Potent and Long-Acting Corticotropin Releasing Factor (CRF) Receptor 2 Selective Peptide Competitive Antagonists

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We present evidence that members of the corticotropin releasing factor (CRF) family assume distinct structures when interacting with the CRF_1 and CRF_2 receptors. Predictive methods, physicochemical measurements, and structure–activity relationship studies have suggested that CRF, its family members, and competitive antagonists such as astressin {cyclo(30–33)-[DPhe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]hCRF$_{(12–41)}$} assume an α-helical conformation when interacting with their receptors. We had shown that α-helical CRF$_{(9–41)}$ and sauvagine showed some selectivity for CRF receptors other than that responsible for ACTH secretion[1] and later for CRF_2.[2] More recently, we suggested the possibility of a helix-turn-helix motif around a turn encompassing residues 30–33[3] that would confer high affinity for both CRF_1 and CRF_2[2,4] in agonists and antagonists of all members of the CRF family.[3] On the other hand, the substitutions that conferred ca. 100-fold CRF_2 selectivity to the antagonist antisauvagine-30 {[DPhe^{11},His^{12}]-sauvagine$_{(11–40)}$} did not confer such property to the corresponding N-terminally extended agonists. We find here that a Glu^{32}-Lys^{35} side chain to side chain covalent lactam constraint in hCRF and the corresponding Glu^{31}-Lys^{34} side chain to side chain covalent lactam constraint in sauvagine yield potent ligands that are selective for CRF_2. Additionally, we introduced deletions and substitutions known to increase duration of action to yield antagonists such as cyclo(31–34)[DPhe^{11},His^{12},CαMeLeu^{13,39},Nle^{17},Glu^{31},Lys^{34}]Ac-sauvagine$_{(8–40)}$ (astressin$_2$-B) with CRF_2 selectivities greater than 100-fold. CRF receptor autoradiography was performed in rat tissue known to express CRF_2 and CRF_1 in order to confirm that astressin$_2$-B could indeed bind to established CRF_2 but not CRF_1 receptor-expressing tissues. Extended duration of action of astressin$_2$-B vs that of antisauvagine-30 is demonstrated in the CRF_2-mediated animal model whereby the inhibition of gastric emptying of a solid meal in mice by urocortin administered intraperitoneally at time zero is antagonized by the administration of astressin$_2$-B but not by antisauvagine-30 at times −3 and −6 h while both peptides are effective when given 10 min before urocortin.

Introduction

Whereas the first members of the CRF (corticoliberin, CRH) family (oCRF, r/hCRF, urotensin, sauvagine, and urocortin) with the possible exception of oCRF (to some extent CRF_1 selective) were found to have similar (within a factor of 10) affinities for cloned CRF receptors CRF_1[5] and CRF_2,[6,7] it is only recently with the discovery of urocortins II[8] and III[9] (N-terminally shortened sequences of stresscopin RP and stresscopin)[10] that CRF_2 selective agonists were identified. At the same time that new members of the CRF family were discovered, novel antagonists were derived from these structures through deletion of the N-terminal 8–11 residues.[11]

These peptide CRF antagonists have played a critical role in unraveling the physiological role and mode of action of CRF. Administered in the periphery, peptide CRF antagonists would not cross the blood–brain barrier, and injected centrally, they would have effects not seen with the administration of CRF antibodies because of their smaller molecular weights and increased ability to diffuse. As such, peptide antagonists are ideal tools for mechanistic studies aimed at distinguishing pharmacological from physiological effects and, pharmacologically, for proof of concept in the search for possible clinical indications. Ideally, these peptide antagonists would have high affinity for the CRF receptors, be or not be selective for the different receptors, be long-acting, and in certain cases, be amenable to selective labeling (carrying radioactive, fluorescent, or cytotoxic markers). SAR studies led to the discovery of several successive generations of antagonists. Chronologically, α-helical CRF$_{(9–41)}$ (the first CRF antagonist to be described),[11] when administered icv, was significantly more potent at abolishing icv CRF-induced elevation of plasma catecholamines and, after iv adminis-

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tration, at preventing CRF-induced hypotension and tachycardia than at blocking CRF-induced elevation of plasma adrenocorticotropin (ACTH) and β-endorphin. This difference in potency in these specific biological systems suggested the existence of more than one CRF receptor.[1] Additionally, α-helical CRF$_{(9-41)}$ was instrumental in demonstrating the physiological role of CRF in stress, evidence that could not be obtained from CRF knockout mice.[12,13]

The next CRF antagonist to be used extensively was [DPhe^{12},Nle^{21,38}]r/hCRF$_{(12-41)}$, itself found to be three times more potent than α-helical CRF$_{(9-41)}$ in the rat anterior pituitary cell culture assay.[14] Finally, it is with the introduction of structural constraints in the form of a lactam ring that astressin {cyclo(30−33)-[DPhe^{12},Nle^{21,38},Glu^{30},Lys^{33}]r/hCRF$_{(12-41)}$} was developed.[15] Astressin was the first antagonist to be a potent inhibitor of ACTH secretion in vitro, being approximately 32 times more potent at inhibiting ACTH secretion in a rat pituitary cell culture assay than were its predecessors. The structure of astressin was further modified to introduce additional structural constraints in the form of methylation of the α-carbon of leucine residues at positions 27 and 40 and extension of the N terminus by the acylated native tripeptide. Astressin B {cyclo(30−33)[DPhe^{12},Nle^{21,40},CαMeLeu^{27,38},Glu^{30},Lys^{33}]-Ac-r/hCRF$_{(9-41)}$} is a very long-acting antagonist with high affinity for both CRF$_1$ and CRF$_2$. Independently, Rühmann et al.[4] described the structure of antisauvagine-30 as being CRF$_2$ selective. Interestingly, we could not derive a CRF$_2$ selective agonist from this structure through extension of the N terminus. We concluded from this observation that the N terminus was responsible for some conformational induction that prevented the C terminus from adopting a favorable CRF$_2$ compatible structure. We knew that the introduction of the Glu^{30}-Lys^{33} cycle in CRF antagonists based on the sequences of hCRF (such as astressin and astressin B) and α-helical CRF (unpublished data) conferred high affinity for CRF$_1$ and CRF$_2$. We observed that the corresponding Glu^{29}-Lys^{32} cycle in sauvagine (this work) or urocortin (unpublished) also conferred high affinity for CRF$_1$ and CRF$_2$ and no selectivity. We also knew that linear antagonists based on the structures of α-helical CRF and sauvagine had limited CRF$_2$ selectivity. When we realized that these analogues had lost their selectivity upon the introduction of a constraint {cycle (30−33) for hCRF and cycle (29−32) for sauvagine}, we concluded that CRF$_1$ or CRF$_2$ selectivity could be modulated by subtle conformational constraints such as lactam bridges. Additionally, we reasoned that the N terminus was critical in stabilizing the bioactive conformation of the C terminus since linear and cyclic agonists had similar potencies whereas the corresponding cyclic antagonist (astressin) had 300-fold greater potency than its linear counterpart.[16] We therefore hypothesized that selectivity in these antagonists could be reinstated if a different constraint was applied on their structures. With the availability of analogues **3**−**12** encompassing a Glu^i, Lys^{i+3} cyclic lactam ring spanning the [DPhe^{12},Nle^{21,38}]hCRF$_{(12-41)}$ sequence over residues 24−35, we assessed their respective affinities for the cloned CRF$_1$ and CRF$_2$ receptors. Data shown in Table 1 demonstrate that CRF$_2$ selectivity can be induced to

different extents by the introduction of the Glu^{32}-Lys^{35} lactam bridge in hCRF$_{(12-41)}$ or the introduction of the corresponding Glu^{31}-Lys^{34} lactam bridge in sauvagine$_{(8-40)}$ and sauvagine$_{(11-40)}$. This investigation ultimately led to the discovery of astressin$_2$-B (**20**), a potent and long-acting CRF$_2$ selective antagonist as determined in several bioassays.

