain

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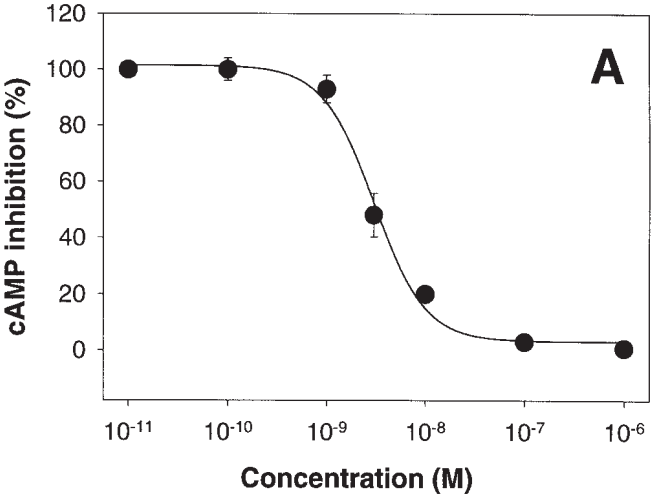

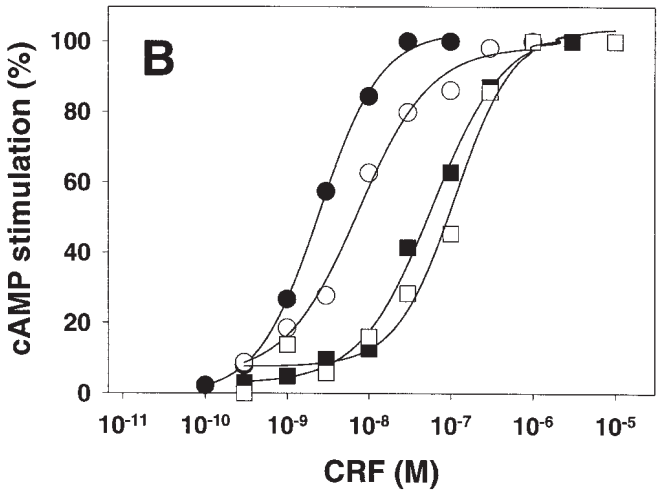

Fig. 2. Antagonism by SSR125543A of CRF-induced cAMP synthesis in Y 79 cells. A, concentration effect of SSR125543A for the inhibition of cAMP synthesis stimulated by 10 nM rat/human CRF. Results are expressed as the percentage of inhibition of the maximal increase induced by CRF. B, concentration-response curves for CRF-stimulated cAMP synthesis in Y 79 cells in the absence (●) or presence of SSR125543A at 3 nM (○), 30 nM (■), and 100 nM (□). Results are expressed as percentage of increase induced by CRF in the presence or absence of competitor. For both experiments, the data are the means of three independent experiments (±S.E.M., Fig. 3A).

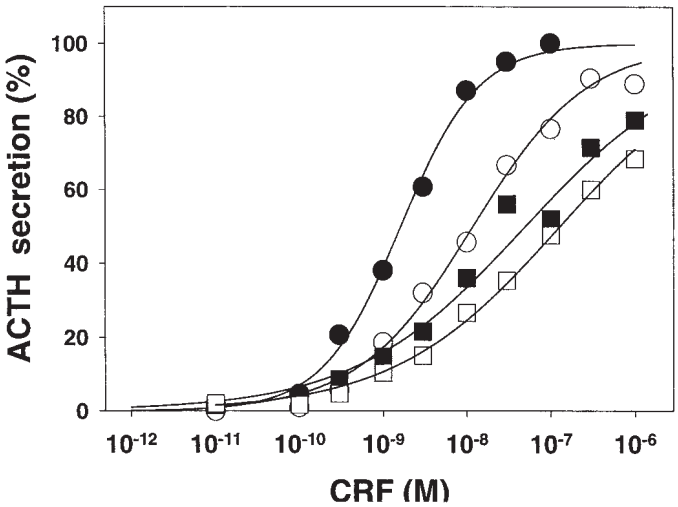

Fig. 3. Antagonism by SSR125543A of CRF-induced ACTH secretion in AtT-20 cells. Concentration-response curves for CRF-stimulated ACTH secretion in AtT-20 cells in the absence (●) or presence of SSR125543A at 3 nM (○), 30 nM (■), and 100 nM (□). The results are expressed as percentage of increase induced by CRF in the presence or absence of SSR125543A. The data are the means of three independent experiments.

