0026-895X/02/6105-1132–1139$7.00
MOLECULAR PHARMACOLOGY
Copyright © 2002 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 61:1132–1139, 2002

Vol. 61, No. 5
1246/976855
Printed in U.S.A.

Five Amino Acids of the *Xenopus laevis* CRF (Corticotropin-Releasing Factor) Type 2 Receptor Mediate Differential Binding of CRF Ligands in Comparison with Its Human Counterpart

FRANK M. DAUTZENBERG, JACQUELINE HIGELIN, OLAF BRAUNS, BRIGITTE BUTSCHA, and RICHARD L. HAUGER

Axovan Ltd. Innovation Center, Allschwil, Switzerland (F.M.D., B.B.); F. Hoffmann-La Roche Ltd., Pharma Division, Preclinical Research, Basel, Switzerland (J.H.); Max Planck Institute of Experimental Medicine, Department for Neuroendocrinology, Göttingen, Germany (O.B.); and University of California at San Diego and Veterans Affairs Medical Center, Department of Psychiatry, La Jolla, California (R.L.H.)

Received July 30, 2001; accepted January 24, 2002

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

The ligand selectivity of human ($hCRF_{2A}$) and *Xenopus laevis* ($xCRF_2$) forms of the corticotropin-releasing factor type 2 (CRF_2) receptor differs. The purpose of this study was to identify amino acids in these two CRF_2 receptors conferring these differences. An amino acid triplet in the third extracellular domain ($Asp^{262}Leu^{263}Val^{264}$ in $hCRF_{2A}$ or $Lys^{264}Tyr^{265}Ile^{266}$ in $xCRF_2$) was found to diverge between both receptors. When binding and signaling characteristics of receptor mutants hR2KYI and xR2DLV were assessed, the tri-amino acid motif replacement produced receptors with binding properties resembling the $xCRF_2$ receptor. The converse mutation created a mutant receptor with a binding pharmacology identical to the profile of the $hCRF_{2A}$ receptor. This effect was most notable for xR2DLV, which possessed a binding affinity for astressin ~15-fold greater for astressin than sauvagine. In contrast, the binding profiles of the $hCRF_{2A}$ receptor and hR2KYI did not differ. These data indicate that another domain of the $xCRF_2$ receptor mediated low-affinity binding of astressin. Two amino acids in the first extracellular domain differ in $xCRF_2$ ($Asp^{69}Ser^{70}$) and $hCRF_{2A}$ ($Glu^{66}Tyr^{67}$) receptors. The $hCRF_{2A}$ receptor mutant (hR2DS-KYI) bound astressin with a low affinity indistinguishable from the $xCRF_2$ receptor. Therefore, these data demonstrate that ligand selectivity differences between amphibian and human forms of the CRF_{2A} receptor are governed by these two motifs of the extracellular domains of the $xCRF_2$ receptor.

Corticotropin-releasing factor (CRF), a 41-amino acid peptide originally isolated from hypothalamus (Vale et al., 1981), is the main integrator of the stress response (Dunn and Berridge, 1990; Arborelius et al., 1999; Hauger and Dautzenberg, 1999). Central and peripheral effects of CRF and its structurally related analogs urocortin (UCN) (Vaughan et al., 1995; Donaldson et al., 1996), fish urotensin I (Lederis et al., 1982), and frog sauvagine (Montecucchi and Henschen, 1981) are mediated by their binding and activation of two CRF receptors (CRF_1 and CRF_2), which belong to the class B subfamily of G protein-coupled receptors (Vale et al., 1997). CRF_1 and CRF_2 receptors are ~70% homologous and couple to stimulatory GTP-binding proteins (reviewed in Dautzenberg et al., 2001a). Three biologically active splice variants (CRF_{2A-C}) have been identified for the CRF_2 receptor (see Kilpatrick et al., 1999).

Despite a high degree of sequence homology, the specificity of CRF agonist binding to CRF_1 and CRF_2 proteins differs to a considerable extent. The mammalian CRF_1 receptor nonselectively recognizes CRF, UCN, urotensin I, and sauvagine. These four CRF peptides bind to the CRF_1 receptor with similar degrees of high affinity and equipotently stimulate intracellular cAMP accumulation (Vaughan et al., 1995; Donaldson et al., 1996; Dautzenberg et al., 1997, 1999; Palchaudhuri et al., 1998). In contrast, the *Xenopus laevis* CRF_1 receptor ($xCRF_1$) selectively binds CRF agonists in a highly selective manner whereby human CRF (hCRF), *X. laevis* CRF (xCRF), urotensin I, and rat UCN are recognized with a significantly higher affinity than the structurally related analogs ovine CRF (oCRF) and sauvagine (Dautzenberg et al., 1997).

The mammalian and *X. laevis* CRF_2 receptors display substrate specificities that differ from the mammalian CRF_1 and the $xCRF_1$ receptor (Donaldson et al., 1996; Dautzenberg et al., 1997, 1999; Ardati et al., 1999; Palchaudhuri et al., 1999). The CRF peptides hCRF, oCRF,

R.L.H. was supported by a Veterans Affairs Merit Review grant, the Veterans Affairs Mental Illness Research, Education and Clinical Center (MIRECC) of VISN22, and the National Institute of Mental Health (PHS MH20914-14) Mental Health Clinical Research Center.

ABBREVIATIONS: CRF, corticotropin-releasing factor; aSVG, anti-sauvagine-30; CRF_1, CRF type 1 receptor; CRF_2, CRF type 2 receptor; UCN, urocortin; hCRF, human CRF; oCRF, ovine CRF; xCRF, *X. laevis* CRF; hUCN, human UCN; EC, extracellular domain; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate.

and xCRF bind with significantly lower affinity than UCN, urotensin I, and sauvagine.

The identification of regions forming the binding pocket or being critical for ligand selectivity of the mammalian CRF_1, $xCRF_1$, and human CRF_{2A} receptors has been the subject of various studies (Liaw et al., 1997a,b; Dautzenberg et al., 1998, 1999; Perrin et al., 1998; Wille et al., 1999; Assil et al., 2001). From those studies, it became evident that both receptors use amino acids that are within the extracellular (EC) domains of the receptor or at the interface between the EC domains and the transmembrane helices. The ligand-selective regions of the CRF_1 and CRF_2 receptor are located, however, in different regions of these two proteins. The major determinant for high-affinity ligand binding of the CRF_1 receptor resides in its N-terminal EC1 domain (Perrin et al., 1998; Wille et al., 1999; Assil et al., 2001), whereas the ligand-selective domains of human CRF_{2A} ($hCRF_{2A}$) have been identified in EC2 and at the junction of EC3 and transmembrane 5 (Liaw et al., 1997a,b). Replacement of the amino acids of $hCRF_1$ with residues at equivalent positions of $hCRF_{2A}$ created a mutated receptor that no longer bound hCRF with high affinity (Liaw et al., 1997a). In agreement with these findings, the agonist-selective domains of the $xCRF_2$ receptor have been mapped to regions other than EC1, indicating that this receptor uses domains similar to its human CRF_{2A} counterpart (Dautzenberg et al., 1999). Recently, we reported the first evidence that the binding modes of mammalian CRF_2 receptors and the $xCRF_2$ receptor differed; furthermore, depending on the radioligand used, rank order binding profiles differed (Dautzenberg et al., 2001b). When competition binding experiments were performed with astressin, a nonselective antagonist (Gulyas et al., 1995), this ligand bound to the $xCRF_2$ receptor with an affinity more than 10-fold higher compared with all other CRF radioligands (Dautzenberg et al., 2001b). Because the ligand-selective domains mapped to the $hCRF_{2A}$ receptor are not well conserved in the $xCRF_2$ receptor (Fig. 1), we speculated that one or more of these regions might be responsible for the differential binding profile of the $xCRF_2$ receptor.