Urocortin has been considered to be the endogenous ligand for CRF$_2$.[17] Urocortin displays similar high affinity for CRF$_{2\alpha}$, found mainly in the brain and for its splice variant, CRF$_{2\beta}$, which predominates in peripheral tissues and brain nonneuronal tissue in rats.[2,17−19] Consistent with the presence of CRF$_{2\beta}$ in the heart, immune cells, and gastrointestinal tract,[18] peripherally administered urocortin exerts diverse cellular and biological effects on cardiovascular, immune, and gastrointestinal systems.[20−22] In particular, we previously reported in rats that urocortin injected iv exhibits greater potency than CRF in delaying gastric emptying of a nonnutrient solution.[22] This action was blocked by the CRF$_1$/CRF$_2$ antagonist astressin but not by the selective CRF$_1$ antagonists, NBI-27914 and antalarmin.[23] In addition, a recent study indicates that ip urocortin-induced delayed gastric emptying in lean and *ob/ob* mice was longer acting than ip CRF-induced effects.[24] These few observations provided indirect pharmacological evidence that peripheral urocortin-inducing delayed gastric emptying may be mediated by CRF$_2$. In addition, we recently showed in mice that the selective CRF$_2$ receptor antagonist, antisauvagine-30,[4] injected ip completely prevented ip urocortin-induced delay of gastric emptying of a solid meal at a w/w ratio of 33:1 providing convincing evidence that the peripheral urocortin-induced gastric stasis is mediated by a CRF$_2$ receptor.[25]

Results and Discussion

Synthesis and Physicochemical Characterization. All analogues shown in Table 1 were synthesized using the solid phase method of Merrifield on a methylbenzhydrylamine resin (MBHA) using the Boc strategy with orthogonal protection of the side chains of the lysine (Fmoc) and glutamic acid (OFm) residues to be cyclized.[15,26,27] Main chain assembly was mediated in most cases by diisopropylcarbodiimide (DIC). The best results were obtained when the peptide chain was assembled in its entirety prior to cleavage of the Fmoc and OFm protecting groups and when the lactam formation was mediated by TBTU or BOP. The peptides were cleaved and deprotected in HF and purified with reversed phase HPLC (RPHPLC). Peptides were characterized using RPHPLC, CZE, and MS.

Receptor Assay. The IC$_{50}$ values given in Table 1 reflect the affinities of the analogues for the cloned type 1 and type 2β CRF receptors. The values were derived from competitive radioligand displacement assays using the nonselective ^{125}I-labeled agonist [Tyr^0,Glu^1,Nle^{17}]-sauvagine as the radioligand. Representative displacement curves (**16**, **18**, **20**, and **23**) are shown in Figure 1.

SAR Studies. The assay consisting of measuring the inhibition of CRF-stimulated release of ACTH from rat pituitary cells in culture was instrumental in identifying astressin as a new lead for potent CRF antagonists since it was 32 times more potent than any

Table 1. Chemical and Biological Characterization of CRF Analogues

R$_2$-selective antagonists

		5	10	15	20	25	30	35	40
r/h CRF[a]		S E E **P P I S** L **D** L T F H **L L R** E V **L E** M A R A **E** Q L A **Q Q A** H S **N R** K L M E I **I-NH₂**							
Sauvagine		*E* G **P P I S** I **D L** S L E **L L R** K M I **E** I E K Q **E** K E K **Q Q A** A N **N R** L L L D T **I-NH₂**							

	compd name	HPLC[b]	CZE[c]	MS calcd[d]	MS found[d]	CRF₁[e] IC₅₀ (nM)	CRF₂[e] IC₅₀ (nM)
1	r/hCRF	>98	96	4755.5	4755.5	0.99 (0.22−4.6)	6.2 (2.0−19)
2	Sau			4596.52	4596.5	0.90 (0.49−1.7)	1.6 (0.7−3.8)
3	cyclo(24−27)[DPhe¹²,Nle²¹,³⁸,**Glu²⁴,Lys²⁷**]-hCRF₍₁₂₋₄₁₎	90	97	3593.02	3592.8	30.6 (16−58)	46 (19−109)
4	cyclo(25−28)[DPhe¹²,Nle²¹,³⁸,**Glu²⁵,Lys²⁸**]-hCRF₍₁₂₋₄₁₎	98	98	3577.06	3577.0	12 (10.4−13.7)	6.2 (2.2−17)
5	cyclo(26−29)[DPhe¹²,Nle²¹,³⁸,**Glu²⁶,Lys²⁹**]-hCRF₍₁₂₋₄₁₎	>98	95	3521.02	3520.9	24 (10−59)	4.9 (2.5−9.8)
6	cyclo(27−30)[DPhe¹²,Nle²¹,³⁸,**Glu²⁷,Lys³⁰**]-hCRF₍₁₂₋₄₁₎	96	95	3535.99	3535.4	119 (64−224)	60 (19−193)
7	cyclo(28−31)[DPhe¹²,Nle²¹,³⁸,**Glu²⁸,Lys³¹**]-hCRF₍₁₂₋₄₁₎	90	82	3635.07	3635.3	14 (5−37)	5.5 (3−11)
8	cyclo(29−32)[DPhe¹²,Nle²¹,³⁸,**Glu²⁹,Lys³²**]-hCRF₍₁₂₋₄₁₎	93	96	3512.02	3512.3	10.2 (5.7−18)	5.0 (1.8−14)
9	cyclo(30−33)[DPhe¹²,Nle²¹,³⁸,**Glu³⁰,Lys³³**]-hCRF₍₁₂₋₄₁₎ astressin	94	96	3562.05	3562.1	0.72 (0.29−1.8)	0.62 (0.49−0.78)
10	linear astressin	96	94	3580.06	3580.1	>100	69 (30−160)
11	cyclo(31−34)[DPhe¹²,Nle²¹,³⁸,**Glu³¹,Lys³⁴**]-hCRF₍₁₂₋₄₁₎	96	97	3592.06	3592.2	>500	>500
12	cyclo(32−35)[DPhe¹²,Nle²¹,³⁸,**Glu³²,Lys³⁵**]-hCRF₍₁₂₋₄₁₎	97	97	3483.98	3483.9	>100	10 (5−22)
13	[DPhe¹¹,His¹²,Nle¹⁷,CαMeLeu¹³,³⁹]-Ac-Sau₍₈₋₄₀₎	>98	>98	4028.30	4028.2	18.4 (17.5−19.4)	0.42 (0.29−0.55)
14	cyclo(27−30)[DPhe¹¹,His¹²,Nle¹⁷,CαMeLeu¹³,³⁹,Glu²⁷,Lys³⁰]Ac-Sau₍₈₋₄₀₎	>98	98	4068.29	4068.2	45 (22−92)	1.3 (0.70−2.5))
15	cyclo(28−31)[DPhe¹¹,His¹²,Nle¹⁷,CαMeLeu¹³,³⁹,Glu²⁸,Lys³¹]Ac-Sau₍₈₋₄₀₎	>98	NA	4068.33	4068.1	7.7 (2.2−27)	0.57 (0.44−0.75)
16	cyclo(29−32)[DPhe¹¹,His¹²,Nle¹⁷,CαMeLeu¹³,³⁹,Glu²⁹,Lys³²]Ac-Sau₍₈₋₄₀₎	>98	NA	4025.32	4025.0	0.24 (0.11−0.54)	1.2 (0.54−2.5)
17	linear[DPhe¹¹,His¹²,Nle¹⁷,CαMeLeu¹³,³⁹,Gln²⁹,Lys(Ac)³²]Ac-Sau₍₈₋₄₀₎	97	NA	4084.36	4084.2	1.9 (1.5−2.3)	0.72 (0.55−0.94)
18	cyclo(30−33)[DPhe¹¹,His¹²,Nle¹⁷,CαMeLeu¹³,³⁹,Glu³⁰,Lys³³]Ac-Sau₍₈₋₄₀₎	95	95	4082.34	4082.1	>500	81 (63−100)
19	linear[DPhe¹¹,His¹²,Nle¹⁷,CαMeLeu¹³,³⁹,Gln³⁰,Lys(Ac)³³]Ac-Sau₍₈₋₄₀₎	97	95	4141.38	4141.1	>100	>100
20	cyclo(31−34)[DPhe¹¹,His¹²,Nle¹⁷,CαMeLeu¹³,³⁹,Glu³¹,Lys³⁴]Ac-Sau₍₈₋₄₀₎	95	NA	4040.28	4040.2	>500	1.3 (0.95−1.7)
21	linear[DPhe¹¹,His¹²,Nle¹⁷,CαMeLeu¹³,³⁹,Glu³¹,Lys³⁴]Ac-Sau₍₈₋₄₀₎	95	NA	4058.30	4058.0	>100	0.92 (0.56−1.5)
22	linear[DPhe¹¹,His¹²,Nle¹⁷,CαMeLeu¹³,³⁹,Gln³¹,Lys(Ac)³⁴]Ac-Sau₍₈₋₄₀₎	>98	>98	4099.32	4099.2	>100	0.60 (0.23−1.6)
23	cyclo(32−35)[DPhe¹¹,His¹²,Nle¹⁷,CαMeLeu¹³,³⁹,Glu³²,Lys³⁵]Ac-Sau₍₈₋₄₀₎	>98	98	4040.30	4040.2	5.9 (2.8−12.4)	0.61 (0.54−0.68)
24	linear[DPhe¹¹,His¹²,Nle¹⁷,CαMeLeu¹³,³⁹,Gln³²,Lys(Ac)³⁵]Ac-Sau₍₈₋₄₀₎	>98	98	4099.33	4099.3	3.4 (1.7−6.7)	0.51 (0.07−3.8)
25	cyclo(33−36)[DPhe¹¹,His¹²,Nle¹⁷,CαMeLeu¹³,³⁹,Glu³³,Lys³⁶]Ac-Sau₍₈₋₄₀₎	>98	98	4040.30	4040.3	>100	>100
26	cyclo(34−37)[DPhe¹¹,His¹²,Nle¹⁷,CαMeLeu¹³,³⁹,Glu³⁴,Lys³⁷]Ac-Sau₍₈₋₄₀₎	98	97	3998.24	3998.2	>100	17.8 (7.7−41)
27	cyclo(31−34)[DPhe¹¹,His¹²,Nle¹⁷,CαMeLeu¹³,³⁹,Glu³¹,Lys³⁴]Ac-Sau₍₁₁₋₄₀₎	96	94	3725.14	3725.1	>500	1.1 (0.59−2.2)
28	linear[DPhe¹¹,His¹²,Nle¹⁷,CαMeLeu¹³,³⁹,Gln³¹,Lys(Ac)³⁴]Ac-Sau₍₁₁₋₄₀₎	>98	95	3784.18	3784.0	>100	1.9 (0.4−9.0)

