Constrained Corticotropin Releasing Factor Antagonists (Astressin Analogues) with Long Duration of Action in the Rat†

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In an earlier report we identified specific modifications and substitutions of corticotropin releasing factor (CRF) that led to the discovery of antagonists with extended duration of action as compared to that of astressin {cyclo(30−33)[DPhe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]hCRF$_{(12-41)}$}. These additional modifications included elongation of the peptide chain by three residues at the N-terminus, its acetylation, and the [CαMeLeu^{27}] substitution to yield cyclo(30−33)[DPhe^{12}, Nle^{21},CαMeLeu^{27},Glu^{30},Lys^{33},Nle^{38}]Ac-hCRF$_{(9-41)}$, which was found to be longer acting than astressin (Rivier, J.; et al. *J. Med. Chem.* **1998**, *41*, 5012−5019). To further increase the efficiency (potency, duration of action, and bioavailability) of this family of antagonists, we introduced two or more CαMe-leucine residues at positions shown in earlier studies to be favorable (Hernandez, J.-F.; et al. *J. Med. Chem.* **1993**, *36*, 2860−2867). Whereas the introduction of CαMe-leucine residues at positions 27 and either 18 (**11**), 37 (**17**), or 40 (**19**) resulted in dramatic increases in duration of inhibitory action in the adrenalectomized (adx) rat after intravenous injection, the same substitution at positions 27 and either 15 (**7**, **8**), 17 (**9**), 19 (**12**, **13**), or 41 (**20**) led to short acting analogues. Other substitutions by CαMeLeu at positions 27 and either 10 (**4**), 13 (**5**), 14 (**6**), 21 (**14**), 24 (**15**), 36 (**16**), or 38 (**18**) yielded analogues with duration of action intermediate between those mentioned above. Cyclo(30−33)[DPhe^{12}, Nle^{21},CαMeLeu^{27},Glu^{30},Lys^{33},Nle^{38},CαMeLeu^{40}]Ac-hCRF$_{(9-41)}$ (astressin B, **19**) was one of the most efficacious analogues of this series (>4 h inhibition of ACTH secretion at 25 μg/adx rat). It was found to be even longer acting via subcutaneous administration in either an aqueous (>24 h inhibition of ACTH secretion at 100 μg/adx rat) or lipid milieu (DMSO/peanut oil, >24 h inhibition of ACTH secretion at 30 μg/adx rat) than after intravenous administration (<12 h inhibition of ACTH secretion at 100 μg/adx rat). We concluded that Cα-methylation at some positions may favor a bioactive conformation while also preventing degradation and/or elimination, resulting in significant extension of duration of action.

Introduction

The existence of corticotropin releasing factor (CRF) was first proposed in 1955 by Guillemin and Rosenberg[3] and Saffran and Schally.[4] Initially isolated from ovine hypothalamus and characterized as a 41 amino acid peptide in 1981,[5] CRF was subsequently characterized from rat hypothalami,[6] and the identical structure was deduced for human CRF on the basis of the cDNA sequence of the human CRF precursor gene.[7] Presently, more than a dozen members of the CRF family (including sauvagine, urotensins, and urocortins) have been described. CRF plays a major role in the maintenance or restoration of homeostasis by stimulating the activity of the hypothalamic-pituitary-adrenal (HPA) axis.[8] It also acts within the brain to control immune,[9] reproductive,[10] and cardiovascular functions,[11,12] as well as catecholamine release,[13] drug withdrawal,[14,15] behavior,[16] mood,[17] and anxiety.[18,19] CRF also acts through the release of adrenal corticosteroids to alter immune parameters[20] and to participate in the regulation of carbohydrate metabolism by enhancing the availability of glucose [reviewed in Dallman et al.[21]]. Finally, the broad central and peripheral distribution of the peptide and its two classes of seven-transmembrane-helix G-protein-coupled CRF receptors[22−28] supports the notion that CRF is also an important local neurotransmitter within the central nervous and immune systems among others.