In this study, we generated mutated $hCRF_2$ and $xCRF_2$ receptors and tested their binding profile using the radioligands ^{125}I-sauvagine and ^{125}I-astressin. In addition, we compared the ability of astressin and anti-sauvagine-30 (aSVG) to inhibit agonist-mediated cAMP accumulation in HEK293 cells stably transfected with the human and amphibian wild-type or mutant receptors.

Experimental Procedures

Materials, Peptides, and Reagents. All cell culture media and reagents were purchased from Invitrogen (Basel, Switzerland). The CRF peptides (purity, >95%) were obtained from Bachem Corp. (Bubendorf, Switzerland), whereas aSVG (purity, >95%) was synthesized in-house according to a previously published method (Rühmann et al., 1996; Brauns et al., 2001).

Radiochemicals. ^{125}I-Astressin (2200 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA), whereas ^{125}I-Tyr^0-sauvagine (^{125}I-sauvagine; 2000 Ci/mmol) was purchased from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK).

Construction of Mutated Receptors. The residues Glu^{66}Tyr^{67} and Asp^{262}Leu^{263}Val^{264} of the $hCRF_{2A}$ receptor were mutated into the corresponding amino acids of the $xCRF_2$ receptors (Asp^{69}Ser^{70}

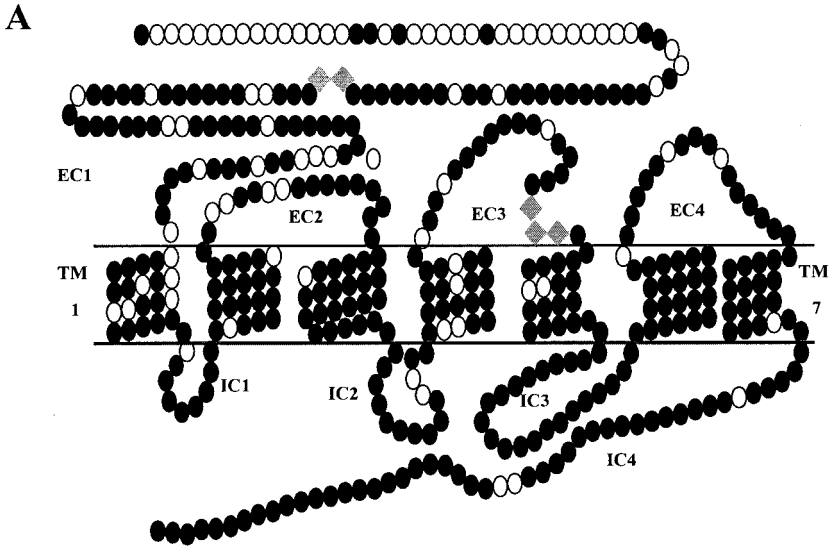

Fig. 1. Two-dimensional model (A) and sequence comparison of the EC3 (B) domain of the $hCRF_{2A}$ and $xCRF_2$ receptors. The conserved amino acids are presented as filled circles, whereas open circles represent divergent residues. The locations of the ligand-selective amino acids of the $hCRF_{2A}$ receptor are highlighted. Sequences surrounding the ligand-selective amino acids in EC3 (B) are shown, and the important triplet of both receptors is highlighted.

and $Lys^{262}Tyr^{263}Ile^{264}$) to create the receptor mutants hR2DS, hR2KYI, and hR2DS-KYI. The residues $Asp^{69}Ser^{70}$ and $Lys^{264}Tyr^{265}Ile^{266}$ of the $xCRF_2$ receptor were mutated to the amino acids occupying these positions in the $hCRF_{2A}$ receptor to generate the xR2EY, xR2DLV, and xR2EY-DLV mutants. Mutagenesis was accomplished using the QuickChange kit (Stratagene, La Jolla, CA) as reported previously (Dautzenberg et al., 1998; Wille et al., 1999). The wild-type and mutant receptors were cloned into the pcDNA3 vector (Invitrogen). Sequences obtained by polymerase chain reaction were verified by DNA sequencing using an ABI 310 DNA sequencer (Applied Biosystems, Weiterstadt, Germany); the GCG software package (Accelrys, Cambridge, UK) was used for analysis.

Cell Transfections and Radioreceptor Binding Assays. cDNAs of $hCRF_{2A}$, $xCRF_2$, and mutated receptors, all inserted into the pcDNA3 vector (2 μg each), were stably transfected into HEK293 cells with the Transfectam reagent (BioSepra, Inc., Villeneuve La Garenne, France) as reported previously (Dautzenberg et al., 2000). Two days after transfection, geneticin selection (500 μg/ml) was initiated, and clones expressing low to moderate receptor levels (500–1200 fmol/mg) were selected.

Membranes from stably transfected HEK293 cells were prepared as described previously (Dautzenberg et al., 1997; Hauger et al., 1997). Scatchard analyses using 0.1 nM ^{125}I-astressin or ^{125}I-sauvagine were performed with 1 to 30 μg of membrane proteins in the scintillation proximity assay format as described previously (Dautzenberg et al., 2000, 2001b). Under these conditions, less than 10% of the total radioactivity was specifically bound by the various receptor constructs. The dissociation constant K_d and the inhibition constant K_i were calculated by the Xlfit program (IDBS, Guildford, UK). Scatchard analyses revealed a one-site model for these four CRF receptors.

cAMP Assays. HEK 293 cells, stably expressing $hCRF_{2A}$, $xCRF_2$, or mutated receptors, were plated at 10,000 to 50,000 cells per well in 96-well dishes. Transfected cells were exposed to CRF peptides for a 10-min stimulation period at 37°C (5% CO_2). During the 10-min incubation period, no desensitization of the cAMP signal was observed. However, longer CRF stimulation periods resulted in considerable desensitization of the cAMP signal, especially at agonist concentrations higher than 100 nM (sauvagine and UCN; unpublished observations). The antagonistic experiments with aSVG and astressin were conducted by simultaneous application of the respective antagonist and sauvagine followed by a 10-min incubation as described above. Measurement of intracellular cAMP levels was performed as described previously (Dautzenberg et al., 2001c). The data were analyzed by two-way analysis of variance, and significance between groups was determined by post hoc analysis using Dunnett's test.

Results

Binding Properties of $hCRF_{2A}$, $xCRF_2$, and Two Mutated CRF_2 Receptors. cDNAs were synthesized to encode the following two different mutations in the EC3 domain of the human and *X. laevis* CRF_2 receptors: hR2KYI (amino acids $Lys^{264}Tyr^{265}Ile^{266}$ of $xCRF_2$) and xR2DLV (residues $Asp^{262}Leu^{263}Val^{264}$ of $hCRF_{2A}$). After these constructs were stably transfected into HEK293 cells, binding profiles using a radiolabeled agonist (^{125}I-sauvagine) and an antagonist (^{125}I-astressin) were determined. Because potential differences in the binding profiles of the mutated receptors could result from differences in G protein-coupling properties (see Kenakin, 1997), we excluded this possibility by assessing binding in the presence of increasing concentrations of GTPγS or Gpp(NH)p. Both GTP analogs potently inhibited ^{125}I-sauvagine binding to the receptor preparations to 56 to 72% (Table 1). In addition, the GTP analog inhibited ^{125}I-sauvagine binding to the CRF_2 receptor preparation with similar affinity (Table 1). In agreement with its antagonist properties, binding of ^{125}I-astressin was not inhibited by Gpp(NH)p and GTPγS (data not shown). Thus, we concluded that the native and mutant CRF_2 receptors exhibited the same degree of coupling to the endogenous Gs proteins.