[a] Bolded residues are identical in both sequences. Sauvagine is 40 residues, and CRF is 41; alignment is shifted by one residue in order to retain correct numbering. [b] Percent purity determined by HPLC using buffer system: A = TEAP (pH 2.5) and B = 60% CH₃CN/40% A with a gradient slope of 1% B/min, at a flow rate of 0.2 mL/min on a Vydac C₁₈ column (0.21 cm × 15 cm, 5-μm particle size, 300 Å pore size). Detection at 214 nm. [c] CZE was done using a Beckman P/ACE System 2050 controlled by an IBM Personal System/2 model 50Z and using a ChromJet integrator. Field strength of 15 kV at 30 °C, mobile phase: 100 mM sodium phosphate (85:15, H₂O:CH₃CN), pH 2.50, on a Supelco P175 capillary (363 μm OD × 75 μm ID × 50 cm length). Detection at 214 nm. [d] The observed *m*/*z* of the monoisotope as compared with the calculated [M + H]⁺ monoisotopic mass. [e] The IC₅₀ values were derived from competitive radioligand displacement assays that reflect the affinities of the analogues for the cloned CRF₁ and CRF₂β receptors using the nonselective ¹²⁵I-labeled agonist [Tyr⁰,Glu¹,Nle¹⁷]sauvagine, as the radioligand. IC₅₀ for antisauvagine-30, αhel-CRF₍₉₋₄₁₎, and [DPhe¹²,Nle²¹,³⁸]hCRF₍₁₂₋₄₁₎ were = 400, 19 (5.5−66), and 19.2 (13.4−27.5) nM for CRF₁ and 1.1 (0.59−2.2), 1.1 (0.99−1.3), and 4.4 (2.0−9.6) nM for CRF₂β, respectively.

of its predecessors {α-hel-CRF₍₉₋₄₁₎ or [DPhe¹²,Nle²¹,³⁸]-hCRF₍₁₂₋₄₁₎}.[28] In the same series, cyclo(26−29)-, cyclo-(28−31)-, and cyclo(29−32)[DPhe¹²,Glu^i,Lys^{i+3},Nle²¹,³⁸]-hCRF₍₁₂₋₄₁₎ were 10 times less potent while cyclo-
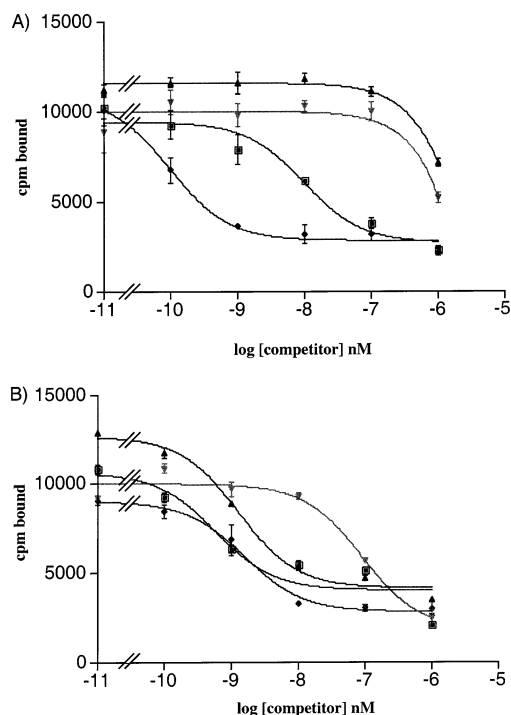
Figure 1. Binding of selected sauvagine analogues to CRF$_1$ and CRF$_{2\beta}$. Competitive displacement of ^{125}I[Tyr^0,Glu^1,Nle^{17}]-sauvagine bound to (A) CRF$_1$ or (B) CRF$_{2\beta}$ by analogues **16** (◆), **18** (▼), **20** (■), or **23** (▲). Data are from one representative assay, repeated at least twice and analyzed by the Prism Graphpad program.

(24−27)-, cyclo(25−28)-, cyclo(27−30)-, cyclo(31−34)-, and cyclo(32−35)[DPhe^{12},Glu^i,Lys^{i+3},Nle^{21,38}]hCRF$_{(12−41)}$ were less than 2% as potent. With the availability of receptor assays for CRF$_1$ and CRF$_2$[2] and as rationalized in the Introduction, this series of Glu^i, Lys^{i+3} cyclic lactams spanning the [DPhe^{12},Nle^{21,38}]hCRF$_{(12−41)}$ sequence was tested for the respective affinity of its members for the two cloned CRF receptors.

Binding data on these analogues gave similar results when correlating CRF$_1$ affinity with in vitro potencies derived from the measure of the inhibition of CRF-induced ACTH stimulation in rat anterior pituitary cells in culture by increasing doses of the antagonist (see Miranda et al. for in vitro data[27] and Table 1 for IC$_{50}$ values at CRF$_1$ and CRF$_2$ for **3−11**). Binding affinities for CRF$_2$, however, diverged significantly in the case of cyclo(32−35)[DPhe^{12},Glu^{32},Lys^{35},Nle^{21,38}]hCRF$_{(12−41)}$ (**12**) whereby it had very low affinity for CRF$_1$ and high affinity for CRF$_2$. It is noteworthy to repeat that astressin (**9**) had high affinity for both receptors while its linear counterpart (**10**) had much lower affinity for both. Because the affinity of **12** for CRF$_2$ was about equal to that of r/hCRF for CRF$_2$ and the affinity of sauvagine for that receptor is about four times greater, we investigated the possibility of generating a potent long-acting CRF$_2$ selective antagonist based on the structure of **13**. Analogue **13** had very high affinity for CRF$_2$ (IC$_{50}$ = 0.42 nM) and was somewhat selective for that receptor (IC$_{50}$ for CRF$_1$ = 18.4 nM). Using this parent compound (**13**), we repeated the scan performed on [DPhe^{12},Nle^{21,38}]hCRF$_{(12−41)}$ (**3−12**). Interestingly, although of medium (5−50 nM) to high (0.2−3) affinity for CRF$_1$ and CRF$_2$, these analogues retained a certain degree of CRF$_2$ selectivity except for **16** and its linear

