Astressin Analogues (Corticotropin-Releasing Factor Antagonists) with Extended Duration of Action in the Rat[‖]

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In earlier reports we identified specific point substitutions (DPhe^{12},Nle^{21,38}), cyclization strategies [in particular, introduction of lactam rings such as that of cyclo(Glu^{30},Lys^{33})], and deletions (residues 1−7) in the CRF molecule that led to agonists. We also noted that further deletions (residues 8−14) produced antagonists such as astressin {cyclo(30−33)[DPhe^{12},Nle^{21,38},Glu^{30}, Lys^{33}]hCRF$_{(12−41)}$} (**1**). We hypothesized that the lactam ring promoted conformational stability to yield analogues with increased potency both in vitro and in vivo as compared to that of their linear counterparts. Additionally, we reported that cyclo(30−33)[DPhe^{12},Nle^{21,38}, Glu^{30},DHis^{32},Lys^{33}]hCRF$_{(12−41)}$ (**3**) and dicyclo(26−36,30−33)[Ac−Asp^{9},DPhe^{12},Nle^{21,38},Cys^{26}, Glu^{30},Lys^{33},Cys^{36}]hCRF$_{(9−41)}$ were ca. twice and 1/100 as potent as astressin, respectively, suggesting a putative turn that encompasses residues 30−33 (previous paper: Koerber et al. *J. Med. Chem.* **1998**, *41*). To increase the potency of **1** and/or **3** in vivo, we extended their chain length by one (**5−8**), two (**9**, **10**), and three (**11**, **12**) residues at the N-terminus and acetylated (**6**, **8**, **10**, **12**). Of the compounds tested for duration of action (**1**, **3−6**, **8**), we found **6** and **8** to be slightly longer-acting than astressin or [DHis^{32}]astressin, while their potencies in vitro were not significantly different from that of **3**. Additionally, we introduced CαMe-leucine residues in lieu of leucine at positions 14, 15, 19, 27, and 37 in [DHis^{32}]astressin. The analogue [CαMe-Leu^{27},DHis^{32}]astressin (**16**) was more potent (although not statistically in all cases) than the other four analogues in vitro. While acetylation of the N-terminus of **16** (i.e., **18**) or of [CαMe-Leu^{27}]astressin (i.e., **19**) did not have a significant effect on in vitro potency, elongation of the N-terminus by one or three residues in addition to acetylation resulted in cyclo(30−33)[DPhe^{12},Nle^{21},CαMe-Leu^{27},Glu^{30},DHis^{32},Lys^{33},Nle^{38}]Ac-hCRF$_{(11−41)}$ (**21**), cyclo(30−33)[DPhe^{12},Nle^{21},CαMe-Leu^{27},Glu^{30},Lys^{33},Nle^{38}]Ac-hCRF$_{(9−41)}$ (**22**), and cyclo(30−33)[DPhe^{12}, Nle^{21},CαMe-Leu^{27},Glu^{30},DHis^{32},Lys^{33},Nle^{38}]Ac-hCRF$_{(9−41)}$ (**23**) that were longer-acting than **6** and **8** (ca. 2 h inhibition of ACTH secretion at 25 μg/adrenalectomized rat). Analogues **22** and **23** were also more potent than astressin at reversing intracisternal CRF- and abdominal surgery-induced delay of gastric emptying in conscious rats.

Introduction

The existence of corticotropin-releasing factor (CRF) was first proposed in 1955 by Guillemin and Rosenberg[2]

and by Saffran and Schally.[3] Initially isolated from ovine hypothalamus and shown to be a 41-amino acid peptide in 1981,[4] CRF was subsequently also characterized from rat hypothalami,[5] and the identical structure was deduced for human CRF on the basis of the cDNA sequence of the human CRF precursor gene.[6] Presently, more than a dozen members of the CRF family (including sauvagine, urotensins, and urocortins) are known. From the point of view of structure−activity relationships (SAR), those native sequences can have less than 50% homology and yet exert similar activities (for example, ACTH release, lowering of blood pressure, and inhibition of gastric emptying) and high potencies, with distinct affinities for the different receptor classes and subtypes.[7−10]

Our interest in understanding the SAR of CRF stems from the hypothesis that conditions characterized by an imbalance of CRF might be alleviated by the administration of CRF/CRF agonists or antagonists (see Chrousos and Gold and references therein[11]). While oCRF and hCRF have been used in the clinic, CRF antagonists