When ^{125}I-sauvagine was used as the competed ligand, the rank orders of CRF ligand binding differed between the $hCRF_{2A}$ and $xCRF_2$ receptor. The $hCRF_{2A}$ receptor bound hUCN, sauvagine, and aSVG with subnanomolar and astressin with low nanomolar affinity, whereas the K_i values for hCRF and oCRF were markedly higher (Table 2). In contrast to $hCRF_{2A}$, the $xCRF_2$ receptor bound hUCN with subnanomolar and sauvagine and aSVG with low nanomolar affinity (Table 2). As reported previously (Dautzenberg et al., 2001b), when ^{125}I-sauvagine was used as the competed ligand, astressin exhibited an 8-fold lower affinity at the $xCRF_2$ compared with the $hCRF_{2A}$ receptor (Fig. 2A, Table 2). hCRF and oCRF competed for ^{125}I-sauvagine binding to the $xCRF_2$ receptor at high nanomolar concentrations similar to their affinities at the $hCRF_{2A}$ receptor (Table 2). Importantly, mutation of the amino acid triplet ($Lys^{264}Tyr^{265}Ile^{266}$) in the $xCRF_2$ receptor, analogous to the sequence of its human counterpart (Asp-Leu-Val) to create the xR2DLV receptor, converted the $xCRF_2$ binding profile to that of the $hCRF_{2A}$ receptor. The xR2DLV receptor exhibited the following binding: hUCN ~ aSVG ~ sauvagine ~ astressin $\gg$ hCRF $\gg$ oCRF (Table 2). Surprisingly, however, mutation of the ligand-selective amino acids of the EC3 domain of the $hCRF_{2A}$ receptor to the corresponding sequence of its amphibian counterpart to form the hR2KYI mutant failed to replicate the full binding profile of the $xCRF_2$ receptor when ^{125}I-sauvagine was used as the competed ligand. Although the hR2KYI mutant, similar to the $xCRF_2$ receptor, bound sauvagine and aSVG with ~6-fold lower affinity than hUCN, the affinity for astressin was ~17-fold higher at the hR2KYI receptor compared with the $xCRF_2$ receptor (Fig. 2A; Table 2).

When ^{125}I-astressin was used as the radioligand instead of

TABLE 1
Inhibition of ^{125}I-sauvagine binding to $hCRF_{2A}$, $xCRF_2$, and two mutated CRF_2 receptors by GTPγS and Gpp(NH)p
The data are means ± S.E.M. from two different binding experiments performed in triplicate using ^{125}I-sauvagine as radioligand.

Receptor	GTPγS		Gpp(NH)p	
	IC_{50}	I_{max}	IC_{50}	I_{max}
	nM	%	*nM*	%
$hCRF_{2A}$	28 ± 5	58 ± 5	188 ± 49	56 ± 8
hR2KYI	36 ± 8	67 ± 7	204 ± 33	64 ± 7
xR2DLV	22 ± 10	61 ± 5	218 ± 51	58 ± 4
$xCRF_2$	26 ± 3	64 ± 6	179 ± 25	61 ± 7

^{125}I-sauvagine, substantial binding differences were observed for the two native and the mutated receptors. For the $hCRF_{2A}$ receptor, significant rightward shifts of the dose-response curves were observed for all agonists except hUCN, which retained affinity <1 nM (Table 3). In contrast, using ^{125}I-astressin, binding affinities of antagonists to the $hCRF_{2A}$ receptor were only minimally affected (Fig. 2B; Table 3). When these agonists and antagonists competed with ^{125}I-astressin at the hR2KYI receptor, the binding profile resembled the $hCRF_{2A}$ profile (Fig. 2B; Table 3). Conversely, the hR2KYI mutant produced the same binding profile as the $xCRF_2$ receptor when ^{125}I-astressin was used as the competed radioligand: hUCN ~ astressin > sauvagine > aSVG > hCRF ≫ oCRF (Table 3). Notably, hCRF was bound with an affinity ~2-fold higher at the hR2KYI and $xCRF_2$ receptors compared with the xR2DLV and $hCRF_{2A}$ receptors when ^{125}I-astressin instead of ^{125}I-sauvagine was the competed radioligand. However, the affinities for sauvagine and aSVG binding to the hR2KYI and $xCRF_2$ receptors were decreased ~6- to 7-fold when the radioligand was the antagonist astressin. The only difference between the $xCRF_2$ and hR2KYI receptor was the binding profile of astressin, which was not affected by the radioligand in the case of the hR2KYI receptor but was 15-fold better at the $xCRF_2$ receptor compared with its affinity to compete for ^{125}I-sauvagine binding (Fig. 2; Tables 2 and 3).

Binding Affinities of Human and Amphibian CRF_2 Receptors Carrying Point Mutations in the EC1 or EC1/EC3 Domains. Mutations in the EC3 domain did not completely reverse the differential binding profiles of the $hCRF_{2A}$ and $xCRF_2$ receptor. Consequently, experiments were performed to compare binding affinities for ligands at human and amphibian CRF_2 receptors carrying point mutations restricted to the EC1 domain or a combination of EC1 and EC3 mutations. Because a two-amino acid motif in the ligand-selective domain of the $xCRF_1$ receptor differs in $hCRF_{2A}$ ($Glu^{66}Tyr^{67}$) and $xCRF_2$ ($Asp^{69}Ser^{70}$) receptors (Dautzenberg et al., 1998), the following four mutated receptors were constructed: hR2DS (EC1 mutation), hR2DS-KYI (EC1/EC3 mutation), xR2EY (EC1 mutation), and xR2EY-DLV (EC1/EC3 mutation). After these mutated receptors were stably transfected into HEK293 cells, their binding properties were characterized using ^{125}I-sauvagine as the radioligand.

The EC1 mutants hR2DS and xR2EY retained the pharmacology of their respective wild-type receptors (Table 4). The rank order of binding affinities for the hR2DS mutant (aSVG ~ hUCN ~ sauvagine > astressin > hCRF ≫ oCRF) was identical to the native $hCRF_{2A}$ receptor (Table 4). The xR2EY mutant displayed a binding rank order (hUCN > aSVG ~ sauvagine > astressin ≫ hCRF > oCRF) identical to the $xCRF_2$ receptor (Tables 2 and 4). In contrast, binding profiles of EC1/EC3 mutants hR2DS-KYI and xR2EY-DLV were shifted completely compared with profiles of the wild-type receptors (Fig. 3). The rank order for the human CRF_{2A} receptor mutant hR2DS-KYI was indistinguishable from the rank order for the $xCRF_2$ receptor (Table 4). Conversely, rank orders for the $xCRF_2$ receptor mutant xR2EY-DLV and the $hCRF_{2A}$ receptor were identical (Table 4). Importantly, similar to binding data for the $hCRF_{2A}$, $xCRF_2$, hR2KYI, and xR2DLV (Table 3), hR2DS, hR2DS-KYI, xR2EY, and xR2EY-DLV bound astressin with equal affinity when ^{125}I-astressin was the competed ligand (Fig. 3).