CRF immunoreactivity and mRNA are found in cell bodies throughout the brain. CRF exerts a number of cellular actions in the brain; it modifies the production of cAMP, regulates the secretion of monoamines and neuropeptides, and modulates membrane potentials and ion currents. When CRF is injected into the brain, the sympathetic nervous system is activated while the parasympathetic nervous system is suppressed, resulting in an increase in heart rate and blood pressure, an

† Abbreviations. IUPAC rules are used for nomenclature of peptides including one-letter codes for amino acids. The following abbreviations are also used: Ac, acetyl; ACTH, adrenocorticotropin hormone; astressin, cyclo(30−33)[DPhe^{12},Nle^{21,38},Glu^{30},Lys^{33}]hCRF$_{(12-41)}$; Boc, *tert*-butyloxycarbonyl; BOP, benzotriazolyloxy-tris(dimethylamino)phosphonium hexafluorophosphate; BSA, bovine serum albumin; CRF, corticotropin releasing factor (o = ovine, h = human); CRFR, CRF receptor; CZE, capillary zone electrophoresis; DCM, dichloromethane; DIC, diisopropylcarbodiimide; DMF, dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HF, hydrogen fluoride; IA, intrinsic activity; MBHA, 4-methylbenzhydrylamine; NMP, *N*-methylpyrrolidone; OFm, *O*-fluorenylmethyl; SAR, structure−activity relationships; TBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; TEAP 2.25, 4.5, 6.5, triethylammonium phosphate pH 2.25, 4.5, 6.5; TFA, trifluoroacetic acid.

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inhibition of digestive functions (upper gastrointestinal tract), and activation of the lower gastrointestinal tract (elimination).[29] After intracerebroventricular administration of low doses, CRF stimulates arousal and increases performance in various learning and memory tests. At higher doses, CRF increases emotionality, induces fear, anxiety, and depression-like symptoms in some species. Antagonists, on the other hand, would have anxiolytic effects in that paradigm. For this reason, a number of pharmaceutical companies have concentrated their efforts toward the identification of non-peptide ligands to the CRF receptors that would be orally active and that would cross the blood brain barrier (BBB). Such non-peptide ligands for CRF-R_1 have been described.[30−37] The fact that they cross the BBB, however, limits their use as investigative tools to dissect the role of CRF in either the CNS or the periphery, as they will be distributed in both compartments.

Additionally, peripheral administration of CRF delays gastric emptying of a non-nutrient liquid meal when injected peripherally in conscious rats, mice, and dogs.[38−45] The inhibition of gastric motor function induced by CRF is mediated by activation of peripheral CRF receptors as shown by the complete inhibition of CRF's inhibitory action by pretreatment with the peptides CRF antagonist α-helical $CRF_{(9-41)}$, [D-Phe^{12}, $Nle^{21,38}$]$CRF_{(12-41)}$, and astressin.[38,46,47] All of these CRF antagonists injected intravenously also prevent gastric stasis induced by abdominal surgery in rats.[38,46,47] Therefore, the development of long acting CRF antagonists will be of relevance in the context of postoperative gastric ileus.

Our interest in understanding the structure−activity relationships (SAR) of CRF stems from the hypothesis that conditions characterized by an imbalance of CRF might be alleviated by the administration of long acting CRF agonists or antagonists. Except for one report describing the use of α-hel-CRF in humans, a weak and safe antagonist,[48] CRF peptide antagonists described so far have been too weak to warrant further clinical investigations. Although we have reported that astressin is longer acting than any earlier CRF antagonists, its duration of action is still too short to warrant its use in chronic studies that would lead to an understanding of the tonic role of peripheral CRF. Here we describe studies that led to the discovery of several CRF peptide antagonists that, due to their high potency, long duration of action, and the unlikelihood that they would cross the BBB, could also be promising clinical candidates to manage a number of conditions thought to be associated with excessive and sustained cortisol secretion. High levels of cortisol have been associated with depression, hypertension, osteoporosis, immunosuppression, and the entire spectrum of metabolic syndrome X (see Chrousos and Gold and references therein[49]). A potent peptide antagonist that would cross the BBB under certain modes of administration (such as intranasal), but not under other peripheral modalities, would also be very useful to manage conditions resulting from central, hypothalamic, and peripheral hypersecretion of CRF. Such states include mental (depression), immune (rheumatoid arthritis, thyroiditis, ulcerative colitis, immune uveitis), and gastrointestinal (ileus, irritable bowel syndrome, etc.) dysfunctions. Finally, excess CRF has also been implicated in inducing premature labor.[50]