homologue **17** that have a bridge corresponding to that found in astressin and showed very high affinity for both receptors. The most striking observation however was the very high affinity of **20** for CRF$_2$ (IC$_{50}$ = 1.3 nM) and its very significant selectivity for that receptor (IC$_{50}$ > 500 nM for CRF$_1$). Such affinity and selectivity are comparable to those of the type 2 selective urocortins, urocortin II[8] and III.[9] The fact that this Glu^{31}-Lys^{34} cycle in sauvagine corresponds to the Glu^{32}-Lys^{35} cycle in r/hCRF, observed to also confer CRF$_2$ selectivity, strongly suggests that this constraint induces a conformation favorable for interaction with CRF$_2$. To gain an appreciation of the role of the Glu^{31}-Lys^{34} lactam bridge in sauvagine on selectivity, the corresponding linear **21** and **22** were also synthesized. The rationale for **21** was that if it was to be active, it could be explained by the formation of a favorable salt bridge. We therefore also synthesized **22** where the charges at both bridge heads were neutralized by amidation and acetylation, respectively. To our surprise, both analogues retained high selectivity (likely not significantly different from that of **20**) and affinity for CRF$_2$. The possibility that the side chains of residues at positions 31 and 34 are either charged (as in **21**) or neutral (as in **22**) and are still interacting cannot be excluded. Another hypothesis is that the side chains of the residues in those positions in both r/hCRF (His^{32} and Arg^{35}) and sauvagine (Ala^{31} and Arg^{34}) may be interaction neutral or mildly repulsive to allow the flexibility needed for CRF$_1$ and CRF$_2$ interactions; flexibility that is now limited by the substitutions reported here. Further extension of the scan toward the C terminus with cyclo(32−35), cyclo-(33−36), and cyclo(34−37) (**23**, **25**, and **26**, respectively) gave unexpected results in that **23** and its linear counterpart **24** have high affinity for both CRF$_1$ and CRF$_2$ with a 5−10-fold greater affinity for the latter. Compounds **25** and **26** are much less potent.

While this work was in progress, Rühmann et al.[4] reported the synthesis and biological characterization of antisauvagine-30, an 11−40 fragment of sauvagine with two substitutions (DPhe^{11} found to increase potency[29] and His^{12} found at the corresponding position in r/hCRF[30]). While Rühmann et al. chose to investigate substitutions in an 11−40 fragment of sauvagine, we favored the N-terminally acetylated 8−40 fragment of sauvagine because of earlier observations that suggested that the longer fragments such as astressin B as compared to astressin were likely to be longer acting in vivo.[31]

We also synthesized the shorter cyclic (**27**) and linear (**28**) forms of **20** that have IC$_{50}$ values of 1.1 and 1.9 nM, respectively, for CRF$_2$ and no measurable affinity for CRF$_1$. Again, the fact that the linear **28** is as potent and as selective as the cyclic **27** raises the question of the importance of the lactam bridge.

To assess the influence of the $i−(i + 3)$ amide bridge on the secondary structure of the [DPhe^{11}, His^{12},CαMeLeu,^{13,39}Nle^{17}]Ac-Sau$_{8−40}$ host, circular dichroism (CD) studies were conducted on **14−17** and **20−22** in aqueous solution and in 12.5% and 50.0% (v/v) solution of TFE (Figure 2A−F). We hypothesized that any differences in the spectra of **16** and **17** and **20** and **21** would be a measure of the contribution of the cycle whereas any difference between the spectra of **16** and
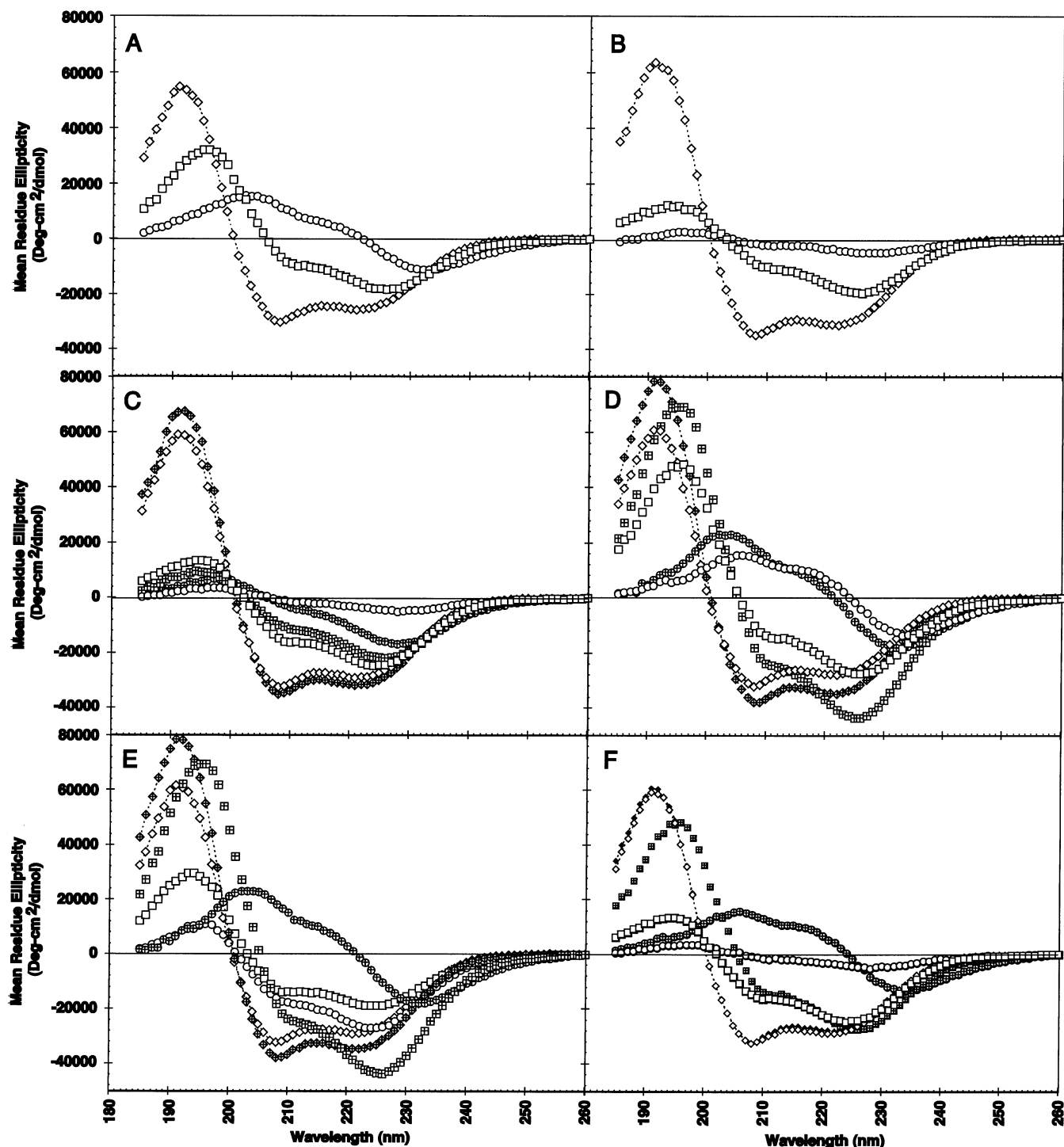
Figure 2. Mean residue ellipticity (deg-cm^2/dmol) vs wavelength; see Experimental Section for collection details. Cases: aqueous (box), 12.5% TFE (circle), 50% TFE (diamond). (A) **14**; (B) **15**; (C) **16** (open symbols), **17** (crossed symbols); (D) **20** (open symbols), **21** (crossed symbols); (E) **21** (crossed symbols), **22** (open symbols); and (F) **16** (open symbols), **20** (crossed symbols).