Stimulation of cAMP Accumulation in HEK293 Cells Expressing $hCRF_{2A}$, $xCRF_2$, and Mutated Receptors. The ability of CRF agonists to stimulate cAMP accumulation was assessed in HEK293 cells stably expressing native or mutated CRF_2 receptors. A similar potency rank order (sauvagine > UCN > hCRF > oCRF) was observed among the four receptors (Table 5). Because sauvagine stimulated intracellular cAMP accumulation with the greatest potency, we assessed the inhibitory effects of the nonselective CRF receptor antagonist astressin and the selective CRF_2 receptor antagonist aSVG on cAMP stimulation produced by 1 nM sauvagine, which is a concentration slightly above the EC_{50} value observed in the four receptor lines (Table 5). When cells expressing the four different CRF_2 receptor proteins were coincubated with 100 nM astressin or aSVG, the sauvagine-stimulated cAMP accumulation was significantly reduced in the four receptor lines, indicating antagonist actions (Figs. 4 and 5).

Next, Schild plots were generated for the antagonist potencies of astressin and aSVG on sauvagine-stimulated cAMP accumulation in the four receptor lines. Astressin and aSVG behaved as competitive antagonists at all CRF_2 receptor-expressing lines (Fig. 5; Table 6). The two antagonists differed, however, in their inhibitory potencies. Astressin markedly inhibited sauvagine-stimulated cAMP accumulation in cells expressing $hCRF_{2A}$, hR2DS, hR2KYI, xR2DLV, and xR2EY-DLV receptors. When Schild plots were calculated for hR2DS-KYI, xR2EY, and $xCRF_2$-expressing cells, 5- to 6-fold higher concentrations of astressin were required to shift the sauvagine dose-response curve to the right (Table 6). In contrast, aSVG was a more potent antagonist than astressin at the $hCRF_{2A}$, hR2DS, xR2DLV, and xR2EY-DLV receptors. However, in HEK293 cells expressing hR2KYI,

TABLE 2
Binding properties of $hCRF_{2A}$, $xCRF_2$, and the two receptor mutants hR2KYI and xR2DLV using ^{125}I-sauvagine as radioligand
The data are means ± S.E.M. from at least three different binding experiments performed in triplicate using ^{125}I-sauvagine as radioligand.

Peptide	$hCRF_{2A}$	hR2KYI	xR2DLV	$xCRF_2$
		K_i, nM		
hCRF	59.1 ± 5.3[a]	107 ± 5[a]	52.5 ± 5.5[a]	129 ± 3[a]
oCRF	242 ± 11[b]	294 ± 48[b]	177 ± 21[b]	490 ± 24[b]
hUCN	0.29 ± 0.04	0.51 ± 0.04	0.57 ± 0.04	0.42 ± 0.06
Sauvagine	0.46 ± 0.04	2.94 ± 0.55	0.84 ± 0.03	2.31 ± 0.39
Astressin	1.51 ± 0.24	0.85 ± 0.07	1.00 ± 0.05	13.7 ± 2.3[c]
aSVG	0.24 ± 0.03	3.14 ± 0.73	0.46 ± 0.02	3.31 ± 0.53

[a] $p < 0.0001$ vs. hUCN, sauvagine, astressin, and aSVG.
[b] $p < 0.0001$ vs. hCRF, hUCN, sauvagine, astressin, and aSVG.
[c] $p < 0.05$ vs. $hCRF_{2A}$, hR2KYI, and xR2DLV.

hR2DS-KYI, xR2EY, or $xCRF_2$ receptors, aSVG at a 10-fold higher concentration than used in the other receptor lines was required to inhibit sauvagine-stimulated cAMP accumulation (Table 6).

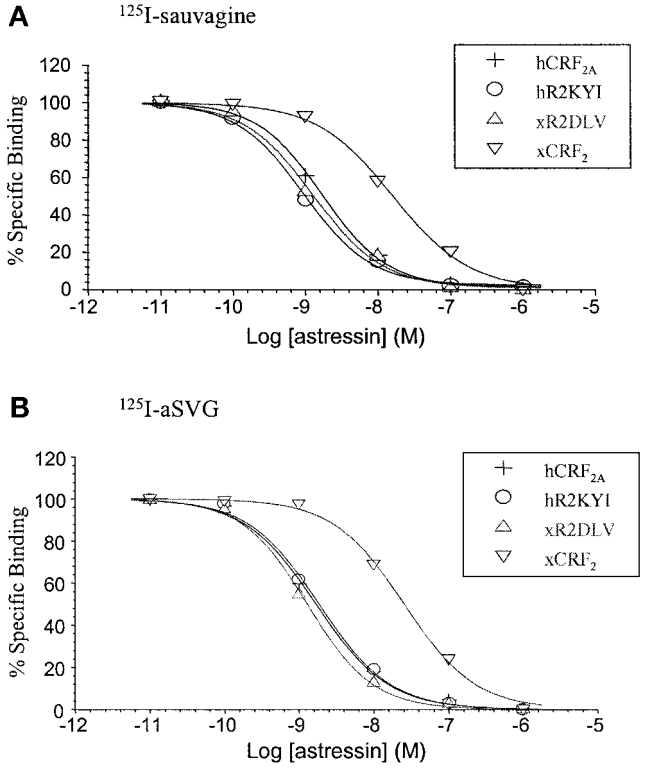

Fig. 2. Competitive binding of astressin to the $hCRF_{2A}$, $xCRF_2$, hR2KYI, and xR2DLV receptors in the presence of either ^{125}I-sauvagine (A) or ^{125}I-astressin (B) as radioligands. Membranes were incubated with increasing concentrations of the CRF antagonists for 120 min at 22°C. The results are representative of three independent binding experiments.

Discussion

The purpose of this study was to identify the residues of the $xCRF_2$ receptor that govern its higher degree of binding selectivity compared with its human counterpart (Dautzenberg et al., 2001). Although the mammalian and amphibian CRF_1 receptor use amino acids located in the N-terminal EC1 domain for high-affinity ligand binding and substrate recognition (Dautzenberg et al., 1998; Perrin et al., 1998; Wille et al., 1999; Assil et al., 2001), the $hCRF_{2A}$ and $xCRF_2$ receptor were reported to use different exofacial domains. For the $hCRF_{2A}$ receptor, three regions within the EC2 and EC3 domain of this receptor have been identified to be critical for selective binding of and activation by CRF agonists (Liaw et al., 1997a). In our recent study, similar regions in EC2 and EC3 seemed to confer its agonist selectivity (Dautzenberg et al., 1999).

Interestingly, the amino acid motifs that probably mediate the ligand selectivity of $hCRF_{2A}$ are not well conserved between the human and *X. laevis* receptors (Dautzenberg et al., 1997, 1999). Moreover, we have recently shown that a histidine residue reported to be located at position 185 and to play a crucial role for the binding specificity of $hCRF_{2A}$ (Liaw et al., 1997a) most likely represents a polymerase chain reaction artifact. Instead, our sequencing experiments identified an arginine residue (Arg^{185}), located at position 185 of the $hCRF_{2A}$ cDNAs, isolated from a variety of tissues, which is conserved in the $hCRF_{2A}$ gene as well as other vertebrate CRF_1 and CRF_2 receptors (Dautzenberg et al., 2000). Furthermore, the second important domain for the ligand selectivity of the $hCRF_{2A}$ receptor, residues $Val^{172}Asp^{173}His^{174}$ (Liaw et al., 1997a), are almost identical to the equivalent region of the $xCRF_2$ receptor ($Ile^{174}Asp^{175}His^{176}$) (Dautzenberg et al., 1997).