In summary, CRF is considered to stimulate many of the functions that help the organism survive (such as locomotor activity and catecholamine release) while inhibiting those that might interfere with an effective stress response. We describe here additional modifications of cyclo(30−33)[DPhe^{12},Nle^{21},CαMeLeu^{27},Glu^{30}, DHis^{32},Lys^{33},Nle^{38}]Ac-$hCRF_{(9-41)}$[1] that led to CRF antagonists with such long duration of action that they may be useful and practical for chronic in vivo studies.

Results and Discussion

All analogues shown in Table 1 were synthesized either manually or automatically on a methylbenzhydrylamine (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.[51−54] 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 BOP/HOBt (pH 9−10, 2 h) and a 3.5-fold excess of Boc-Cα-methyl leucine.[2,55] In case of incomplete couplings, monitored by Kaiser's ninhydrin test,[56] 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.[52] 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.[1,52,54,57−59] 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 within 100 ppm of 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.[5,60,61] Duration of action was assessed in the adrenalectomized rat according to published procedures.[60]

In this article we describe SAR studies designed to identify additional residues to those shown earlier to increase the duration of action of astressin analogues in the adrenalectomized (adx) rat. The approach consisted of introducing CαMeLeu residues in addition to that in position 27 (**2**) which had already been found to be favorable.[1] The rationale for such substitutions was that CαMeLeu is compatible with retention of α-helical structure and is likely to be more resistant to the action of endopeptidases than Leu. Positions that were selected for such substitutions included those positions where leucine is found in native members of the CRF family. In CRF, these include positions 10, 14, 15, 19, and 37, in addition to position 27 that was shown to be a favorable substitution.[1] Additionally, Leu is a native substitution in the 40 amino acid human urocortin at positions 13, 14, 17, 18, and 20, which translates into positions 14, 15, 18, 19, and 21 in the 41 amino acid hCRF. In the 40 amino acid sauvagine, leucine exists at positions 9, 11, 13, 14, 35, 36, and 37, and corresponds

Table 1

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

ID no.	CRF antagonists	HPLC[a]	CZE[b]	MS (mono)[c] calcd	MS (mono)[c] found	rel. potencies[d] in vitro	IA[e]	dur. of action[f]	refs
1	cyclo(30−33)[DPhe^{12},Nle^{21},Glu^{30},DHis^{32}, Lys^{33},Nle^{38}]hCRF$_{(12-41)}$	97	>97	3562.05	3562.4	1.00	25	SA	59
2	cyclo(30−33)[DPhe^{12},Nle^{21},CαMeLeu^{27},Glu^{30},Lys^{33}, Nle^{38}]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	1
3	cyclo(30−33)[DPhe^{12},Nle^{21},CαMeLeu^{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	1