20 and **17** and **21** would be a measure of a contribution of the shift in position of the bridge. Results in aqueous solutions, 12.5% aqueous TFE, and aqueous 50% TFE are presented in Table 2 and Figure 2A–F. These analogues demonstrate moderate to high α-helical character in aqueous solution spanning the range of deconvoluted α-helix contributions (Table 2) from 34.3 (**15**) to 93.7% (**21**) as judged by the neural network deconvolution method of Böhm.[32] The strikingly large value for the α-helical component in aqueous solution for **21** may be somewhat overestimated because the

reference compounds in the method of Böhm are composed of physiological amino acids, and the Cα-methyl functionality may have a small effect on the observed CD spectra. However, this effect would presumably be present in all of the compounds investigated in this set. In general, all of the analogues tested can assume complete α-helical structure in 50% TFE.

A comparison of the spectra of **14** and **15**, Figure 2A–B, suggests that the shift of the amide bridge from (27–30) to (28–31) modifies the manifold of secondary structural motifs available to the compounds. In aque-

Table 2. Deconvolution of CD Spectra of **14**−**17** and **20**−**22** by the Method of Böhm et al.[32] as Obtained with the CDNN Program, Version 2.1[a]

	compd						
	14	**15**	**16**	**17**	**20**	**21**	**22**
Aqueous							
helix	0.45	0.34	0.43	0.35	0.72	0.94	0.50
antiparallel	0.01	0.06	0.03	0.06	0.05	0.01	0.02
parallel	0.08	0.09	0.07	0.09	0.05	0.01	0.07
β-turn	0.13	0.16	0.15	0.16	0.06	0.02	0.13
random coil	0.41	0.38	0.29	0.37	0.10	0.03	0.29
total sum	1.08	1.02	0.97	1.02	0.98	1.00	0.99
12.5% TFE							
helix	0.17	0.19	0.20	0.25	0.34	0.12	0.44
antiparallel	0.18	0.31	0.25	0.12	0.11	0.87	0.03
parallel	0.19	0.13	0.06	0.13	0.10	0.07	0.07
β-turn	0.18	0.20	0.18	0.17	0.12	0.07	0.15
random coil	0.68	0.46	0.40	0.49	0.57	0.14	0.27
total sum	1.40	1.29	1.08	1.16	1.23	1.27	0.95
50% TFE							
helix	0.81	0.89	0.86	0.90	0.85	0.94	0.86
antiparallel	0.00	0.00	0.00	0.00	0.00	0.00	0.00
parallel	0.02	0.01	0.02	0.01	0.02	0.01	0.02
β-turn	0.10	0.09	0.09	0.08	0.09	0.07	0.09
random coil	0.06	0.04	0.05	0.03	0.05	0.02	0.05
total sum	0.99	1.02	1.01	1.03	1.01	1.04	1.02

[a] Entries are a fractional population of each component structure type.

ous solution, **14** and **15** have comparable structures dominated by the helix but also including significant β-turn and random coil contributions; see Table 2. At 12.5% TFE, the spectra of these compounds are significantly different. We were unable to deconvolute these spectra at 12.5% TFE, as judged by the total component percentages differing so markedly from 100%, due most likely to a rapid equilibrium of transient structures available to these compounds under these solvent conditions. For example, the CD spectrum of **15** virtually disappears at 12.5% TFE, whereas that of **14** is observable if not novel with a maximum at 213 nm and a minimum at 233 nm. At 50% TFE, both compounds are almost fully helical. It is noteworthy that such spectral differences may not translate in major biological shifts in affinity or selectivity since both analogues are slightly (about 10-fold) CRF$_2$ selective.

To determine the effect of the bridge per se, we compared the CD spectra of **16** (cyclic analogue) with that of **17** (linear analogue unable to cyclize or form a salt bridge because both carboxylic and amino side chain functionalities are blocked by an amide functionality at Gln^{29} and an acetyl at Lys^{32}(Ac)). Spectra are shown in Figure 2C. The amide bridge seems to facilitate α-helical conformation. In the intermediate range of organic cosolvent, the conformational states available to **16** and **17** are different, as judged by differences in the CD, suggesting that the bridge can modulate the overall structure of the host. Both analogues have high affinity for CRF$_1$ and CRF$_2$ and exhibit no selectivity.

Figure 2D,E compare **20** with **21** and **21** with **22**, respectively. Again, as observed with **14**−**17**, the location and nature of the bridging residues at positions 31−34 determine the secondary structure available in 12.5% TFE. Additionally, there is a significant increase in the α-helical component upon the breaking of the 31−34 bridge (see Table 2). This can be interpreted as the 31−34 bridge actually breaking a longer otherwise contiguous section of helix with the introduction of a

turn. Such a turn has been postulated to be part of the active conformation of astressin (cyclo(30−33) as compared to cyclo(29−32) in a sauvagine analogue).[3,16] To demonstrate that the chemical nature of the side chains forming the bridge in active cyclic CRF antagonists can modulate structure, the CD spectra of **22** were collected and were compared with that of **21** in Figure 2E. These compounds differ in that the side chains at residues 31 (carboxyl) and 34 (amino) are free in **21** and blocked in **22** (amide and acetylation, respectively). If the rationale were that a salt bridge could form in **21**, such secondary structure would not be available in **22**. Clearly, the structural consequences of this substitution influence not only the manifold of structures available in the 12.5% TFE case but also the position and intensity of the positive exciton split $\pi^*-\pi$ peak usually observed in the region of 195 nm. Interestingly, the biological consequences are not significant in that all three analogues have high affinity for CRF$_2$ and very low affinity for CRF$_1$ suggesting that despite the fact that different structures are observed in different solvent systems, a bioactive conformation recognizing CRF$_2$ is available to all three analogues. If, on the other hand, we compare the CD spectra of **16** (a nonselective, potent ligand to CRF$_1$ and CRF$_2$) to that of **20** (a potent CRF$_2$ selective ligand), we see spectral differences that may account for the differences in selectivity (Figure 2F). Analogue **20**, with a (31−34) Glu-Lys amide bridge, is highly helical (72%, Table 2) in aqueous solution as compared to **16** with a (29−32) bridge and a deconvoluted helical content of 43% in the aqueous case. Clearly, the transition case of 12.5% TFE facilitates completely different structural properties on these compounds (Figure 2F, open and crossed circle symbols). Interestingly, the CD spectra of **16** and **20** are virtually superimposable in 50% TFE, suggesting that equivalent secondary structure is available to both compounds. It appears that the presence of high helical structure (i.e., > ∼50% by deconvolution) in aqueous solution correlates with high CRF$_2$ selectivity as demonstrated by helical component percentages of 72, 94, and 50% for compounds **20**−**22**, respectively. Whether this observation can be refined into a working hypothesis with a clear-cut basis for the secondary structural preferences suggested awaits an analysis of the CD spectra of CRF$_1$ selective analogues when available and more refined physical, biochemical characterization (such as NMR studies.) Additionally, because the largest difference in CD spectra of **16** and **20** is observed in 12.5% TFE while there is none in 50% TFE, one could conclude that if the receptor-induced ligand structure is responsible for selectivity, then 12.5% TFE is a better mimic of the receptor environment than water alone or 50% TFE.