The above findings led us to first focus our investigation on

TABLE 3

Binding properties of $hCRF_{2A}$, $xCRF_2$, and the receptor mutants hR2KYI and xR2DLV using ^{125}I-astressin as radioligand

The data are mean K_i values ± S.E.M. from at least three different binding experiments performed in triplicate using ^{125}I-sauvagine as radioligand.

Peptide	$hCRF_{2A}$	hR2KYI	xR2DLV	$xCRF_2$
		nM		
hCRF	125 ± 19[a]	57.7 ± 8.2[a]	166 ± 17[a]	69.8 ± 5.1[a]
oCRF	949 ± 108[b]	416 ± 11[b]	1020 ± 58[b]	604 ± 80[b]
hUCN	0.79 ± 0.05	1.36 ± 0.19	1.14 ± 0.09	0.29 ± 0.06
Sauvagine	26.6 ± 2.9	20.6 ± 1.8	24.7 ± 3.1	14.5 ± 2.3
Astressin	1.26 ± 0.04	1.33 ± 0.09	0.99 ± 0.07	0.89 ± 0.15
aSVG	1.94 ± 0.24	17.4 ± 0.8[c]	1.37 ± 0.15	24.6 ± 3.3[c]

[a] $p < 0.0001$ vs. hUCN, sauvagine, astressin, and aSVG.
[b] $p < 0.0001$ vs. hCRF, hUCN, sauvagine, astressin, and aSVG.
[c] $p < 0.005$ vs. $hCRF_{2A}$ and xR2DLV.

TABLE 4

Binding properties of $hCRF_{2A}$, $xCRF_2$, and the two receptor mutants hR2KYI and xR2DLV using ^{125}I-sauvagine as radioligand

The data are mean K_i values ± S.E.M. from at least three different binding experiments performed in triplicate using ^{125}I-sauvagine as radioligand.

Peptide	hR2DS	hR2DS-KYI	xR2EY	xR2EY-DLV
		nM		
hCRF	44.9 ± 3.1[a]	134 ± 25[a]	151 ± 29[a]	52.5 ± 5.5[a]
oCRF	198 ± 19[b]	306 ± 61[b]	405 ± 39[b]	177 ± 21[b]
hUCN	0.36 ± 0.09	0.44 ± 0.05	0.36 ± 0.04	0.57 ± 0.04
Sauvagine	0.63 ± 0.11	2.94 ± 0.55	2.01 ± 0.42	0.84 ± 0.03
Astressin	1.76 ± 0.32	19.8 ± 2.1[c]	13.1 ± 2.2[c]	1.00 ± 0.05
aSVG	0.19 ± 0.02	2.12 ± 0.67	3.93 ± 0.69	0.18 ± 0.05

[a] $p < 0.002$ vs. hUCN, sauvagine, astressin, and aSVG.
[b] $p < 0.0001$ vs. hCRF, hUCN, sauvagine, astressin, and aSVG.
[c] $p < 0.02$ vs. hR2DS and xR2EY-DLV.

the third ligand-selective domain, residues $Asp^{262}Leu^{263}Val^{264}$ of the $hCRF_{2A}$ receptor (Liaw et al., 1997a). The equivalent domain of the $xCRF_2$ receptor, residues $Lys^{264}Tyr^{265}Ile^{266}$ (Dautzenberg et al., 1997), differ strongly from its human counterpart.

Indeed, mutagenesis of these three amino acids altered the binding pharmacology of the two mutants. The xR2DLV mutant, encoding the residues of the $hCRF_{2A}$ receptor, displayed the same binding preferences with both radioligands, ^{125}I-sauvagine and ^{125}I-astressin, as the human receptor. For the human mutant hR2KYI, the presence of the $Lys^{264}Tyr^{265}Ile^{266}$ triplet of the $xCRF_2$ receptor resulted in a pharmacology profile closely resembling the amphibian CRF_2 receptor. The largest differences in binding-affinity profiles were observed for the agonists hCRF and sauvagine and for the antagonists aSVG and astressin. Interestingly, the hR2KYI mutant and the $xCRF_2$ receptor bound hCRF with low affinity when ^{125}I-sauvagine was the competed radioligand. For the xR2DLV and $hCRF_{2A}$ receptor, a low binding affinity for hCRF was observed in the presence of ^{125}I-astressin, consistent with the concept that binding of an agonist is reduced in the presence of a radiolabeled antagonist (Sleight et al., 1996; Dautzenberg et al., 2001b). Furthermore, sauvagine binding in the presence of ^{125}I-sauvagine occurred with a lower affinity to the $xCRF_2$ and hR2KYI receptor compared with other ligands, whereas sauvagine bound with high affinity to the $hCRF_{2A}$ and xR2DLV receptor. However, in the presence of ^{125}I-astressin, the agonist showed no differences in binding to the four receptors. The CRF_2-selective antagonist aSVG, like sauvagine, bound with a lower affinity to the $xCRF_2$ and hR2KYI receptor than to the $hCRF_{2A}$ receptor and the xR2DLV mutant. However, unlike classical antagonists, which bind independent of the agonistic or antagonistic nature of the competed radioligand (Sleight et al., 1996; Perrin et al., 1999), the affinity of aSVG was shifted to the right with all four receptors. This effect was strongest for the $xCRF_2$ receptor and hR2KYI mutant, which showed only a moderate affinity of ~20 nM for the antagonist. This unusual behavior for a receptor antagonist suggests that aSVG binding selectively depends on the agonistic or antagonistic nature of the competed radioligand. Nevertheless, both the nonradioactive and the iodinated form of the peptide have been shown to behave as antagonists (Rühmann et al., 1998; Higelin et al., 2001).

Notably, the mutagenesis approach within the EC3 domain did not unravel completely the unusual binding profile of astressin to the $xCRF_2$ receptor. Although incorporation of the amino acid triplet Asp-Leu-Val into the $xCRF_2$ receptor created a mutant with binding preferences indistinguishable from that of the $hCRF_{2A}$ receptor, the converse result was not obtained when the corresponding human sequence was replaced by the triplet Lys-Tyr-Ile in the hR2KYI mutant. This mutant bound astressin with high affinity in the presence of ^{125}I-sauvagine and thus differed from the $xCRF_2$ receptor, which showed a lower affinity for astressin when ^{125}I-sauvagine was the competed radioligand (Dautzenberg et al., 2001b).