have not been administered to humans, except for one study with α-helical-CRF$_{(9-41)}$[12] which suggested the need for analogues with increased potency and duration of action. Several observations were made that were critical to the discovery of CRF antagonists with increased potencies. Structural homology (α-helicity) of CRF, sauvagine, and urotensin was predicted by Chou–Fassman analysis[13] and documented by circular dichroism (CD) spectrophotometry.[14] Nuclear magnetic resonance (NMR) spectroscopy confirmed that CRF assumed significant helicity in a mixed organic/aqueous milieu (TFE/water).[15,16] Earlier, we hypothesized that α-helicity might be essential to receptor binding, and we synthesized α-helical-CRF that contained, at most positions, the amino acids with the highest P_α[13] values, found in the then known members of the CRF family. Because α-helical-CRF was found to be 3 times as potent as CRF,[17] we concluded that the bioactive conformation of CRF must be at least in part helical. Systematic deletion of residues at the N-terminus led to the identification of the first CRF antagonist, α-helical-CRF$_{(9-41)}$.[17] In a systematic scan of the oCRF sequence it was discovered that the [DPhe^{12}] substitution resulted in a 2-fold increase in potency over that of oCRF.[18] Introduction of DPhe at position 12 in [Nle^{21,38}]rCRF$_{(12-41)}$ yielded our most potent antagonist, which has been used as standard in our assays for several years [12 times as potent as α-helical-CRF$_{(9-41)}$].[18] Although Cα-methylation of selected leucine and alanine residues resulted in analogues that were somewhat more potent and marginally longer-acting than [DPhe^{12},Nle^{21,38}]-rCRF$_{(12-41)}$,[19] it was only with the discovery of cyclo-(30–33)[DPhe^{12},Nle^{21,38},Glu^{30},Lys^{33}]hCRF (astressin, **1**), a compound that is 32 times more potent than [DPhe^{12}, Nle^{21,38}]rCRF$_{(12-41)}$,[7,8,20] that two important structural issues could be addressed: i.e., is the cyclo(30–33) cycle promoting α-helicity or is it stabilizing a turn around residues 30–33 suggested by computer analysis?[14] We know that inversion of chirality of certain residues within turns is often well-tolerated, whereas it may destabilize helices.[1] Because [DHis^{32}]astressin is ca. twice as potent as astressin (although not statistically different), and because the weakly potent dicyclo(26–36,30–33)[Ac-Asp^9,DPhe^{12},Nle^{21,38},Cys^{26},Glu^{30},Lys^{33},-Cys^{36}]hCRF$_{(9-41)}$ is, according to CD data, also highly helical,[1] we concluded that a turn around residues 30–33 could not be excluded and that only NMR data of a highly potent dicyclic analogue could resolve that issue (work in progress). In this paper, we reexamine the effect on potency and duration of action of N-terminal lengthening and acetylation and of Cα-methylation of the endogenous leucine residues in astressin analogues. End points were ACTH release [measured in dispersed rat pituitary cells or the adrenalectomized (adx) rat] or gastric emptying (measured in the intact rat). Potency was assessed in the isolated cells, while duration of action was assessed in the adx rat.

Results and Discussion

All analogues shown in Table 1 were synthesized manually or automatically on a methylbenzdrylamine (MBHA) resin using the Boc strategy with orthogonal protection of the side chains of the lysine (Fmoc) and glutamic acid (OFm) residues to be cyclized.[8,20–22] Main-chain assembly was mediated in most cases by diisopropylcarbodiimide (DIC). The solid-phase assembly of the Cα-substituted CRF analogues presented some difficulties that were remedied using a 1:1 mixture of benzotriazolyloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP)/1-hydroxybenzotriazole (HOBt) [pH 9–10, 2 h and the usual excess (3.5-fold) of Boc-Cα-methylamino].[19,23] In case of incomplete couplings monitored by Kaiser's ninhydrin test,[24] couplings were repeated followed by acetylation. Cleavage of the Fmoc and OFm protecting groups was achieved using piperidine after complete assembly of the peptide resin; lactam formation was mediated by TBTU or BOP.[20] The peptide resins were treated with HF to liberate the fully deblocked crude peptides which were purified using reverse-phase HPLC and three different buffer systems in the following order: TEAP 2.25, TEAP 4.5, or TEAP 6.5 and 0.1% TFA.[20,22,25,26] The critical step in obtaining CRF analogues of high purity was that in which we used a TEAP buffer at a pH higher than 4.5 as described earlier.[1] The highly purified final products were isolated as the trifluoroacetate salts and characterized using HPLC, CZE, and liquid secondary ionization mass spectrometry (LSIMS) (Table 1). The measured masses obtained using LSIMS were in agreement with those calculated for the protonated molecule ions. The compounds were tested for their ability to antagonize CRF-induced release of ACTH by rat pituitary cells in culture.[4,17,27] Duration of action and relative in vivo potency were assessed in the rat in three models: the adx rat for inhibition of ACTH release and the conscious rat to reverse the ic CRF- and abdominal surgery-induced delay of gastric emptying according to published protocols (Table 2).[10] We used the rat pituitary cell assay and the adx rat model for the screening of our analogues. With analogues **1–12** we identified the optimum peptide length for maximal potency in vitro; in analogues **13–24** we introduced one CαMe-leucine as a means to increase structural and biostability.