Stress-Induced ACTH Secretion in Rats. A 15-min restraint stress caused a marked elevation of ACTH plasma levels (4- or 6-fold stimulation) in rats. This increase was significantly antagonized by oral administration of 10 (but not 3) mg/kg SSR125543A (given 1 h before the stress session) ($p < 0.01$, $n = 9$) (Fig. 6). Under similar experimental conditions, 30 mg/kg antalarmin also produced a significant decrease of the CRF response, which was lesser in extent than that of SSR125543A ($p < 0.05$, $n = 10$).

CRF-Induced Hippocampal ACh Release in Rats. In anesthetized rats, the i.c.v. injection of 1 μg/2 μl CRF produced a rapid increase in extracellular ACh levels measured in dialysates from the hippocampus (Fig. 7A). In both groups, a significant increase was observed at 60 min ($p < 0.01$) after CRF injection, which persisted up to 120 min postinjection with a maximal effect at 90 min (+135 ± 28 and + 84 ± 14%, $p < 0.01$, $n = 9$ and 8, respectively). SSR125543A (20 mg/kg) injected i.p. 3 h before the administration of CRF ($p < 0.05$, $n = 7$) partially antagonized the CRF-evoked hippocampal ACh release as measured by the area under the curve during the 120-min sampling period after CRF injection (Fig. 7B). Similarly, 30 mg/kg antalarmin injected i.p. 30 min before CRF significantly ($p < 0.01$, $n = 6$) reduced the peptide response.

CRF-Induced Forepaw Treading in Gerbils. The results presented in Table 1 show that forepaw treading produced by i.c.v. injection of CRF was significantly attenuated by pretreatment with SSR125543A ($\chi^2 = 29.32$, $p < 0.01$) and antalarmin ($\chi^2 = 27.66$, $p < 0.01$) at the doses of 10 and 30 mg/kg, respectively, 60 and 15 min before CRF injection (Table 4).

Discussion

This study describes the biochemical and pharmacological properties of SSR125543A, a novel nonpeptide antagonist of CRF_1 receptors. SSR125543A inhibits the specific binding of [^{125}I-Tyr^0] ovine CRF to the CRF_1 receptor cloned from human brain and stably expressed in CHO cells (hCRF_1-CHO), with a nanomolar affinity close to that of the natural ligand rat/human CRF and of antalarmin, another nonpeptide CRF_1 receptor antagonist. The high affinity of SSR125543A for the native human CRF_1 receptor was demonstrated in membrane preparations obtained from cultured human retinoblastoma Y 79 cells that constitutively express CRF_1 receptors. SSR125543A recognized with the same affinity CRF_1 receptors from rat, mouse, and gerbil brain, demonstrating no species difference between human and rodent. This compound is 1000-fold more selective for human CRF_1 than for human $CRF_{2\alpha}$ receptors or human CRF binding protein. The selectivity of SSR125543A for the CRF_1 receptor was also demonstrated by its lack of activity, when tested at high concentrations (1 or 10 μM, in a panel of binding assays for