Thus, we concluded that an unidentified region of the $xCRF_2$ receptor interacts with the amino acid triplet $Lys^{264}Tyr^{265}Ile^{266}$ to negatively regulate astressin binding. Similarly, a recent study demonstrated that the binding of astressin differed from CRF and UCN at rat CRF_1 receptor (Perrin et al., 1998). Although astressin binding to the rat

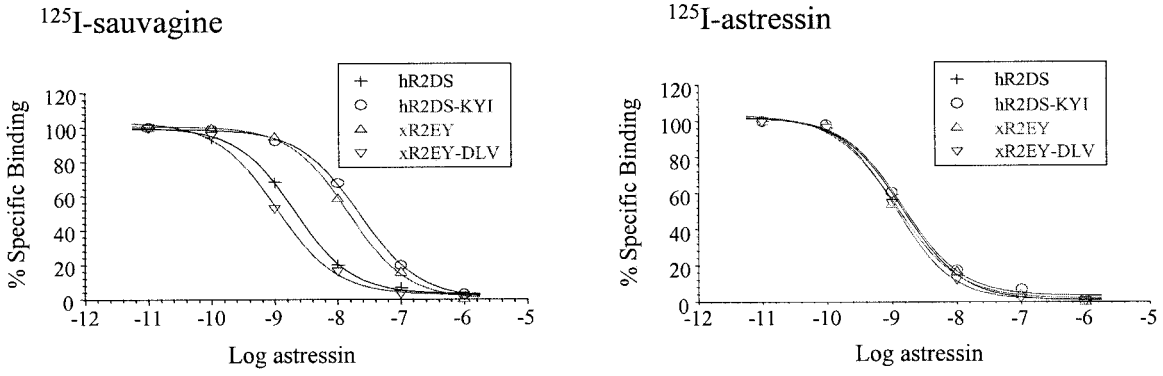

Fig. 3. Competitive binding of astressin to the mutated CRF_2 receptors hR2DS, hR2DS-KYI, xR2EY, and xR2EY-DLV using ^{125}I-sauvagine as the radioligand. Membranes were incubated with increasing concentrations of the CRF antagonists for 120 min at 22°C. The results are representative of three independent binding experiments.

TABLE 5

Stimulation of cAMP production in HEK293 cells, stably transfected with cDNAs coding for $hCRF_{2A}$, $xCRF_2$, or mutated CRF_2 receptors

The data are mean EC_{50} values ± S.E.M. of at least three cAMP stimulations.

Receptor	hCRF	oCRF	hUCN	Sauvagine
		nM		
$hCRF_{2A}$	7.95 ± 0.24[a]	28.7 ± 2.1[b]	1.95 ± 0.31	0.37 ± 0.09
hR2DS	8.63 ± 0.48[a]	30.1 ± 2.9[b]	2.43 ± 0.29	0.31 ± 0.03
hR2KYI	10.1 ± 0.9[a]	31.5 ± 1.6[b]	2.69 ± 0.44	0.29 ± 0.06
hR2DS-KYI	9.01 ± 0.91[a]	32.0 ± 3.9[b]	3.01 ± 0.51	0.34 ± 0.12
xR2EY	7.91 ± 0.26[a]	27.1 ± 1.4[b]	2.16 ± 0.19	0.26 ± 0.02
xR2DLV	9.09 ± 0.56[a]	30.3 ± 3.1[b]	2.91 ± 0.41	0.38 ± 0.03
xR2EY-DLV	8.98 ± 0.64[a]	31.9 ± 4.2[b]	2.47 ± 0.23	0.22 ± 0.01
$xCRF_2$	9.65 ± 0.46[a]	29.9 ± 3.6[b]	1.88 ± 0.16	0.25 ± 0.04

[a] $p < 0.005$ vs. hUCN and sauvagine.
[b] $p < 0.0001$ vs. oCRF, hUCN and sauvagine.

CRF_1 receptor only requires the EC1 domain, the EC4 domain is also needed for binding of CRF and UCN (Perrin et al., 1998). Furthermore, a microheterogeneity of agonist binding to the $hCRF_{2A}$ receptor was also reported (Liaw et al., 1997b). Although introduction of the ligand-selective regions of the $hCRF_{2A}$ receptor into the sequence of the $hCRF_1$ receptor strongly impaired CRF and sauvagine binding, UCN binding was insensitive to this mutation (Liaw et al., 1997b). These findings are not only restricted to the CRF receptor system because a similar observation was reported for another member of the class B G protein-coupled receptor subfamily, the parathyroid hormone receptor, which shows involvement of different amino acids for the binding of different parathyroid hormone variants (Lee et al., 1995).

We focused our search for the additional extracellular region that impairs binding of astressin to the $xCRF_2$ receptor on the N-terminal EC1 domain. Recently, we identified an amino acid doublet within the ligand-binding site of the mammalian CRF_1 receptor (Wille et al., 1999; Assil et al., 2001) in a parallel study investigating the site for astressin binding to the $xCRF_1$ receptor (S. Wille, J. Higelin, and F. M. Dautzenberg, manuscript in preparation). This doublet is conserved in $xCRF_1$ ($Glu^{70}Tyr^{71}$) and $hCRF_{2A}$ ($Glu^{66}Tyr^{67}$) but diverges in $hCRF_1$ ($Ala^{70}Phe^{71}$) and the $xCRF_2$ receptor ($Asp^{69}Ser^{70}$). Interestingly, mutagenesis of this amino acid doublet did not alter binding of astressin to the hR2DS and xR2EY receptors. In contrast, replacement of EC1/EC3 residues of the $hCRF_{2A}$ receptor with the corresponding *X. laevis* motifs resulted in a $xCRF_2$ pharmacology. Likewise, replacement of EC1/EC3 residues of the $xCRF_2$ receptor with the corresponding human motifs shifted its ligand rank order to an hCRF2A profile.

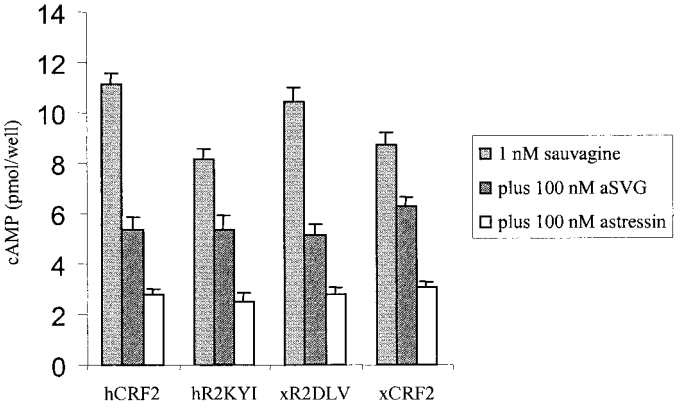

Fig. 4. Inhibition of sauvagine-mediated cAMP accumulation in HEK293 cells stably expressing the $hCRF_{2A}$, $xCRF_2$, hR2KYI, or xR2DLV receptors by aSVG astressin. Cells were incubated in the presence or absence of 1 nM sauvagine with or without 10 nM antagonist for 10 min at 37°C. Each bar represents the mean ± S.E.M. of three independent stimulation experiments conducted in quadruplicate.

Rank orders for sauvagine-stimulated cAMP accumulation resembled rank orders obtained with binding studies where ^{125}I-sauvagine was used as the radioligand. Although astressin and aSVG were highly potent and competitive antagonists at the $hCRF_{2A}$, hR2DS, xR2DLV, and xR2EY-DLV receptors, both antagonists were significantly less potent at the hR2DS-KYI, $xCRF_2$, and xR2EY receptors. However, astressin was a more potent antagonist than aSVG in hR2KY-expressing cells in agreement with astressin possessing a higher binding affinity than aSVG at the $hCRF_{2A}$ receptor. Thus, the binding properties of both antagonists correspond closely when ^{125}I-sauvagine rather than ^{125}I-astressin is used as the radioligand. Furthermore, these data demonstrate that the binding pocket of $xCRF_2$ for astressin differs from its binding pockets for agonists and other antagonists. Importantly, the slopes for the antagonism of aSVG and astressin of sauvagine-stimulated cAMP accumulation were ~1. Although our aSVG data agree with the findings of a recent study (Brauns et al., 2001), the slopes for astressin