ID no.	with two CαMeLeu substitutions	HPLC[a]	CZE[b]	MS (mono)[c] calcd	MS (mono)[c] found	rel. potencies[d] in vitro	IA[e]	dur. of action[f]
4	cyclo(30−33)[CαMeLeu^{10},DPhe^{12},Nle^{21},CαMeLeu^{27},Glu^{30}, Lys^{33},Nle^{38}]Ac-hCRF$_{(9-41)}$	94	97	3961.2	3961.3	0.18 (0.07−0.41)	34	MA
5	cyclo(30−33)[DPhe^{12},CαMeLeu^{13},Nle^{21},CαMeLeu^{27},Glu^{30}, Lys^{33},Nle^{38}]Ac-hCRF$_{(9-41)}$	95	94	3937.3	3937.3	0.8 (0.35−1.9)	9	MA
6	cyclo(30−33)[DPhe^{12},CαMeLeu^{14},Nle^{21},CαMeLeu^{27},Glu^{30}, Lys^{33},Nle^{38}]Ac-hCRF$_{(9-41)}$	98	98	3961.2	3961.3	0.68 (0.42−1.1)	0	MA
7	cyclo(30−33)[DPhe^{12},CαMeLeu^{15},Nle^{21},CαMeLeu^{27},Glu^{30}, DHis^{32},Lys^{33},Nle^{38}]Ac-hCRF$_{(11-41)}$	96	95	3733.14	3733.1	1.1 (0.62−2.0)	0	SA
8	cyclo(30−33)[DPhe^{12},CαMeLeu^{15},Nle^{21},CαMeLeu^{27},Glu^{30}, Lys^{33},Nle^{38}]Ac-hCRF$_{(9-41)}$	94	99	3961.2	3961.3	0.55 (0.26−1.2)	3	SA
9	cyclo(30−33)[DPhe^{12},CαMeLeu^{17},Nle^{21},CαMeLeu^{27},Glu^{30}, Lys^{33},Nle^{38}]Ac-hCRF$_{(9-41)}$	83	79	3945.3	3945.3	0.10 (0.03−0.26)	0	SA
10	cyclo(30−33)[DPhe^{12},CαMeLeu^{18},Nle^{21},CαMeLeu^{27},Glu^{30}, DHis^{32},Lys^{33},Nle^{38}]Ac-hCRF$_{(11-41)}$	97	100	3747.15	3746.9	1.9 (0.97−4.1)	9	MA
11	cyclo(30−33)[DPhe^{12},CαMeLeu^{18},Nle^{21},CαMeLeu^{27},Glu^{30}, Lys^{33},Nle^{38}]Ac-hCRF$_{(9-41)}$	93	95	3975.3	3975.8	0.64 (0.26−1.6)	18	LA
12	cyclo(30−33)[DPhe^{12},CαMeLeu^{19},Nle^{21},CαMeLeu^{27},Glu^{30}, DHis^{32},Lys^{33},Nle^{38}]Ac-hCRF$_{(11-41)}$	98	99	3733.14	3733.2	0.57 (0.36−0.90)	5	SA
13	cyclo(30−33)[DPhe^{12},CαMeLeu^{19},Nle^{21},CαMeLeu^{27},Glu^{30}, Lys^{33},Nle^{38}]Ac-hCRF$_{(9-41)}$	94	99	3961.2	3961.3	0.066 (0.030−0.14)	15	SA
14	cyclo(30−33)[DPhe^{12},CαMeLeu^{21,27},Glu^{30},Lys^{33}, Nle^{38}]Ac-hCRF$_{(9-41)}$	93	96	3961.25	3961.0	4.3 (0.95−23)	0	MA
15	cyclo(30−33)[DPhe^{12},Nle^{21},CαMeLeu^{24,27},Glu^{30},Lys^{33}, Nle^{38}]Ac-hCRF$_{(9-41)}$	98	97	4003.3	4003.3	0.77 (0.29−2.0)	0	MA
16	cyclo(30−33)[DPhe^{12},Nle^{21},CαMeLeu^{27},Glu^{30},Lys^{33}, CαMeLeu^{36},Nle^{38}]Ac-hCRF$_{(9-41)}$	95	97	3946.24	3946.3	1.7 (0.53−5.8)	0	MA
17	cyclo(30−33)[DPhe^{12},Nle^{21},CαMeLeu^{27},Glu^{30},Lys^{33}, CαMeLeu^{37},Nle^{38}]Ac-hCRF$_{(9-41)}$	98	99	3961.2	3961.2	0.51 (0.29−0.89)	5	LA
18	cyclo(30−33)[DPhe^{12},Nle^{21},CαMeLeu^{27},Glu^{30},Lys^{33}, CαMeLeu^{38}]Ac-hCRF$_{(9-41)}$	99	97	3961.2	3961.3	0.27 (0.16−0.45)		
19	cyclo(30−33)[DPhe^{12},Nle^{21},CαMeLeu^{27},Glu^{30},Lys^{33}, Nle^{38},CαMeLeu^{40}]Ac-hCRF$_{(9-41)}$ (astressin B)	98	99	3961.2	3961.3	0.45 (0.25−0.80) 1.0 (0.47−2.3) 1.1 (0.50−2.5)	13 0 12	LA
20	cyclo(30−33)[DPhe^{12},Nle^{21},CαMeLeu^{27},Glu^{30},Lys^{33}, Nle^{38},CαMeLeu^{41}]Ac-hCRF$_{(9-41)}$	96	99	3961.2	3961.3	0.020 (0.009−0.040) 0.037 (0.013−0.092)	14	SA