Further In Vitro Characterization. In in vitro receptor autoradiography experiments using the universal radioligand ^{125}I[Tyr^0,Glu^1,Nle^{17}]sauvagine, we observed a complete displacement in the nanomolar range of the radioligand by the unlabeled sauvagine analogue as well as astressin$_2$-B (Figure 3; Table 3) in two established CRF$_2$-expressing tissues, namely, the choroid plexus and blood vessels. In CRF$_1$-expressing tissue, namely, cortex and cerebellum, displacement of the radioligand in the nanomolar range was observed with the unlabeled sauvagine analogue but not with
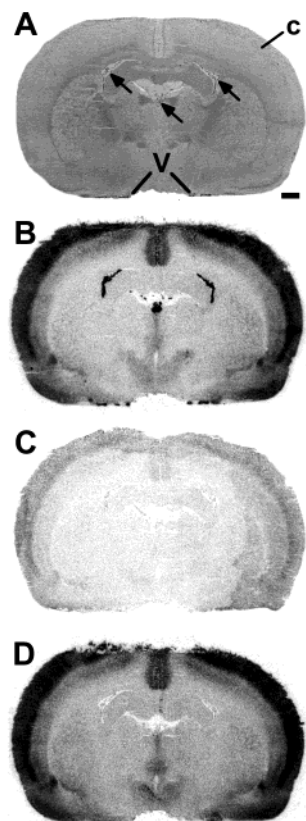
Figure 3. In vitro receptor autoradiography showing the selective binding of astressin$_2$-B to CRF$_2$ receptors in the choroid plexus and blood vessels but not to CRF$_1$ receptors in the rat cortex. (A) Hematoxylin-eosin-stained section of the rat brain showing the cortex (c), the choroid plexus (arrow), and blood vessels (v). Bar = 1 mm. (B) Autoradiogram showing total binding of ^{125}I[Tyr^0,Glu^1,Nle^{17}]sauvagine. Strong labeling is seen in cortex (c), vessels (v), and the choroid plexus (arrows). (C) Autoradiogram showing binding of ^{125}I[Tyr^0,Glu^1,Nle^{17}]-sauvagine in the presence of 50 nM of the unlabeled peptide. The radioligand is displaced in the cortex, blood vessels, and in the choroid plexus. (D) Autoradiogram showing binding of ^{125}I[Tyr^0,Glu^1,Nle^{17}]sauvagine in the presence of 50 nM astressin$_2$-B. The radioligand is completely displaced in the CRF$_2$-expressing choroid plexus and blood vessels but not in the CRF$_1$-expressing cortex.

Table 3. IC$_{50}$ (nM; Mean ± SEM) for the Universal Ligand [Tyr^0,Glu^1,Nle^{17}]sauvagine and the CRF$_2$ Selective Astressin$_2$-B in CRF$_1$- and CRF$_2$-Expressing Rat Tissues

	IC$_{50}$ (nM) mean ± SEM	
	[Tyr^0,Glu^1,Nle^{17}]-sauvagine	astressin$_2$-B
CRF$_2$-expressing tissues (choroid plexus, blood vessels) $n = 3$	2.07 ± 0.13	0.57 ± 0.11
CRF$_1$-expressing tissues (cortex, cerebellum) $n = 4$	11.9 ± 1	>300

astressin$_2$-B (Figure 3; Table 3). These autoradiographic data show that the CRF analogue astressin$_2$-B binds only with high affinity to CRF$_2$ structures such as blood vessels or the choroid plexus.

In Vivo Studies. Previous studies in mice and rats showed that peripheral administration of CRF or CRF-related peptides inhibits gastric emptying of a liquid or solid meal through the activation of CRF$_2$ receptors.[22,25,33,34] Therefore, the potency and duration

of astressin$_2$-B (**20**) to antagonize peripheral urocortin-induced inhibition of gastric emptying was investigated and compared with that of antisauvagine-30 and **28**.

After ip injection of urocortin (3 μg/kg), only 10.9 ± 4.9% of the food ingested after an 18 h fast had emptied from the stomach 2 h later as compared with 58.5 ± 7.7% in ip vehicle-treated mice. These results are consistent with previous demonstrations that peripheral urocortin inhibits gastric emptying in mice and rats.[22,24,25] Dose−response studies showed that astressin$_2$-B (**20**) was more potent than antisauvagine-30 and compound **28** to antagonize the inhibitory effect of urocortin (Figure 4). Astressin$_2$-B (3, 10, or 30 μg/kg) injected ip 10 min before ip urocortin increased dose-dependently gastric emptying and induced a complete reversal of urocortin inhibitory action at 10 μg/kg (Figure 4A). Antisauvagine-30 had no antagonist effect at 3 μg/kg while at 10 and 30 μg/kg, it increased gastric emptying to 38.0 ± 6.4 ($P > 0.05$) and 44.0 ± 8.0% ($P < 0.05$), respectively, as compared with the vehicle + urocortin group (Figure 4A). Likewise, in another study, antisauvagine-30 injected ip at 30 μg/kg antagonized partially, and at 100 μg/kg completely, ip urocortin-induced delayed gastric emptying in mice.[25] Compound **28** (3, 10, or 30 μg/kg) did not significantly prevent the urocortin inhibitory effect although there was a tendency to enhance gastric emptying of a solid meal at the two highest doses (Figure 4A). Astressin$_2$-B, antisauvagine-30, or **28** injected ip at 30 μg/kg 10 min before ip vehicle did not influence gastric emptying of the solid meal (56.1 ± 5.0%, $n = 9$; 50.6 ± 6.4%, $n = 6$; and 55.3 ± 9.0%, $n = 6$, respectively).

Time−course studies showed that astressin$_2$-B is longer acting than antisauvagine-30 (Figure 4B). In mice pretreated sc with vehicle at 3 or 6 h before the ip injection of vehicle, gastric emptying was not significantly different (56.3 ± 8.2 and 63.7 ± 6.6%, respectively). By contrast, in similar vehicle-pretreated groups, ip injection of urocortin (3 μg/kg) decreased gastric emptying to 8.2 ± 3.9 and 11.7 ± 3.4%, respectively (Figure 4B). Astressin$_2$-B (100 μg/kg) injected sc at 3 or 6 h before urocortin (3 μg/kg, ip) blocked urocortin inhibitory action while antisauvagine-30 (100 μg/kg, sc) had no significant antagonist effect under these conditions (Figure 4B). Astressin$_2$-B or antisauvagine-30 (100 μg/kg, sc) did not influence significantly food intake during the 1 h feeding period starting at 2 h (0.30 ± 0.04 and 0.37 ± 0.02 g/1 h, respectively, vs vehicle: 0.29 ± 0.03; $n = 12$/group) or 5 h (0.25 ± 0.03 and 0.32 ± 0.02 g/1 h, respectively, vs vehicle 0.37 ± 0.04 g; $n = 11−13$/group) after peptide injection. Under the same experimental conditions, the peptide antagonists injected alone (100 μg, sc) did not influence gastric emptying of a solid meal (Figure 4B).

These data strengthen the notion that peripheral urocortin-induced delayed gastric emptying is CRF$_2$ receptor-mediated in rodents. This is supported by the blockade of urocortin action by two selective CRF$_2$ antagonists, antisauvagine-30[4] and astressin$_2$-B (present study). In other in vivo biological systems, we also established that astressin$_2$-B acts selectively to antagonize CRF$_2$-mediated inhibition of gastric emptying while not influencing CRF$_1$-mediated stimulation of colonic motor function induced by intravenous CRF in rats.[34]
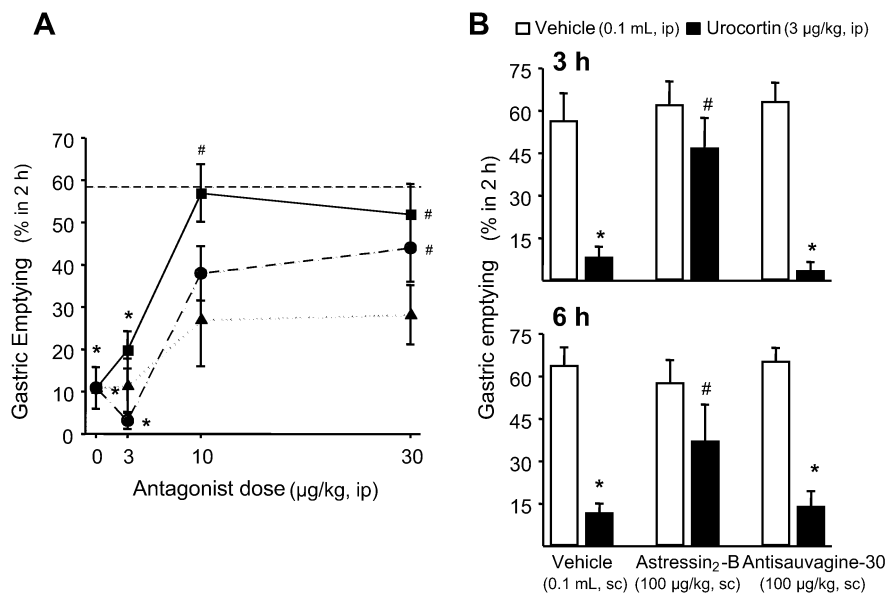
A

B □ Vehicle (0.1 mL, ip) ■ Urocortin (3 µg/kg, ip)

Figure 4. Influence of astressin$_2$-B, antisauvagine-30, and **28** on urocortin-induced inhibition of gastric emptying of a solid meal in conscious mice. (A) Dose response; food and water were given to fasted mice for a 1 h period and removed. Vehicle or urocortin (3 µg/kg) was then injected ip and gastric emptying of the ingested meal was monitored 2 h later. Peptide antagonists were injected ip 10 min before urocortin or vehicle. Each point represents the mean ± SEM of 6−7 mice/group. #P < 0.05 as compared with vehicle + urocortin, *P < 0.05 as compared with vehicle + vehicle. Astressin$_2$-B, ■; antisauvagine-30, ●; **28**, ▲; broken straight line, vehicle + vehicle, 58.5 ± 7.7%, n = 7. (B) Time course; similar protocols detailed in A were followed except that vehicle, astressin$_2$-B, or antisauvagine-30 was injected sc at 3 or 6 h before the ip injection of saline or urocortin. Each bar represents the mean ± SEM of 5−7 mice/group. *P < 0.05 as compared with vehicle + vehicle; # compared with vehicle + urocortin or antisauvagine-30 + urocortin.