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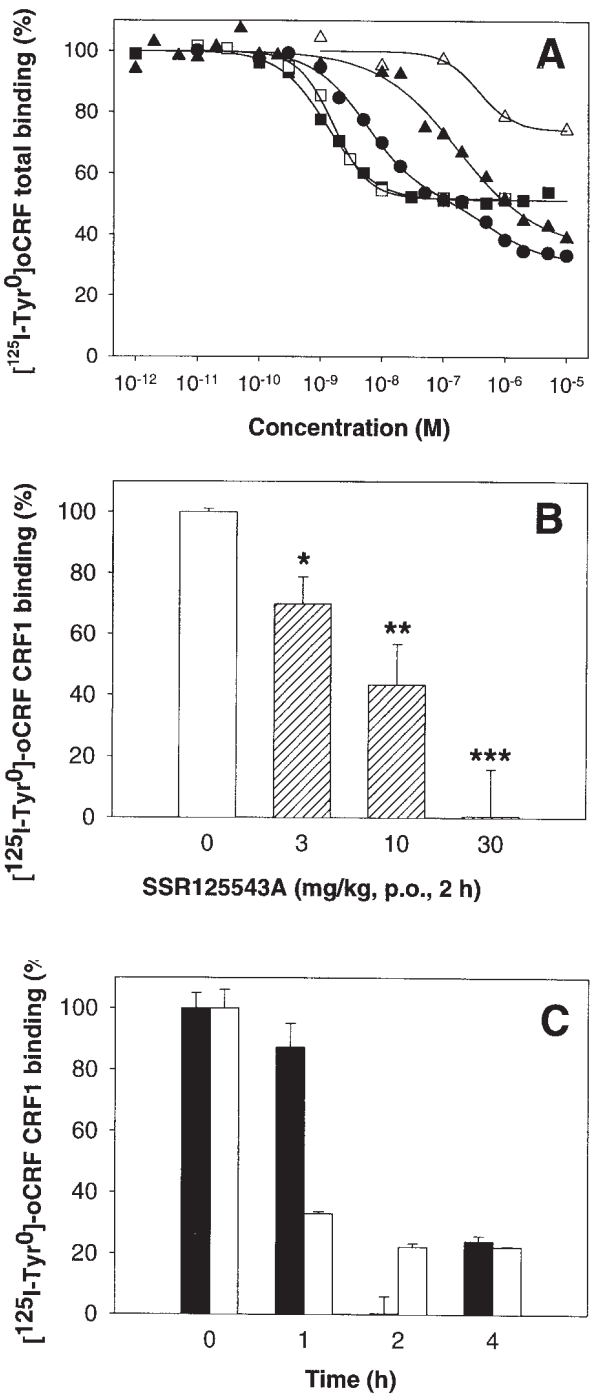

Fig. 4. Ex vivo binding results. A, inhibition by ovine CRF, antalarmin, and SSR125543A of [^{125}I-Tyr^0] ovine CRF specific binding to rat brain homogenates. Membranes were incubated in the presence of [^{125}I-Tyr^0] ovine CRF with increasing concentrations of ovine CRF (●), antalarmin (□), and SSR125543A (■), antisauvagine-30 (▲), and rat/human $CRF_{(6-33)}$ (△). Results are expressed as percentage of [^{125}I-Tyr^0] ovine CRF total binding and are representative of three experiments performed in triplicate. B, dose-dependent occupation by SSR125543A of CRF_1 receptors in brain homogenates after 2-h oral treatment evidenced by [^{125}I-Tyr^0] ovine CRF binding assay. C, comparison of the time course occupation by SSR125543A of brain and pituitary CRF_1 receptors. The compound was administered orally, at the dose of 30 mg/kg, at various time periods (1, 2, and 4 h) before brain and pituitary removal. Results of both studies (B and C) are expressed as the mean percentage and S.E.M. values of [^{125}I-Tyr^0] ovine CRF specific binding, with in vitro determination of the nonspecific binding by using a 100 nM concentration of the selective CRFi receptor antagonist antalarmin. ■, brain; □, pituitary. ★★, $p < 0.01$; ★★★, $p < 0.001$ versus CRF control group.

TABLE 3

Competition by ovine CRF, antalarmin, SSR125543A, antisauvagine-30 and rat/human $CRF_{(6-33)}$ of [^{125}I-Tyr^0] ovine CRF binding to rat brain homogenates

pK_i values from binding study performed in triplicate.

Compound	CRF_1 Binding Sites	Non-CRF_1 Binding Sites
	50% of total binding	*30% of total binding*
Antalarmin	8.77	Not displaced
SSR125543A	8.89	Not displaced
Ovine CRF	8.35	6.23
Asvg-30	6.77 (n = 0.67)	
Rat/human $CRF_{(6-33)}$	Not displaced	6.43

neurotransmitters and their transporters, peptides and hormones, or enzymes and ion channels.

The binding of CRF to CRF_1 receptors has been reported to increase adenylate cyclase activity and in turn cAMP levels in cells transfected with the CRF_1 receptor and to stimulate ACTH secretion from pituitary corticotrophs. The present study clearly demonstrates that SSR125543A behaves as a nanomolar CRF_1 receptor antagonist, devoid of agonist properties. It concentration dependently inhibited CRF-induced cAMP production in the human retinoblastoma Y 79 cell line in a competitive manner. The compound also inhibited ACTH secretion induced by CRF in the pituitary tumor AtT-20 cell line. However, the observed rightward shift was associated with a concentration-dependent diminution of secretion level, which suggested a noncompetitive inhibition in these murine cells.