Structural elements that define members of the astressin family include a D-phenylalanine substitution at position 12 and an optimized 30–33 cycle with an L- or D-residue at position 32 of CRF$_{(12-41)}$. This scaffold, discussed in detail in an earlier paper,[1] was used as a template for further improvements in potency. Before the discovery of astressin, it was accepted that antagonists which varied in length from CRF$_{(9-41)}$ to CRF$_{(12-41)}$ were essentially equipotent. Structural considerations led us to question whether this trend would remain with the introduction of the 30–33 cycle.[7] More precisely, we had postulated that the structural function of the 30–33 cycle was to stabilize (in the antagonist series) an α-helical conformation that would otherwise be fulfilled (in the agonist series) by the addition of several residues at the N-terminus. We therefore sought to optimize the length of the sequence that would also bind to the nonactivated form of the receptor(s).[9] Additionally, we surmised that acetylation of the N-terminus would be beneficial as a means of helix stabilization and to block aminopeptidase activity.[28] We next incrementally increased the size of astressin by the addition of one, two, and three residues with (**6**, **10**, **12**) and without (**5**, **9**, **11**) acetylation, having shown earlier that the addition of four residues resulted in agonists with essentially full intrinsic activity.[1] In the same series, two pairs of

Table 1. Characterization of CRF Antagonists by MS, HPLC, CZE, and in Vitro Relative Potency

	5	10	15	20	25	30	35	40

Human/Rat CRF 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 NH$_2$

no.	CRF antagonists	HPLC^a	CZE^b	MS (mono)^c calcd	found	relative potencies in vitro^d	IA^e	duration of action^f	ref
1	cyclo(30−33)[DPhe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]hCRF$_{(12−41)}$ (astressin)	95	97	3562.13	3562.2	32‡	0−5	SA	7
2	cyclo(30−33)[DPhe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]Ac-hCRF$_{(12−41)}$	95	96	3604.06	3604.1	37 (19−67)‡	3		1
3	cyclo(30−33)[DPhe^{12},Nle^{21},Glu^{30},DHis^{32},Lys^{33},Nle^{38}]hCRF$_{(12−41)}$	97	>97	3562.05	3562.4	56‡ 1.00‡‡	30 1 25	SA	1
4	cyclo(30−33)[DPhe^{12},Nle^{21},Glu^{30},DHis^{32},Lys^{33},Nle^{38}]Ac-hCRF$_{(12−41)}$	94	90	3604.06	3604.1	1.2 (0.41−3.3)‡‡	10	SA	1
5	cyclo(30−33)[DPhe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]hCRF$_{(11−41)}$	95	97	3663.10	3663.2	40 (28−72)‡	18	SA	
6	cyclo(30−33)[DPhe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]Ac-hCRF$_{(11−41)}$	>97	>97	3705.11	3705.1	64 (33−129)‡ 4.4 (2.7−7.1)‡‡ 2.0 (0.87−4.7)‡‡	6 17 14	SA+	
7	cyclo(30−33)[DPhe^{12},Nle^{21},Glu^{30},DHis^{32},Lys^{33},Nle^{38}]hCRF$_{(11−41)}$	95	80	3663.10	3663.2	0.78 (0.41−1.5)‡‡	12		
8	cyclo(30−33)[DPhe^{12},Nle^{21},Glu^{30},DHis^{32},Lys^{33},Nle^{38}]Ac-hCRF$_{(11−41)}$	95	90	3705.11	3705.2	3.1 (1.1−7.8)‡‡ 1.7 (0.88−3.4)‡‡	11 7	SA+	
9	cyclo(30−33)[DPhe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]hCRF$_{(10−41)}$	>97	>97	3776.18	3776.1	17 (9.1−31.3)‡ 32 (14−65)‡	18 27		
10	cyclo(30−33)[DPhe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]Ac-hCRF$_{(10−41)}$	96	>97	3818.19	3818.1	32 (16−69)‡ 2.5 (1.5−4.0)‡‡	22 17		
11	cyclo(30−33)[DPhe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]hCRF$_{(9−41)}$	97	>97	3891.21	3891.7	0.27 (0.13−0.53)‡‡	28 21		
12	cyclo(30−33)[DPhe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]Ac-hCRF$_{(9−41)}$	>97	>97	3933.22	3933.0	172 (68−522)‡ 0.77 (0.40−1.5)‡‡ 2.0 (1.1−3.7)‡‡	0 24 18		
13	cyclo(30−33)[DPhe^{12},CαMe-Leu^{14},Nle^{21},Glu^{30},DHis^{32},Lys^{33},Nle^{38}]hCRF$_{(12−41)}$	93	92	3576.07	3576.1	15 (7.5−29)‡	12		
14	cyclo(30−33)[DPhe^{12},CαMe-Leu^{15},Nle^{21},Glu^{30},DHis^{32},Lys^{33},Nle^{38}]hCRF$_{(12−41)}$	93	99	3576.07	3576.1	20 (12−37)‡	11		
15	cyclo(30−33)[DPhe^{12},CαMe-Leu^{19},Nle^{21},Glu^{30},DHis^{32},Lys^{33},Nle^{38}]hCRF$_{(12−41)}$	100	98	3576.07	3576.0	4.5 (1.9−9.7)‡	4.7		
16	cyclo(30−33)[DPhe^{12},Nle^{21},CαMe-Leu^{27},Glu^{30},DHis^{32},Lys^{33},Nle^{38}]hCRF$_{(12−41)}$	96	98	3576.07	3576.1	54 (26−112)‡ 0.89 (0.42−1.8)‡‡	7.4 0	SA	
17	cyclo(30−33)[DPhe^{12},Nle^{21},Glu^{30},DHis^{32},Lys^{33},CαMe-Leu^{37},Nle^{38}]hCRF$_{(12−41)}$	88	84	3576.07	3570.0	16 (9.3−26)‡	17		
18	cyclo(30−33)[DPhe^{12},Nle^{21},CαMe-Leu^{27},Glu^{30},DHis^{32},Lys^{33},Nle^{38}]Ac-hCRF$_{(12−41)}$	99	99	3618.08	3618.0	0.54 (0.20−1.2)‡‡	0		
19	cyclo(30−33)[Nle^{21},CαMe-Leu^{27},Glu^{30},Lys^{33},Nle^{38}]Ac-hCRF$_{(12−41)}$	90	97	3618.08	3618.0	1.7 (0.6−4.1)‡‡ 0.42 (0.18−0.92)‡‡	0 6		
20	cyclo(30−33)[DPhe^{12},Nle^{21},CαMe-Leu^{27},lu^{30},DHis^{32},Lys^{33},Nle^{38}]hCRF$_{(11−41)}$	95	90	3677.11	3677.2	1.3 (0.74−2.2)‡‡		SA	
21	cyclo(30−33)[DPhe^{12},Nle^{21},CαMe-Leu^{27},Glu^{30},DHis^{32},Lys^{33},Nle^{38}]Ac-hCRF$_{(11−41)}$	98	NA too phobic	3719−12	3719.2	1.4 (0.74−2.7)‡‡	10	MA	
22	cyclo(30−33)[DPhe^{12},Nle^{21},CαMe-Leu^{27},Glu^{30},Lys^{33},Nle^{38}]Ac-hCRF$_{(9−41)}$	93	92	3947.23	3947.9	1.7 (0.6−4.2)‡‡ 0.67 (0.41−1.1)‡‡	1.4 12	MA	
23	cyclo(30−33)[DPhe^{12},Nle^{21},CαMe-Leu^{27},Glu^{30},DHis,^{32}Lys^{33},Nle^{38}]Ac-hCRF$_{(9−41)}$	100	NA	3947.23	3947.2	3.9 (2.1−7.5)‡‡ 0.49 (0.30−0.79)‡‡	1.3 23	MA	
24	cyclo(30−33)[DPhe^{12},CαMe-Leu^{15},Nle^{21},Glu^{30},DHis(im-Bzl),^{32}Lys^{33},Nle^{38}]hCRF$_{(12−41)}$	96	96	3666.11	3666.2	24 (14−39)‡	13		