Astressin$_2$-B was found to be more potent and longer acting than antisauvagine-30. At the antagonist:agonist ratio of 3:1, astressin$_2$-B abolished urocortin action by 100% as reported using astressin B, the nonselective CRF$_1$/CRF$_2$ peptide antagonist tested under the same conditions.[25] By contrast, antisauvagine-30 at the antagonist:agonist ratio of 3:1 had no significant effect and at 10:1 and 33:1 ratios, it induced a 75 (present study) and 100%[25] reversal of urocortin inhibitory action. Astressin$_2$-B, unlike sauvagine, displayed a long duration of action (over 6 h). Other in vivo effects of the long-acting astressin B and astressin$_2$-B in response to several stressors have been[31] or will be reported elsewhere (C. Rivier; et al. Manuscript in preparation). Last, gastric emptying of a solid meal was not altered by astressin$_2$-B injected ip or sc at 30−100 µg/kg showing that the peptide did not display agonist activity and that CRF$_2$ receptors do not play a role in the basal regulation of digestive motor function. Taken together, these findings provide biological evidence that astressin$_2$-B is a selective, potent, and long-acting CRF$_2$ antagonist, which will be a valuable tool to assess the role of the CRF$_2$ receptor under stress conditions.[33]

In conclusion, we present evidence that members of the CRF family assume distinct structures when interacting with the CRF$_1$ and CRF$_2$ receptors and that these structures can be preferentially induced and stabilized by the introduction of lactam bridges. Hence, whereas a Glu^{30}-Lys^{33} side chain to side chain covalent lactam constraint increased affinity of linear CRF antagonists for CRF$_1$ and CRF$_2$, we found that a Glu^{32}-Lys^{35} side chain to side chain covalent lactam constraint in hCRF fragments and the corresponding Glu^{31}-Lys^{34} side chain to side chain covalent lactam constraint in sauvagine fragments yield potent ligands that are highly selective for CRF$_2$. We demonstrated this selectivity (>100-fold)

in both cloned receptor cell lines and in receptor autoradiography studies on rat brain slices. Additionally, we identified unique substitutions (CαMeLeu) that conferred these analogues long duration of action in vivo. We selected one member of this family of CRF$_2$ selective analogues, astressin$_2$-B, for further in vivo testing and expect it to become a valuable tool to assess the role of CRF$_2$ receptor activation under acute and chronic stress conditions.

Materials and Methods

Peptide Synthesis and Characterization. All analogues shown in Table 1 were synthesized on a MBHA with a substitution varying from 0.25 to 0.5 mequiv/g using the Boc strategy with orthogonal protection of the side chains of the lysine (Fmoc) and glutamic acid (OFm) residues to be cyclized.[15,26,27,35] Amino acid derivatives Boc-Ala, Boc-Arg(Tos), Boc-Asn(Xan), Boc-Asp(cHex), Boc-Gln(Xan), Boc-Glu(cHex), Boc-His(Tos), Boc-Ile, Boc-Met, Boc-Leu, Boc-Phe, Boc-Pro, Boc-Ser(Bzl), Boc-Thr(Bzl), Boc-Tyr(2,6-Br$_2$-Bzl), and Boc-Val were obtained from Bachem Inc. (Torrance, CA). Boc-Glu(Ofm) and Boc-Lys(Fmoc) were synthesized as described earlier.[36] All solvents were reagent grade or better. TFA, 60% in DCM, was used to remove the Boc group. Main chain assembly was mediated by DIC. The coupling time was 90−120 min followed by acetylation (excess acetic anhydride in DCM for 15 min). Three-fold excess protected amino acid was used based on the original substitution of the methyl benzhydrylamine resin. Deprotection of the Fmoc group was achieved using a freshly prepared solution of 20% piperidine/DMF (2 × 10 min) followed by sequential washes with DMF, MeOH, 10% TEA/DCM, and DCM. Lactam formation was mediated using TBTU or HBTU in DMF or NMP. Best results were obtained when the peptide chain was assembled in its entirety prior to cleavage of the Fmoc and Ofm protecting groups and cyclization as shown earlier.[27] The peptides were cleaved and deprotected in HF at 0 °C for 1 h in the presence of a scavenger. HF was removed under vacuum and collected in a trap cooled in liquid nitrogen. Crude peptides were washed with ether and extracted with aqueous TFA (1%) in the presence of 30% acetonitrile (Acn).

After lyophilization, the crude peptide was purified with HPLC using linear gradients of Acn in three aqueous buffers (TEAP 2.25, TEAP 4.5, or TEAP 6.5 and 0.1% TFA).[15,26,37,38] The critical step in obtaining highly purified CRF analogues was the use of a TEAP buffer at a pH equal to or higher than 4.5 depending on the solubility of the peptide at these pH values. Under those conditions, impurities in amounts close to 30% that were difficult to detect in other buffer systems were eliminated. Peptides were characterized as shown in Table 1. Most analogues were determined to be greater than 95% pure using RPHPLC and CZE criteria. CZE was done using a Beckman P/ACE System 2050 controlled by an IBM Personal System/2 model 50Z and using a ChromJet integrator. Electrophoresis was performed in 0.1 M sodium phosphate (pH 2.5).

Mass Spectroscopy. LSIMS mass spectra were measured with a JEOL JMS-HX110 double-focusing mass spectrometer (JEOL, Tokyo, Japan) fitted with a Cs^+ gun. An accelerating voltage of 10 kV and a Cs^+ gun voltage between 25 and 30 kV were employed.[15] The calculated values for protonated molecular ions were in agreement with those obtained using LSIMS.

CD Spectropolarimetry (Figure 2). CD spectropolarimetry was conducted using an Aviv model 62DS spectropolarimeter (Aviv Associates, NJ) under control of the manufacturer's 60DS software. Constant conditions: wavelength span, 185–260 nm; collection frequency, 1.0 nm/point; integration time, 1.0 s; spectral bandwidth, 1.5 nm; data acquisition, 5 repetitions/spectrum; path length, 0.05 cm. Solvent cases: (a) aqueous, 0.01 M sodium phosphate buffer, pH 7.0; (b) 12.5% (v/v) TFE, 0.01 M sodium phosphate; (c) 50% (v/v) TFE, 0.005 M sodium phosphate. Peptide concentrations were calculated from the mass of the lyophilized powder assuming 8% water content corrected for TFA salt formation with cationic functions. Final peptide concentrations fell in the range of 0.30–0.35 mg/mL. Ellipticities are reported as mean residue ellipticity (deg-cm^2/dmol) based on the calculated peptide concentration. No postcollection data smoothing was employed. Spectral deconvolution employed the neural network back-propogation method of Böhm et al. (CDNN V2.1[32]) obtained from the Halle site (http://bioinformatik.biochemtech.uni-halle.de/cd_spec).