The ability of SSR125543A to reach the pituitary and the brain has been demonstrated in ex vivo binding assays performed in rats after both oral and i.p. administration.

First, the relative population of CRF_1 binding sites present in crude homogenates was determined in [^{125}I-Tyr^0] ovine CRF binding studies performed in vitro, on naïve brain and pituitary tissues. Competition by ovine CRF revealed two populations of sites (representing 50 and 30% of total binding). Only 50% of sites displaced by the two selective CRF_1 receptor antagonists antalarmin and SSR125543A were relevant to CRF_1 receptors. The antisauvagine-30 weakly competed with the ligand (pK_i = 6.77) and did not discriminate between these two populations. Its low affinity suggests that the non-CRF_1 component recognized by this peptide does not correspond to CRF_2 binding sites. Rat/human $CRF_{(6-33)}$ concentration dependently inhibited only the second (30%) population of binding sites. The measured pK_i = 6.44 is in agreement with the lower affinity of this competitor for ovine CRF used as radioligand in our study. The non-CRF_1 component, not displaced by antalarmin and SSR125543A, may correspond to the CRF-BP present in rat brain crude homogenate. It is worth noting that in similar experimental conditions, CRF_1 binding (90% of the total binding) to rat pituitary homogenates was totally displaced by 100 nM antalarmin and corresponded to the specific binding. Taking into account the CRF_1 receptor selectivity of antalarmin, 100 nM this compound was used to define CRF_1 specific binding in subsequent studies.

In ex vivo binding experiments, SSR125543A dose dependently prevented the binding of radiolabeled CRF to CRF_1 binding sites in tissue homogenates, suggesting its presence in the pituitary and in the brain, respectively, at 1 and 2 h after oral administration. Moreover, the inhibitory effects of

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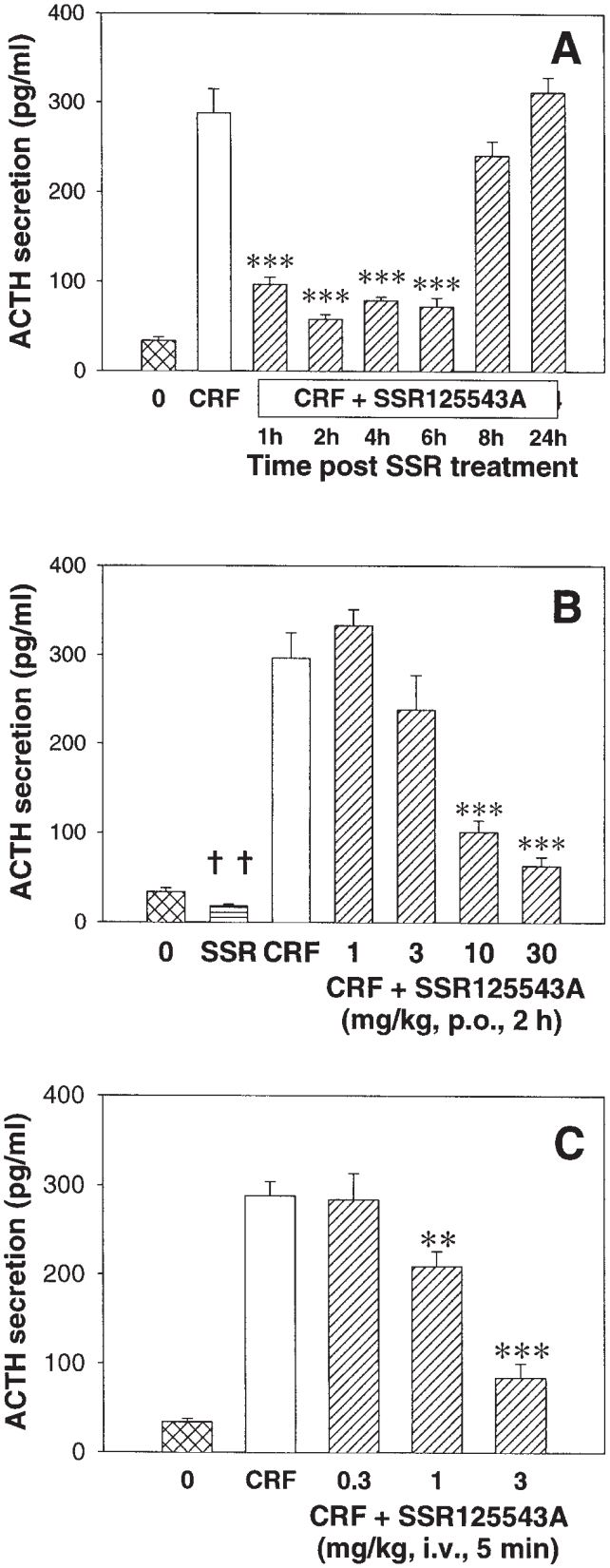