^{a} Percent purity determined by HPLC using buffer system: A = TEAP (pH 2.5) and B = 60% CH$_3$CN/40% A with a gradient slope of 1% B/min, at a flow rate of 0.2 mL/min on a Vydac C$_{18}$ column (0.21 × 15 cm, 5-μm particle size, 300-Å pore size), detection at 214 nm. ^{b} Capillary zone electrophoresis (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 H$_2$O−CH$_3$CN), pH 2.50, on a Supelco P175 capillary (363-μm o.d. × 75-μm i.d. × 50-cm length), detection at 214 nm. ^{c} The observed m/z of the monoisotope compared with the calculated [M + H]$^+$ monoisotopic mass. ^{d} Antagonist potencies are relative to that of [DPhe^{12},Nle^{21,38}]hCRF$_{(12−41)}$‡ or to that of cyclo(30−33)[DPhe^{12},Nle^{21},Glu^{30},DHis^{32},Lys^{33},Nle^{38}]hCRF$_{(12−41)}$‡‡ (**3**) in the in vitro rat pituitary cell culture assay, with 95% confidence limits in parentheses. Potencies of **1** and **3** relative to that of [DPhe^{12},Nle^{21,38}]hCRF$_{(12−41)}$‡ are average of three assays. ^{e} The percent intrinsic activity (IA) of each of the antagonists is calculated by determining the level of secretion caused by the highest dose of antagonist (in the absence of oCRF) minus basal secretion, dividing that number by the level of secretion of 1 nM oCRF minus basal secretion, and multiplying the result by 100. IA values for **1** and **3** are the average of three assays. ^{f} Definition of duration of action in the adx rat after iv administration of 25 μg of peptide/rat (100 μg/kg): short acting (SA), duration of action less than 90 min; medium acting (MA), shows complete inhibition at 120 min and no detectable standard error, with partial recovery at 180 min.

analogues with [DHis^{32}] (**3** and **7** are the nonacetylated homologues of **4** and **8**) were also synthesized and tested. It should be noted that the introduction of the [DHis^{32}] substitution resulted in two cases in doubling in in vitro potency over that of the corresponding analogues without [DHis^{32}] (**3** and **4** are approximately twice as potent as the corresponding antagonists **1** and **2** although not statistically different) and in two cases resulted in no improvement of potency (the two pairs of analogues **5** and **7** and **6** and **8** being equipotent). Elongation of astressin by one residue (**5**, **7**) has no effect on potency, while additional acetylation (**6**, **8**) may

Table 2. Comparison of CRF Antagonists' ED$_{50}$'s To Block Intracisternal CRF- and Abdominal Surgery-Induced Inhibition of Gastric Emptying in Conscious Rats

CRF antagonist	CRF	abdominal surgery
[DPhe^{12},Nle^{21,38}]hCRF$_{12-41}$	6.3 (4.7−8.5) 0.994	5.9 (4.0−8.8) 0.994
astressin	1.5 (0.8−2.9) 0.973	1.3 (0.41−4.4) 0.970
22	0.11 (0.092−0.14) 0.999	0.11 (0.075−0.15) 0.997
23	0.14 (0.12−0.16) 0.999	0.17 (0.085−0.33) 0.977