Radioreceptor Assay. [Tyr^0,Glu^1,Nle^{17}]sauvagine was radioiodinated using the Chloramine-T method as previously described.[39] Membrane proteins were prepared from stable Chinese hamster ovary (CHO) cell lines expressing hCRF$_1$[5] or mCRF$_{2\beta}$.[40] Clonal cell lines for each receptor type were established as previously described,[41] and crude plasma membranes were prepared as described.[42] Prepared membranes were stored in 10% sucrose at −80 °C until use. Test peptides and radioligand, ^{125}I[Tyr^0,Glu^1,Nle^{17}]sauvagine (~10 000 cpm), diluted in assay buffer (20 mM Hepes, 2 mM EGTA, 0.1% bovine serum albumin, 10% sucrose, pH 7.6) were combined with receptor in MAHV microtiter plates (Millipore) precoated with 0.1% polyethyleneimine. The reaction mixture was incubated for 90 min at room temperature followed by rapid washing twice with assay buffer and filtration. The radioligand complex was quantified by γ-counting. Inhibitory binding constants were determined using the Prism program.

CRF Receptor Autoradiography. In vitro CRF receptor autoradiography was performed in selected areas of the rat brain in order to evaluate whether CRF$_2$ selective analogues bind selectively in situ to CRF$_2$-expressing tissues. For this purpose, CRF receptor autoradiography was performed in sections of rat tissue containing CRF$_1$-expressing cortex or cerebellum and CRF$_2$-expressing blood vessels and choroid plexus, according to the method of de Souza and Kuhar[43] with minor modifications, using the universal CRF radioligand ^{125}I-[Tyr^0,Glu^1,Nle^{17}]sauvagine.[41] Its displacement by increasing concentrations of the unlabeled ^{125}I[Tyr^0,Glu^1,Nle^{17}]sauvagine and of the CRF$_2$ selective analogue astressin$_2$-B was analyzed on successive tissue sections, as described previously with other peptide receptors[44] and IC$_{50}$ values calculated for both peptides.

Measurements of Gastric Emptying. Male mice C57BL/6 (8–12 weeks, 20–25 g; Harlan, San Diego, CA) were housed in group cages with free access to food (Purina Chow) and tap water under controlled conditions of light:dark cycle (6:30 AM: 6:30 PM), temperature (21–23 °C), and humidity (30–35%). Experiments were conducted under the VA Animal Component of Research protocol number 99-070-04 in mice fasted for 18–20 h.

Rat urocortin, astressin$_2$-B, antisauvagine-30, and **28** were stored in powder form at −80 °C and dissolved in pyrogen free distilled water immediately before use. The ip and sc injections were performed in 5 mL/kg.

Gastric emptying of the nutrient solid meal was measured as described previously.[45,46] After an 18–20 h food but not water deprivation, mice were given Purina chow ad libitum for 1 h, and then, food and water were removed. Two hours later, mice were euthanized by cervical dislocation, the abdominal cavity was opened, and the stomach was removed and weighed, its content was washed out with tap water, and the gastric wall was wiped with gauze and weighed. The amount (g) of food contained in the stomach was quantified as the difference between the total weight of the stomach with the content and the weight of the gastric wall. The amount of food ingested by the mice was determined by the difference between the total weight of the Purina chow before feeding and the weight of the remaining Purina chow and spills at the end of the 1 h feeding period. The gastric emptying during the experimental period was calculated according to the following equation: gastric emptying (%) = [1 − (wet weight of food recovered from the stomach/weight of food intake)] × 100.

For the dose-related effects of CRF$_2$ antagonists, Purina chow and water were given for a 1 h period to fasted mice and then removed for the duration of the experiment. Urocortin (3 μg/kg) or vehicle (distilled water, 0.1 mL/mouse) was injected ip immediately at the end of the 1 h feeding period. Antisauvagine-30, astressin$_2$-B, **28** (3, 10, or 30 μg/kg in 0.1 mL), or vehicle (distilled water, 0.1 mL/mouse) was injected ip 10 min before urocortin or vehicle. Mice were euthanized by cervical dislocation 2 h after ip injection of urocortin or vehicle, and gastric emptying was monitored. To assess the time–course of CRF$_2$ antagonists, astressin$_2$-B (100 μg/kg), antisauvagine-30 (100 μg/kg), or vehicle (distilled water, 0.1 mL/mouse) was injected sc at either 3 or 6 h before the ip injection of urocortin (3 μg/kg) or vehicle (distilled water, 0.1 mL/mouse). The 1 h feeding period started at 2 or 5 h after the sc injection of vehicle, astressin$_2$-B, or antisauvagine-30. Gastric emptying was measured as described above 2 h after ip injection of vehicle or urocortin.

Abbreviations Used

The abbreviations for amino acids are in accord with the recommendations of the IUPAC–IUB Joint Commission on Biochemical Nomenclature (*Eur. J. Biochem.* **1984**, *138*, 9–37). Additional abbreviations: Adx rats, adrenalectomized rats; CRF, corticotropin releasing factor; CRF$_1$, CRF receptor 1; CRF$_2$, CRF receptor 2; CZE, capillary zone electrophoresis; DCM, dichloromethane; DMF, dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, *O*-(benzotriazol-l-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate; HPLC, high performance liquid chromatography; ip, intraperitoneal; LSIMS, liquid secondary ion mass spectrometry; MeOH, methanol; NMP, *N*-methylpyrrolidone; Ofm, O-fluorenylmethyl; SAR, structure activity relationships; sc, subcutaneous; TBTU, *O*-(benzotriazol-l-yl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate; TEAP, triethylammonium phosphate; TEA, triethylamine; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; Xaa, any amino acid.

Nomenclature

In 1995, we defined astressin as being a potent, long-acting human CRF (corticoliberin, CRH) competitive

antagonist. Astressin was the successor of earlier CRF antagonists named α-helical CRF$_{(9-41)}$[11] and [DPhe^{12}, Nle^{21,38},CαMeLeu^{37}]hCRF[14] that were incrementally more potent in their ability to inhibit CRF-induced release of ACTH from rat pituitary cells in culture. In that assay, astressin and astressin B were equipotent and 30 times more potent than the former.[31] Whereas α-helical CRF$_{(9-41)}$ is CRF$_2$ selective to a certain extent, neither astressin nor astressin B are receptor selective (Table 1 legend).

Here, we describe structural modifications that led to a new generation of CRF$_2$ selective, structurally constrained, and long-acting peptide antagonists. For the purpose of facilitating literature searches, we propose to name selected structurally constrained CRF/urocortin/sauvagine/urotensin peptide antagonists by the generic name of astressins. To facilitate the identification of the most useful analogues, we suggest that the next generations of nonselective constrained CRF antagonists be called astressin C, D, etc. whereas receptor selective astressins be called astressin$_x$-Y where x is either 1 or 2 (with the possible addition of α or β) for receptor identification and Y = B, C, etc. as identified above. This follows the guidelines of the CRF receptor subcommittee of the International Union of Pharmacology (IUPHAR). Similarly constrained synthetic peptide agonists could be called by the general name "stressins" with the first optimized CRF$_1$ selective agonist called stressin$_1$-A. We believe that this general term distinguishes native molecules with similar activities (ACTH-releasing activity) such as sauvagine in the frog, urotensins in the fish, and CRF, urocortin, urocortin II, and urocortin III in mammals, from synthetic analogues containing multiple substitutions by natural or unnatural amino acids. A literature search for stressin would therefore identify peptide analogues of the CRF family (agonists and antagonists), a literature search for astressin would identify peptide antagonists of the CRF family (receptor selective and nonselective), and a literature search for astressin$_x$ would identify peptide antagonists of the CRF family selective for receptor x (1, 2, 2β). Finally, a literature search for astressin$_2$-B would identify a single peptide antagonist of the CRF family selective for receptor 2 structurally related to astressin B. Obviously, IUPAC-recommended nomenclature should apply for closely related astressin analogues such as [DTyr^4]astressin$_2$-B.

We acknolwledge some limitations to this proposed nomenclature: although consistent, it suffers from the fact that both stressins and astressins imply a function associated with stress originally defined as the response to any real or perceived threat resulting in activation of the HPA axis. In reality, activation of the HPA axis is not representative of all of the CRF actions (in this particular case, it does not consider all CRF$_2$-mediated responses). Such misnomers however are frequent (corticoliberin being an example) and are the result of our limited but ever evolving knowledge.

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