Fig. 5. Effects of SSR125543A on CRF-stimulated ACTH secretion in awake rats. A, time course of the inhibition by SSR125543A of ACTH secretion induced by CRF (4 μg/kg i.v.). The compound was administered orally, at the dose of 30 mg/kg, at various time periods (1, 2, 4, 6, 8, and 24 h) before the CRF challenge. B, dose-dependent inhibition induced by oral administration of SSR125543A on ACTH secretion stimulated by CRF injection (4 μg/kg i.v.). Increasing doses of SSR125543A (1–30 mg/kg) were administered orally 2 h before the CRF challenge. C, dose-dependent inhibition by i.v. injection of SSR125543A of CRF-induced ACTH secretion. Increasing doses of SSR125543A (0.3–3 mg/kg) were administered i.v. 5 min before CRF injection. In both cases, blood samples were collected 30 min after the CRF injection for ACTH plasma levels determination. All results are expressed as means and S.E.M. values of ACTH plasma levels expressed in picograms per milliliter. Control group (receiving only the corresponding vehicles) and SSR125543A group (receiving only the 30-mg/kg dose) are represented, respectively, by column 0 in A, B, and C and column SSR in B. ††, $p < 0.01$ versus control group, and ★★, $p < 0.01$, ★★★, $p < 0.001$ versus CRF group.

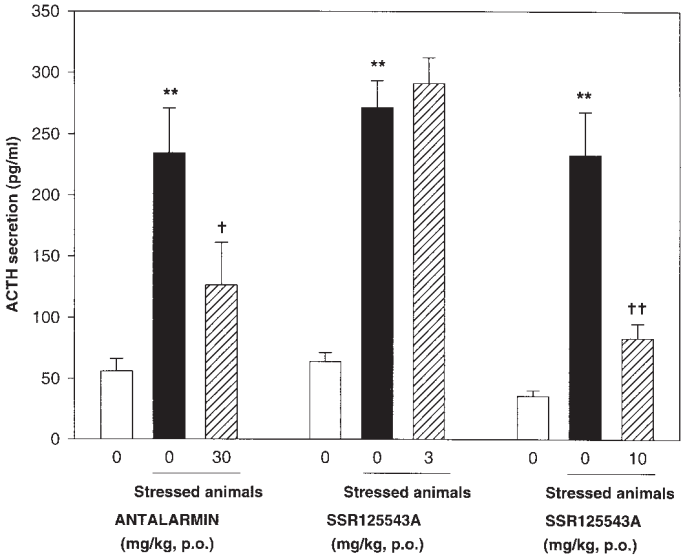

Fig. 6. Effects of SSR125543A and antalarmin on ACTH secretion induced by restraint stress in awake rats. Oral administration of 30 mg/kg antalarmin or 10 mg/kg SSR125543A, 1 h before a 15-min restraint stress in rats prevents the increase in ACTH secretion. At the dose of 3 mg/kg, SSR125543A does not modify the endocrine effect of restraint stress. Results are expressed as means and S.E.M. values of ACTH plasma levels (pg/ml). ★★, $p < 0.01$ versus nonstressed animal (□); †, $p < 0.05$ and ††, $p < 0.01$ versus vehicle-treated stressed rats (■).

30 mg/kg p.o. SSR125543A in both organs was sustained for 24 h.