^{a} Data represent ED$_{50}$ values (μg/rat, ic) with the respective 95% confidence interval (in parentheses) and the r^2 according to a nonlinear regression model.

double potency. In comparing the potency of **5** to that of **7** (each tested against a different standard), we assume that **3** is 56 times more potent than [DPhe^{12}, Nle^{21,38}]hCRF$_{(12−41)}$ and that the potency of **7** against the same standard as the one used for **5** would be 0.78 × 56 = 44. We have found this converting factor to be quite accurate in some cases when the same analogues were tested against the two standards (**16**) and less so in others (**6**, **10**, **12**). It would take several assays of the same compounds against the two standards to reconcile these data. Further elongation without acetylation results in loss of potency (**9**, **11**), yet acetylation of these analogues may restore potency (**10**, **12**). These observations suggest that our in vitro assay is remarkably sensitive to minor changes in both primary and secondary structure of the analogues. The question, however, can be raised whether those differences in potencies (such as doubling) which are not statistically different (overlapping 95% confidence limits) can be used for the identification of useful substitutions. Data from our laboratory support that such trends observed in several series are generally indicative of a real effect that confirms intuitive (although statistically not significant) rankings in potency.

In the adx rat, astressin (**1**) significantly ($P < 0.01$) lowered plasma ACTH levels for at least 60 min (Figure 1A,B). Compounds **3** and **4** were only effective ($P < 0.01$) for 30 and 60 min, respectively (Figure 1A), while **5**, **6**, and **8** were effective ($P < 0.05−0.01$) for at least 90 min (**5**, Figure 1B) or 120 min (**6** and **8**, Figure 1B). By our definition, we consider **6** and **8** to still be short-acting. In summary, while in earlier studies, we had identified the Glu^{30}−Lys^{33} cycle and the [DPhe^{12}] and [DHis^{32}] substitutions as being favorable, the above data indicated that acetylation of the N-terminus of CRF antagonists may also have favorable effects on potency and duration of action.

At this point we decided to capitalize on the past observation that substitutions by CαMe-Leu residues had measurable effects on the potency and duration of action of CRF antagonists.[19] We therefore substituted leucines at positions 14, 15, 19, 27, and 37 of astressin and [DHis^{32}]astressin with CαMe-Leu (structures shown in Table 1). We hypothesized that this residue might block the action of endopeptidases in ways that could not be achieved with the introduction of D-residues alone.[18]

In a first step, we substituted leucines at positions 14, 15, 19, 27, and 37 of **3** with CαMe-Leu to yield **13**–**17** with in vitro potencies of 15, 20, 4.5, 54, and 16,
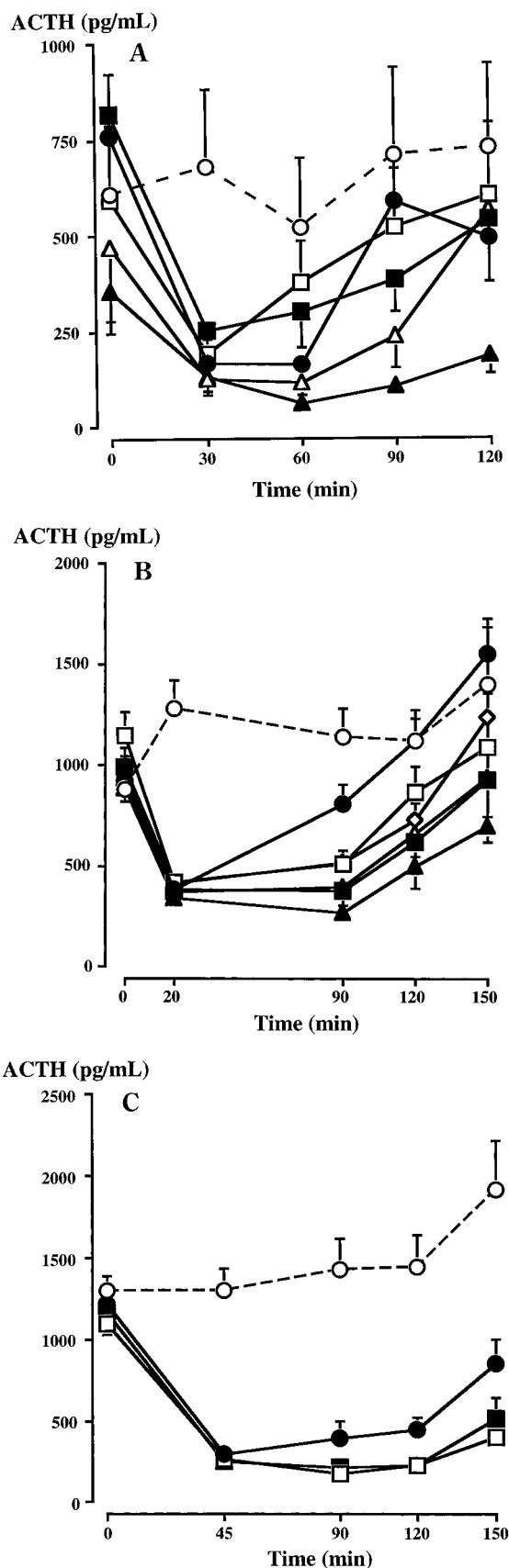