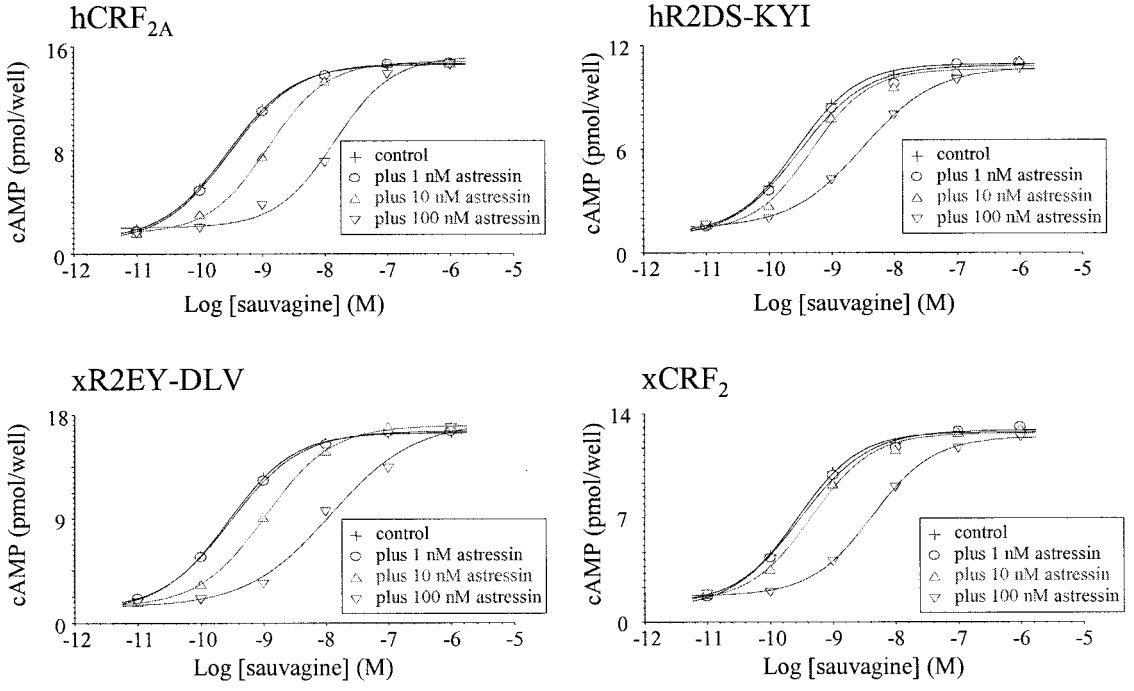

Fig. 5. Antagonist properties of astressin with the two natural CRF_2 receptors $hCRF_{2A}$ and $xCRF_2$ and two mutated receptors. Full dose-response curves for sauvagine-mediated cAMP accumulation were conducted in the presence of increasing concentrations (1–100 nM) of astressin. Data represent triplicates of one representative experiment repeated three times.

TABLE 6

Functional antagonism of the agonist potency of sauvagine by astressin and aSVG on HEK293 cells stably expressing the $hCRF_{1A}$, $xCRF_2$, and mutated receptors

The data are means ± S.E.M. of three cAMP stimulation experiments performed in duplicate using sauvagine as agonist.

Receptor	Astressin	aSVG
	pA_2	
$hCRF_{2A}$	8.39 ± 0.05	9.04 ± 0.04
hR2DS	8.22 ± 0.09	9.11 ± 0.02
hR2KYI	8.24 ± 0.02	7.94 ± 0.12
hR2DS-KYI	7.61 ± 0.08[a]	8.06 ± 0.06
xR2EY	7.66 ± 0.04[a]	7.86 ± 0.03
xR2DLV	8.21 ± 0.03	8.84 ± 0.12
xR2EY-DLV	8.30 ± 0.05	9.08 ± 0.13
$xCRF_2$	7.70 ± 0.11[a]	7.91 ± 0.08

[a] Statistically significant differences: $p < 0.0001$ vs. $hCRF_{2A}$, hR2DS, hR2KYI, xR2DLV, and xR2EY-DLV; $p < 0.0001$ vs. $hCRF_{2\alpha}$, hR2DS, xR2DLV, and xR2EY-DLV. Note: Schild analyses revealed slopes for astressin- and aSVG-mediated cAMP inhibition close to unity (0.8–1.3) at all receptors.

antagonism differ. Because our transfected cell lines express CRF_2 receptors at a level significantly lower than the CRF_2 receptor expression level used by Brauns et al. (2001), our data may reflect more native cell lines endogenously expressing CRF receptors.

In conclusion, our site-directed mutagenesis experiments have identified two important regions mediating the differential binding of CRF analogs to the amphibian CRF_2 receptor: a) an amino acid triplet $Lys^{264}Tyr^{265}Ile^{266}$ in the EC3 domain and b) a two-amino acid motif, $Asp^{69}Ser^{70}$, in the EC1 domain of the $xCRF_2$ receptor. Replacement of this region by the corresponding amino acids of the $hCRF_{2A}$ receptor ($Glu^{66}Tyr^{67}$ and $Asp^{262}Leu^{263}Val^{264}$) generated a mutant with a binding pharmacology indistinguishable from that of the $hCRF_{2A}$ receptor. The converse replacement of this region in the human CRF_{2A} receptor with the corresponding *X. laevis* sequence shifted the binding profile to that of the $xCRF_2$ receptor. Finally, antagonism of sauvagine-stimulated cAMP accumulation by astressin and aSVG followed competitive binding data using ^{125}I-sauvagine as a radioligand. Therefore, microheterogeneity within the ligand-binding pocket seems to be present in the amphibian CRF_2 receptor.

References

Arborelius L, Owens MJ, Plotsky PM, and Nemeroff CB (1999) The role of corticotropin-releasing factor in depression and anxiety disorders. *J Endocrinol* **160:**1–12.

Ardati A, Goetschy V, Gottowick J, Henriot S, Valdenaire O, Deuschle U, and Kilpatrick GJ (1999) Human CRF_2 α and β splice variants: pharmacological characterization using radioligand binding and a luciferase gene expression assay. *Neuropharmacology* **38:**441–448.

Assil IQ, Qi LJ, Arai M, Shomali M, and Abou-Samra AB (2001) Juxtamembrane region of the amino terminus of the corticotropin releasing factor receptor type 1 is important for ligand interaction. *Biochemistry* **40:**1187–1195.

Brauns O, Liepold T, Radulowic J, and Spiess J (2001) Pharmacological and chemical properties of astressin, antisauvagine-30 and alpha-helCRF: significance for behavioral experiments. *Neuropharmacology* **41:**507–516.

Dautzenberg FM, Dietrich K, Palchaudhuri MR, and Spiess J (1997) Identification of two corticotropin-releasing factor receptors with high ligand selectivity from *Xenopus laevis*: unusual pharmacology of the type 1 receptor. *J Neurochem* **69:**1640–1649.

Dautzenberg FM, Huber G, Higelin J, Py-Lang G, and Kilpatrick GJ (2000) Evidence for the abundant expression of arginine 185 containing human $CRF_{2\alpha}$ receptors and the role of position 185 for receptor-ligand selectivity. *Neuropharmacology* **39:**1368–1376.

Dautzenberg FM, Kilpatrick GJ, Hauger RL, and Moreau J-L (2001a) Molecular biology of the CRH receptors–in the mood. *Peptides* **22:**753–760.