Growing evidence implicates elevated HPA function and CRF-mediated neurotransmission in human anxiety and depression. Because of its selective stimulation of the corticotrope cells of the pituitary gland, CRF can lead to exacerbated ACTH secretion. In humans, CRF injection elevates both plasma ACTH and cortisol levels, increases the respiratory drive, and results in hypotension and flushing. Currently, the clinical uses of CRF ("CRF challenge") are limited to the diagnosis of HPA dysfunctions (Chrousos et al., 1984). Because the pituitary is located outside the blood-brain barrier, the HPA axis is better activated by systemic rather than by central administration of CRF.

In a similar animal model (Rivier et al., 1982) the effect of SSR125543A on ACTH secretion induced by i.v. injection of CRF was studied under nonstressful conditions in freely moving rats. Treatment with SSR125543A 2 h before CRF injection dose dependently prevented the stimulating effect of CRF on ACTH secretion, regardless of the route of administration (p.o. or i.v.). Furthermore, these results demonstrated that the compound is orally active and are in accordance with the presence of the compound within the pituitary as revealed by the ex vivo binding assays.

The hormonal response to stress is triggered by in-

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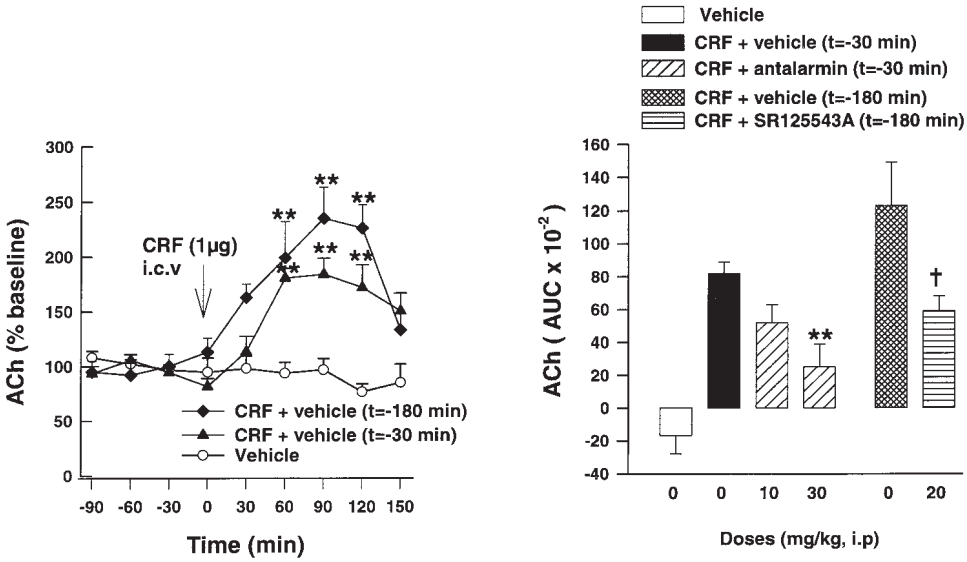

Fig. 7. Effects of SSR125543A and antalarmin on acetylcholine release evoked in rat hippocampus by i.c.v. injection of 1 μg of CRF. A, effect of CRF (1 μg i.c.v.) on hippocampal ACh release. The changes in ACh levels are expressed as the percentage of the mean value of the four basal samples before CRF application. Each data point represents the mean value ± S.E.M of eight to nine animals. ⋆, $p < 0.05$, ⋆⋆, $p < 0.01$ compared with vehicle control group by ANOVA with repeated measures and the Dunnett's t test. B, reversal of CRF-induced ACh release by SSR125543A and antalarmin injected i.p., 180 and 30 min, respectively, before CRF injection. The data are the mean area under the curve ± S.E.M of five to nine animals for the 120 min after CRF injection. ⋆, $p < 0.05$; ⋆⋆, $p < 0.01$; and †, $p < 0.05$ compared with CRF respective control group by ANOVA followed by Student's t test or Dunnett's t test.

TABLE 4
Effects of SSR125543A and antalarmin on CRF (1 μg i.c.v.)-induced forepaw treading in gerbils
Data represent mean ± S.E.M. No forepaw treading was seen in control animals (n = 20) receiving the vehicle alone i.c.v.