Figure 1. Effect of CRF antagonists (25 μg/rat iv) on ACTH secretion in adx rats. Each point represents the mean ± SEM of 3−7 animals. When not visible, SEM are encompassed within the symbol; control, ○. 1A: **1**, ●; **3**, □; **4**, ■; **16**, △; **21**, ▲. 1B: **1**, ●; **5**, □; **6**, ■; **8**, △; **21**, ▲; **20**, ◇. 1C: **21**, ●; **22**, □; **23**, ■.

respectively, that are relative to that of [DPhe^{12}, Nle^{21,38}]hCRF$_{(12-41)}$ (potency = 1). When tested against the parent analogue **3**, these peptides were approximately 0.25, 0.33, 0.08, 1.0, and 0.25 as potent as **3**. These results suggested that CαMe-Leu^{27} at position 27 was well-tolerated and that the other leucines (at positions 14, 15, 19, and 37) were particularly sensitive to modifications as minor as the introduction of a CαMe group which is predicted to favor helicity. This substitution by itself, however, had a minor beneficial effect on duration of action [**16** is longer-acting than **3** (Figure 1A), although both are considered short-acting by our definition]. It is noteworthy that substitutions of leucines at positions 14, 15, 19, 27, and 37 in oCRF by norleucines yielded analogues that, except for [Nle^{14}]-oCRF (with decreased binding affinity), had similar affinities for the CRF receptor as CRF[29] suggesting that the CαMe-Leu substitution may be more discriminating than a norleucine substitution. This may point to the enhanced susceptibility of the receptor for conformational rather than for steric parameters at these positions. Acetylation of the N-terminus of **16** and retention of the L-configuration of His^{32} yielded **18** and **19** that were essentially equipotent in vitro to their closest homologue **16**. Since **6** may be longer-acting than **5** (Figure 1B), we synthesized **20** and **21** which also showed an improvement in duration of action. While in the adx rat, **1** significantly ($P < 0.01$) lowered plasma ACTH levels for 60–90 min (Figure 1A,B), **16**, **20**, and **21** were effective ($P < 0.05-0.01$) for at least 90 min (**16**, Figure 1A), 120 min (**20** Figure 1B), or 150 min (**21**, Figure 1A–C), and **22** and **23** were effective for >150 min (Figure 1C). Although being equipotent in vitro, **21** inhibited ACTH secretion in the adx rat for at least 2.5 h which was almost twice the duration of action of **20** (Figure 1B) under the same experimental conditions. Although no in vivo data are available for **18** and **19**, comparison of the duration of action of **20** and **21** may suggest that **18** would be longer-acting than **16** (Figure 1A). When comparing the duration of action of **22** and **23**, it was evident that acetylation of the N-terminus was more important than the presence of either L- (**22**) or D-histidine (**23**) at position 32. Addition of CαMe-Leu^{27} and at least one residue (Ac-Thr^{11}) (**21**) or three residues (Ac-Asp^9,Leu^{10},Thr^{11}) (**23**) at the N-terminus of **1** had a marked effect on duration of action, extending it by a factor of at least 3 over that of **1**. It is noteworthy that **1** and **21**, tested in two/three consecutive in vivo assays (respectively), consistently gave the same duration of action (Figure 1A–C) suggesting that the subtle differences in duration of action seen between analogues is likely reflecting a unique property of each analogue. It is on the basis of a number of such observations that we have gained confidence in using what appeared as modifications with marginal effects in the design of ever more potent and longer-acting analogues.

We then sought to confirm the results obtained with the adx rats in two additional bioassays. We had previously shown that, after injection in the cerebrospinal fluid (CSF) at the level of the cisterna magna, CRF antagonists reversed CRF- and stress-induced gastrointestinal (GI) motor alterations.[10] In Table 2 we compare the ED$_{50}$'s of **22** and **23** injected into the CSF, to block CRF- and abdominal surgery-induced inhibition of gastric emptying in conscious rats, to that of [DPhe^{12},Nle^{21,38}]hCRF$_{(12-41)}$ and astressin. Both antagonists showed similar high potency and long-lasting activity in these assays. Both compounds were, based on their ED$_{50}$'s, about 10 times more potent than the related compound astressin and about 40 times more potent than the previously developed CRF antagonist [DPhe^{12},Nle^{21,38}]hCRF$_{(12-41)}$ in these systems. These results further strengthen the important role of CRF in stress-related alterations of gastrointestinal motor functions and the potential therapeutic interest of CRF antagonists to prevent postoperative ileus.