Dautzenberg FM, Kilpatrick GJ, Wille S, and Hauger RL (1999) The ligand-selective domains of corticotropin-releasing factor type 1 and type 2 receptors reside in different extracellular domains: generation of chimeric receptors with a novel ligand-selective profile. *J Neurochem* **73:**821–829.

Dautzenberg FM, Py-Lang G, Higelin J, Fischer C, Wright MB, and Huber G (2001b) Different binding modes of amphibian and human CRF type 1 and type 2 receptors: evidence for evolutionary differences. *J Pharmacol Exp Ther* **296:**113–120.

Dautzenberg FM, Wichmann J, Higelin J, Py-Lang G, Kratzeisen C, Malherbe P, Kilpatrick G, and Jenck F (2001c) Pharmacological characterization of the novel non-peptide orphanin FQ/nociceptin receptor agonist Ro-64–6198: rapid and reversible desensitization of the ORL1 receptor *in vitro* and lack of tolerance *in vivo*. *J Pharmacol Exp Ther* **298:**812–819.

Dautzenberg FM, Wille S, Lohmann R, and Spiess J (1998) Mapping of the ligand-selective domain of the *Xenopus laevis* corticotropin-releasing factor receptor 1: implications for the ligand-binding site. *Proc Natl Acad Sci USA* **95:**4941–4945.

Donaldson C, Sutton S, Perrin MH, Corrigan AZ, Lewis KA, Rivier J, Vaughan JM, and Vale WW (1996) Cloning and characterization of human urocortin. *Endocrinology* **137:**2167–2170.

Dunn AJ and Berridge CW (1990) Physiological and behavioral responses of corticotropin releasing factor administration: is CRF a mediator of anxiety or stress responses? *Brain Res Rev* **15:**71–100.

Gulyas J, Rivier C, Perrin M, Koerber C, Sutton S, Corrigan A, Lahrichi SL, Craig W, Vale W, and Rivier J (1995) Potent, structurally constrained agonists and competitive antagonists of corticotropin-releasing factor. *Proc Natl Acad Sci USA* **92:**10575–10579.

Hauger RL and Dautzenberg FM (1999) Regulation of the stress response by corticotropin-releasing factor receptors, in *Neuroendocrinology in Physiology and Medicine* (Conn PM and Freedman ME, eds.) pp. 261–286, Humana Press Inc., Totowa, NJ.

Hauger RL, Dautzenberg FM, Flaccus A, Liepold T, and Spiess J (1997) Regulation of corticotropin-releasing factor receptor function in human Y-79 retinoblastoma cells: rapid and reversible homologous desensitization but prolonged recovery. *J Neurochem* **6:** 2308–2316.

Higelin J, Py-Lang G, Paternoster C, Ellis G, Patel A, and Dautzenberg FM (2001) ^{125}I-Antisauvagine-30 a novel and specific high-affinity radioligand for the characterization of corticotropin-releasing factor type 2 receptors. *Neuropharmacology* **40:**114–122.

Kenakin T (1997) Differences between natural and recombinant G protein-coupled receptor systems with varying receptor/G protein stoichiometry. *Trends Pharmacol Sci* **18:**456–464.

Kilpatrick GJ, Dautzenberg FM, Martin GR, and Eglen RM (1999) G-protein coupled receptors–the splicing on the cake. *Trends Pharmacol Sci* **20:**294–301.

Lederis K, Letter A, McMaster D, and Moore G (1982) Complete amino acid sequence of urotensin-I, a hypotensive and corticotropin-releasing neuropeptide from *Catostomus*. *Science (Wash DC)* **218:**162–164.

Lee CW, Luck MD, Jüppner H, Potts JT Jr, Kronenberg HM, and Gardella TJ (1995) Homolog-scanning mutagenesis of the parathyroid hormone (PTH) receptor reveals PTH-(1–34) binding determinants in the third extracellular loop. *Mol Endocrinol* **9:**1269–1278.

Liaw CW, Grigoriadis DE, Lorang MT, De Souza EB, and Maki RA (1997b) Localization of agonist- and antagonist-binding domains of human corticotropin releasing factor receptors. *Mol Endocrinol* **11:**2048–2053.

Liaw CW, Grigoriadis DE, Lovenberg TW, De Souza EB, and Maki RA (1997a) Localization of ligand-binding domains of human corticotropin-releasing factor receptor: a chimeric receptor approach. *Mol Endocrinol* **11:**980–985.

Montecucchi PC and Henschen A (1981) Amino acid composition and sequence analysis of sauvagine, a new active peptide from the skin of *Phyllomedusa sauvagei*. *Int J Pept Protein Res* **18:**113–120.

Palchaudhuri MR, Hauger RL, Wille S, Fuchs E, and Dautzenberg FM (1999) Isolation and pharmacological characterization of two functional splice variants of corticotropin-releasing factor type 2 receptor from *Tupaia belangeri*. *J Neuroendocrinol* **11:**419–428.

Palchaudhuri MR, Wille S, Mevenkamp G, Spiess J, Fuchs E, and Dautzenberg FM (1998) Corticotropin-releasing factor receptor type 1 from *Tupaia belangeri*: cloning, functional expression and tissue distribution. *Eur J Biochem* **258:**78–84.

Perrin MH, Sutton S, Bain D, Berggren WT, and Vale WW (1998) The first extracellular domain of corticotropin-releasing factor-R1 contains major binding determinants for urocortin and astressin. *Endocrinology* **139:**566–570.

Perrin MH, Sutton SW, Cervini LA, Rivier JE, and Vale WW (1999) Comparison of an agonist, urocortin, and an antagonist, astressin, as radioligands for characterization of corticotropin-releasing factor receptors. *J Pharmacol Exp Ther* **288:**729–734.

Rühmann A, Bonk I, Lin CR, Rosenfeld MG, and Spiess J (1998) Structural requirements for peptidic antagonists of the corticotropin-releasing factor receptor (CRFR): development of CRFR2β-selective antisauvagine-30. *Proc Natl Acad Sci USA* **95:**15264–15269.

Rühmann A, Kopke AKE, Dautzenberg FM, and Spiess J (1996) Synthesis and characterization of a photoactivatable analog of corticotropin-releasing factor for specific receptor labeling. *Proc Natl Acad Sci USA* **93:**10609–10613.

Sleight AJ, Stam NJ, Mutel V, and Vanderheyden PM (1996) Radiolabeling of the human 5-HT2A receptor with an agonist, a partial agonist and an antagonist: effects on apparent agonist affinities. *Biochem Pharmacol* **51:**71–76.

Vale W, Spiess J, Rivier C, and Rivier J (1981) Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and β-endorphin. *Science (Wash DC)* **213:**1394–1397.

Vale W, Vaughan J, and Perrin M (1997) Corticotropin-releasing factor (CRF) family of ligands and their receptors. *The Endocrinologist* **7:**S3–S9.

Vaughan J, Donaldson C, Bittencourt J, Perrin MH, Lewis K, Sutton S, Chan R, Turnbull AV, Lovejoy D, Rivier C, et al. (1995) Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. *Nature (Lond)* **378:**287–292.

Wille S, Sydow S, Palchaudhuri MR, Spiess J, and Dautzenberg FM (1999) Identification of amino acids in the N-terminal domain of corticotropin-releasing factor receptor 1 that are important determinants of high affinity ligand binding. *J Neurochem* **72:**388–395.

Address correspondence to: Dr. Frank M. Dautzenberg, Axovan Limited, Gewerbestrasse 16, CH-4070 Allschwil, Switzerland. E-mail: frank.dautzenberg@axovan.com