Compound	Dose	Cumulative Forepaw Treading Time
	mg/kg	*s*
SSR125543A p.o.	0	33.4 ± 3.8
	10	16.4 ± 4.5*
	30	9.3 ± 1.8*
Antalarmin i.p.	0	29.6 ± 3.9
	10	9.4 ± 3.3*
	30	5.6 ± 2.1*

* $p < 0.05$ versus vehicle with CRF, Mann-Whitney U test with α adjustment of Holm (n = 9–10).

creased CRF secretion from the paraventricular neurons of the hypothalamus into the hypothalamic-pituitary-portal system, leading to enhanced ACTH secretion by pituitary corticotropes and subsequent corticosteroid secretion by the adrenal gland (cortisol in primates and corticosterone in rats) (Owens and Nemeroff, 1991). The above-mentioned changes in hormone secretion are commonly observed in animals under stress conditions. Indeed, ACTH release appears to be a constant feature of the response to most types of stressors (Gibbs, 1984) and changes in pituitary-adrenal tone reflect the intensity of stimulation to which an organism is exposed (Hennessy and Levine, 1978). Restraint and immobilization stress in rats, which requires the animal's cognitive appraisal of the situation, is often considered as a model of psychological stimuli that in turn stimulate the HPA axis (De Souza and Van Loon, 1985). Consequently, a 15-min restraint period has been used to evaluate the ability of SSR125543A to antagonize HPA axis hyperactivity. This stress procedure induces a 10-fold elevation of plasma ACTH levels 13 min after the initiation of stress. Stress-induced ACTH release was antagonized by oral administration of the two CRF_1 receptor antagonists SSR125543A (10 mg/kg) and antalarmin (30 mg/kg), 1 h before the stress session. These results confirm that SSR125543A is able to antagonize the hormonal stimulating effect of endogenously produced CRF in a situation that reproduces the clinical condition seen in patients suffering from affective disorders.

In addition to the hormonal role played by CRF in the activation of the HPA axis, this peptide also behaves as a neurotransmitter and/or neuromodulator in the central nervous system and promotes various physiological and behavioral changes (Dunn and Berridge, 1990; Menzaghi et al., 1993). Moreover, neurochemical studies have demonstrated that acute experimental stress in rats increases the synthesis and release of brain noradrenaline, dopamine, and 5-hydroxytryptamine. Centrally acting CRF increases arousal as defined behaviorally (Sutton et al., 1982) and electroencephalographically (Ehlers et al., 1983). Based on the effects of CRF on cognitive tasks and the fact that pathological stress can be considered as a "toxic" effect of excessive arousal (Hennessy and Levine, 1979), CRF has been proposed to be a positive regulator of cholinergic tone in the CNS. In support of this view, i.c.v. CRF injection has been demonstrated to increase ACh release in the rat hippocampus (Day et al., 1998). Microdialysis experiments showed that i.p. injection of 10 mg/kg SSR125543A, 3 h before i.c.v. CRF challenge, antagonized ACh release evoked by CRF in the rat hippocampus. Similar results were obtained with 30 mg/kg antalarmin administered i.p., 30 min before the peptide.

In addition to an increase in arousal, i.c.v. CRF injection elicits a number of behavioral responses that depend on the testing conditions. For example, the threshold dose of CRF to induce grooming may be related to stress, because rats exposed to a novel environment display increased grooming behavior that habituates with repeated exposure to the same environment (Colbern et al., 1978). When i.c.v. injected in several animal species, CRF elicits a high frequency tremor of the forelimbs called forepaw treading or piano-playing. This behavior is specifically linked to CRF_1 receptor stimulation because it is not seen after i.c.v. urotensin injection (Britton et al., 1984). In gerbils, CRF induces a smaller effect on grooming behavior than in rats, which allows the observation of forepaw treading. Oral administration of SSR125543A and antalarmin before CRF injection was found to significantly and dose dependently (10–30 mg/kg) reduce piano-playing behavior in gerbils.

In summary, SSR125543A is a potent and selective CRF_1 receptor antagonist coming from a new chemical family. It is orally active, has a long duration of action, and readily

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crosses the blood-brain barrier. SSR125543A will be a useful tool for better understanding the role of CRF in anxiety, depression, and cognitive function. As shown in the accompanying article (Griebel et al., 2002), SSR125543A reduces anxiety and depressive-related responses in several animal models, and as such, has a potential in the treatment of depression and some forms of anxiety.

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