One further attempt to increase duration of action stemmed from an earlier observation that histidine-containing peptides become significantly less water-soluble at physiological pH as compared to their aqueous solubility at acidic pH. We have shown that this property can be further enhanced by the introduction of a benzyl group on the imidazole ring.[30] Since potency can be influenced by steric effects and duration of action can be modulated by hydrophobicity, we introduced DHis(im-Bzl) at position 32 of **14**. The observation that **24** is equipotent to **14** demonstrates that bulky D-residues can be accommodated at that position, as suggested by model studies in which a turn encompassing residues 30–33 has been introduced.[1]

In conclusion, extension of astressin's and [DHis^{32}]-astressin's sequence by one to three residues, acetylation of the N-terminus, and introduction of [CαMe-Leu^{27}] yielded a family of analogues with increased potency and extended duration of action as compared to that of astressin. It is suspected that Leu^{27} is either sensitive to enzymatic cleavage (which is blocked by the introduction of the CαMe group) or unique in its ability to maintain the overall bioactive conformation of CRF and is therefore likely to be helical around that residue. We have shown that the adx rat model and the reversal of CRF- or stress-induced delay of gastric emptying are three unusually reliable tools for SAR studies and optimization of pharmacokinetics in the rat. As in general the rat is a good model of human physiology, our data suggest that potent and long-acting CRF antagonists may be obtainable for use in the clinic. Yet, the limited duration of action of even the longest-acting known CRF antagonists (**21**–**23**) (as compared to that of other peptides such as GnRH antagonists[31] or melanotropin derivatives[32]) indicates that there may be other (biochemically or structurally) unrecognized sensitive regions in **21**–**23** that could be optimized to yield extended duration of action. Interestingly, the amino acids of the CRF molecule successfully modified in this report (positions 12, 21, 27, 30, 32, 33, and 38) to yield relatively long-acting CRF antagonists are not those shown to be sensitive to brain enzymes (positions 1, 11, 13, 15, 23, 35, and 36).[33] We acknowledge that, although these results suggest that CRF and CRF antagonists may have different sensitivities to central and peripheral enzymatic extracts, further data (including receptor affinity and peptide diffusion/transport) need to be obtained on the effect of substitutions of the sensitive amino acids at positions 1, 11, 13, 15, 23, 35, and 36 by unnatural ones and, possibly most importantly, on the effect of acetylation of the N-terminus.

Experimental Section

Synthesis of CRF Analogues. All analogues shown in Table 1 were synthesized either manually or on a Beckman 990 peptide synthesizer using the solid-phase approach, the MBHA resin,[20] and the Boc strategy with orthogonal protection (Fmoc and OFm) of the side chains of residues to be cyclized.[21] 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-Br-Cbz), and Boc-Val were obtained from Bachem Inc. (Torrance, CA), Chem-Impex International (Wood Dale, IL), and Calbiochem (San Diego, CA). Boc-C$^\alpha$Me-$_L$Leu,[19] Boc-Glu(OFm), and Boc-Lys(Fmoc)[34] were synthesized (and resolved where applicable) as described earlier. All solvents were reagent grade or better. TFA, 50–60% in DCM (1% *m*-cresol), was used to remove the Boc group. Main-chain assembly was mediated by DIC. Three-fold excess protected amino acid was used based on the original substitution of the MBHA resin. When the synthesis was carried out on a synthesizer, coupling time was 90–120 min followed by recoupling after residue 32 (with the exception of glycine and alanine residues which were not recoupled). Automatic acetylation (excess acetic anhydride in DCM for 15 min) was carried out after addition of each amino acid. When synthesized manually, recouplings were carried out only when necessary and acetylations only when recoupling could not yield negative ninhydrin tests.[24] Deprotection of the Fmoc group was achieved using a fresh solution of 20% piperidine/DMF or NMP (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.[20] The peptides were cleaved and deprotected in HF in the presence of anisole (5–10%, v/v) and purified using RP-HPLC and three solvent systems (TEAP at pH 2.25, TEAP at pH 4.5–6.5, and 0.1% TFA, successively).[25,26]

Characterization of CRF Analogues. Peptides were characterized as shown in Table 1 and below. Most analogues were greater than 95% pure with no impurity greater than 1% using independent HPLC and CZE criteria and had expected masses.

A. RP-HPLC. In addition to determining the purity of the peptides in an acidic system (see Table 1 legend), most of the analogues were also analyzed using 0.05% TEAP at pH 6.8 and a Vydac C$_8$ column (0.21 × 15 cm) at a flow rate of 0.2 mL/min with slightly varying gradient slopes. Percent purity was in the range of that found with CZE or with HPLC under acidic conditions.

B. Capillary Zone Electrophoresis (CZE). CZE was carried out using a Beckman P/ACE System 2000 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) except for α-hel-CRF$_{(9-41)}$ which was measured in 0.1 M sodium borate (pH 8.5). Acetonitrile (30%) was added to the buffers in order to gain sharp elution profiles.[35]

C. 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 Cs$^+$ gun voltage of 25 kV were employed; for further details, see ref 20. Calculated values for protonated molecule ions were in agreement with those observed using liquid secondary ion mass spectrometry.

D. In Vitro Pituitary Cell Culture Assay. Rat anterior pituitary glands from male Sprague–Dawley rats were dissociated by collagenase treatment and plated (0.16 × 10^6 cells/well in 48-well plates) in medium containing 2% fetal bovine serum (FBS).[27] Three days after plating, the cells were washed three times with fresh medium containing 0.1% bovine serum albumin (BSA) and incubated for 1 h. Following the 1-h preincubation, the cells were washed once more and the test peptides were applied. At the end of a 3-h incubation period, the media were collected and the level of ACTH was determined by radioimmunoassay (Diagnostic Products Corp.). When not mentioned, IA = 100%.