ID no.	with three CαMeLeu substitutions	HPLC[a]	CZE[b]	MS (mono)[c] calcd	MS (mono)[c] found	rel. potencies[d] in vitro	IA[e]	dur. of action[f]
21	cyclo(30−33)[DPhe^{12},CαMeLeu^{14},Nle^{21},Nle^{27},Glu^{30}, Lys^{33},CαMeLeu^{37},Nle^{38},CαMeLeu^{40}]Ac-hCRF$_{(9-41)}$	96	97	3975.2	3975.3	0.50 (0.23−1.0)	6	MA
22	cyclo(30−33)[DPhe^{12},CαMeLeu^{21},CαMeLeu^{27},Glu^{30}, Lys^{33},Nle^{38},CαMeLeu^{40}]Ac-hCRF$_{(9-41)}$	98	96	3975.27	3975.34	0.43 (0.19−0.93)	13	LA
23	cyclo(30−33)[DPhe^{12},Nle^{21},CαMeLeu^{27},Glu^{30},Lys^{33}, CαMeLeu^{37},Nle^{38},CαMeLeu^{40}]Ac-hCRF$_{(9-41)}$	90	90	3975.3	3975.2	0.75 (0.37−1.5)	19	LA

ID no.	with four CαMeLeu substitutions	HPLC[a]	CZE[b]	MS (mono)[c] calcd	MS (mono)[c] found	rel. potencies[d] in vitro	IA[e]	dur. of action[f]
24	cyclo(30−33)[DPhe^{12},CαMeLeu^{14},Nle^{21}, CαMeLeu^{27},Glu^{30},Lys^{33},CαMeLeu^{37},Nle^{38}, CαMeLeu^{40}]Ac-hCRF$_{(9-41)}$	97	96	3989.3	3989.4	0.60 (0.33−1.1)	7	LA
25	cyclo(30−33)[DPhe^{12},CαMeLeu^{17},Nle^{21}, CαMeLeu^{27},Glu^{30},Lys^{33},CαMeLeu^{37},Nle^{38}, CαMeLeu^{40}]Ac-hCRF$_{(9-41)}$	98	95	3973.32	3973.3	0.22 (0.08−0.55)	0	SA

[a] Percent purity determined by HPLC using the following buffer system: A = TEAP (pH 2.5) and B = 60% CH_3CN/40% A with a gradient slope of 1% B/min, at flow rate of 0.2 mL/min on a Vydac C_{18} column (0.25 × 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_2O:CH_3CN) pH 2.50, on a Supelco P175 capillary (363 μm OD × 75 μm ID × 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 cyclo(30−33)[DPhe^{12},Nle^{21},Glu^{30},DHis^{32},Lys^{33},Nle^{38}]hCRF$_{(12-41)}$ in the in vitro rat pituitary cell culture assay, with 95% confidence limits in parentheses. [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 and dividing that number by the level of secretion of 1 nM oCRF minus basal secretion and multiplying the result by 100. [f] Definition of duration of action in the adrenalectomized rat after iv administration of 25 μg peptide/rat. Short acting: duration of action less than 90 min. Medium acting: shows complete inhibition at 120 min and no detectable standard error, with partial recovery at 180 min. Long acting: shows complete inhibition at 240 min and no detectable standard error, with partial recovery at 300 min.