E. In Vivo Adx Rat Assay. Adult male rats were adrenalectomized under halothane anesthesia 8 days prior to the experiments. Their diet was supplemented with oranges, and their water contained 0.9% NaCl. They were equipped with indwelling jugular cannulae[36] 48 h prior to the iv injection of the vehicle or the antagonists. All protocols were approved by the Salk Institute IACUC. Astressin was first diluted in sterile distilled water, and the pH was adjusted to 7.0. Further dilutions were made in 0.04 M phosphate buffer, pH 7.4, containing 0.1% BSA and 0.01% ascorbic acid. Blood samples were obtained immediately before treatment as well as 15–120 min later. Decanted plasma were frozen until assayed for ACTH concentrations with a commercially available kit (Allegro Kit, Nichols Institute, San Juan Capistrano, CA).[36]

F. Reversal of Intracisternal CRF- and Abdominal Surgery-Induced Delay of Gastric Emptying. Animals: Adult male Sprague–Dawley rats (Harlan, San Diego, CA) weighing 250–280 g were used. Animals were housed in group cages with free access to food (Purina Rat Chow) and tap water and maintained at a 12:12-h light–dark cycle, under controlled temperature (21–23 °C). Animals were fasted, but had free access to water, for 18–20 h before experiments. All experimental protocols were approved by the Animal Use Committee of the West Los Angeles VA Medical Center.

Treatments: Peptides **22** and **23** were dissolved in doubly distilled HPLC water at pH 7.0 warmed to 37 °C. Peptides (at doses of 0.1, 0.3, and 1 μg/rat, in 5- or 10-μL volume as appropriate) were injected immediately before either CRF (0.6 μg/rat, 5 μL, ic) administration or abdominal surgery. Intracisternal injections were performed acutely under short enflurane anesthesia (2–3 min) by puncture of the occipital membrane with a Hamilton syringe, in rats, placed in ear bars of a stereotaxic equipment. Presence of CSF in the Hamilton syringe upon aspiration before injection ensured correctness of needle placement in the cisterna magna.

Measurement of Gastric Emptying: Gastric emptying was determined by the phenol red method, as previously described.[10] A suspension of continuously stirred 1.5% methyl cellulose (Sigma Chemical Co., St. Louis, MO) and phenol red (0.5%, Sigma Chemical Co.) was given intragastrically (1.5 mL) to conscious rats. After a 20-min period, rats were euthanized by CO$_2$ inhalation. The abdominal cavity was opened, the gastroesophageal junction and the pylorus were clamped, and the stomach was removed, rinsed in 0.9% saline, placed in 100 mL of 0.1 N NaOH, and homogenized (Polytron, Brinkmann Instruments). The suspension was allowed to settle for 1 h at room temperature, and then, 5 mL of the supernatant was added to 0.5 mL of 20% trichloroacetic acid (wt/vol) and then centrifuged at 3000 rpm at 4 °C for 20 min. The supernatant was mixed with 4 mL of 0.5 N NaOH and the absorbance of the sample read at 560 nm (Shimazu 260 spectrophotometer). Phenol red recovered from animals euthanized immediately after the administration of the methyl cellulose solution was used as standard (0% emptying). Percent emptying in the 20-min period was calculated according to the following equation:

$$\% \text{ emptying} = (1 - \text{absorbance of text sample}) \times 100/\text{absorbance of standard}$$

Experimental Protocols: 1. Intracisternal CRF-Induced Inhibition of Gastric Emptying. Under short enflurane anesthesia either [$_D$Phe^{12},Nle^{21,38}]CRF$_{(12-41)}$, astressin, **22**, **23** (0.1–10 μg/rat), or vehicle (5 μL) was injected ic immediately before the ic injection of either CRF (0.6 μg/rat) or vehicle (5 μL) in fasted rats. The ic dose of CRF was selected to produce a 60% inhibition of gastric emptying when tested under the same experimental conditions in our previous dose–response studies.[10] After ic injections, animals were returned to their home cages, and 10 min later, the marker used for the measurement of gastric emptying of noncaloric liquid meals was administered to awake rats. The rate of gastric emptying was determined 20 min after the administration of the marker.

2. Abdominal Surgery-Induced Inhibition of Gastric Emptying.

Under 10-min exposure of enflurane anesthesia (5.5% vapor concentration in O_2; Ethrane-Anaquest) either vehicle (10 μL) or astressin (1, 3, or 10 μg/rat) was injected ic and abdominal surgery with cecal manipulation was performed. Abdominal surgery consisted of a medial celiotomy (3−4 cm) and cecal exteriorization with cecal handling for a period of 1 min in gauze soaked with saline. The cecum was then returned to the abdominal cavity. The linea alba and the skin were sutured separately with 3−0 silk suture. The 20-min rate of gastric emptying was measured at 160 min after the completion of abdominal surgery.

G. Statistical Analysis. ED_{50}, defined as the dose of peptide antagonist inducing a 50% reversion of gastric ileus compared with the rate of emptying in vehicle-treated rats (taken as 0% reversion), was determined by nonlinear regression to a sigmoidal equation with variable slope (Prism, version 2.0; GraphPad, San Diego, CA).

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