to residues 10, 12, 14, 15, 36, 37, and 38 in hCRF. Additionally, Leu is found at position 40 of tilapia CRF. We also introduced CαMeLeu at position 13 instead of histidine and at position 41 instead of Ile where Beyermann et al. had found it to be compatible.[62]

In vitro potencies relative to that of cyclo(30−33)-[DPhe^{12},Nle^{21},Glu^{30},DHis^{32},Lys^{33},Nle^{38}]hCRF$_{(12-41)}$ (**1**) resulting from the introduction of CαMeLeu at positions 10 (**4**), 13 (**5**), 14 (**6**), 15 (**7**, **8**), 17 (**9**), 18 (**10**, **11**), 19 (**12**, **13**), 21 (**14**), 24 (**15**), 36 (**16**), 37 (**17**), 38 (**18**), 40 (**19**), and 41 (**20**) of **2** are given in Table 1. Additionally three related analogues were synthesized that were shorter by two residues (**7**, **10**, **12**) to test the hypothesis that improved biological properties brought about by the introduction of CαMeLeu would compensate for the loss of duration of action in the adx rat, observed when residues 9 and 10 were deleted.[1]

While the relative in vitro potencies varied from 2 to 4% (**20**) to 430% (**14**), introduction of CαMeLeu at those different positions similarly resulted in analogues with durations of action that varied from shorter to longer than that of the parent analogue **2**. We arbitrarily decided that an analogue would be identified as having an intermediate duration of action when its subcutaneous injection to adx rats at a dose of 100 μg/kg in 200 μL buffer would maximally inhibit ACTH secretion in all rats (4−7 rats per point) for 2 h with partial recovery at 3 h. Analogues that had a duration of action shorter than that of **2** were those with CαMeLeu at positions 15 (**7**, **8**), 17 (**9**), 19 (**12**, **13**), and 41 (**20**) (Figure 1A−C). There does not seem to be any correlation between potencies in vitro and duration of action in vivo. Indeed, there is as much as a 50-fold difference between the in vitro potency of the most (110% for **7**) and least (2−4% for **20**) potent analogues of this series of short acting analogues, which is not surprising in view of the expected complexity of the pharmacokinetics of these relatively large peptides.

While **20** may be the least potent member of this series in vitro and also short acting for steric reasons (native substitutions in the CRF family include Ala, Ile, Val, and Phe, yet [DAla^{41}]oCRF is 100 times less potent than oCRF[63]), it is not possible to rationalize why **7**, **8**, and **12**, which are statistically equipotent to **2** in vitro, would be shorter acting in vivo. It should be noted, when examining Figure 1A, that the distinction between the qualitatives short, medium, and long acting (defined in the legend of Table 1) is arbitrary and is only used for the purpose of the discussion. In Figure 1A for example, the durations of action of analogues **20**, **15**, and **4** are certainly not different. In Figure 1C on the other hand, analogues are more clearly grouped in three distinct categories. Analogues that had duration of action similar to that of **2** are those with CαMeLeu at positions 10 (**4**), 13 (**5**), 14 (**6**), 18 (**10**), 21 (**14**), 24 (**15**), 36 (**16**), and 38 (**18**). Again, there does not seem to be much correlation between in vitro potencies spanning from 18% (**4**) to 430% (**14**) in this series of analogues exhibiting intermediate duration of action. We conclude from these data that the CαMeLeu substitution at these positions has no effect on any of the parameters suspected to influence duration of action (secondary structure that may influence affinity and/or resistance to enzymatic degradation). Clearly of more interest are those positions where CαMeLeu substitutions resulted in increased duration of action. These positions are 18 (**11**), 37 (**17**), and 40 (**19**). In this case, in vitro potencies are

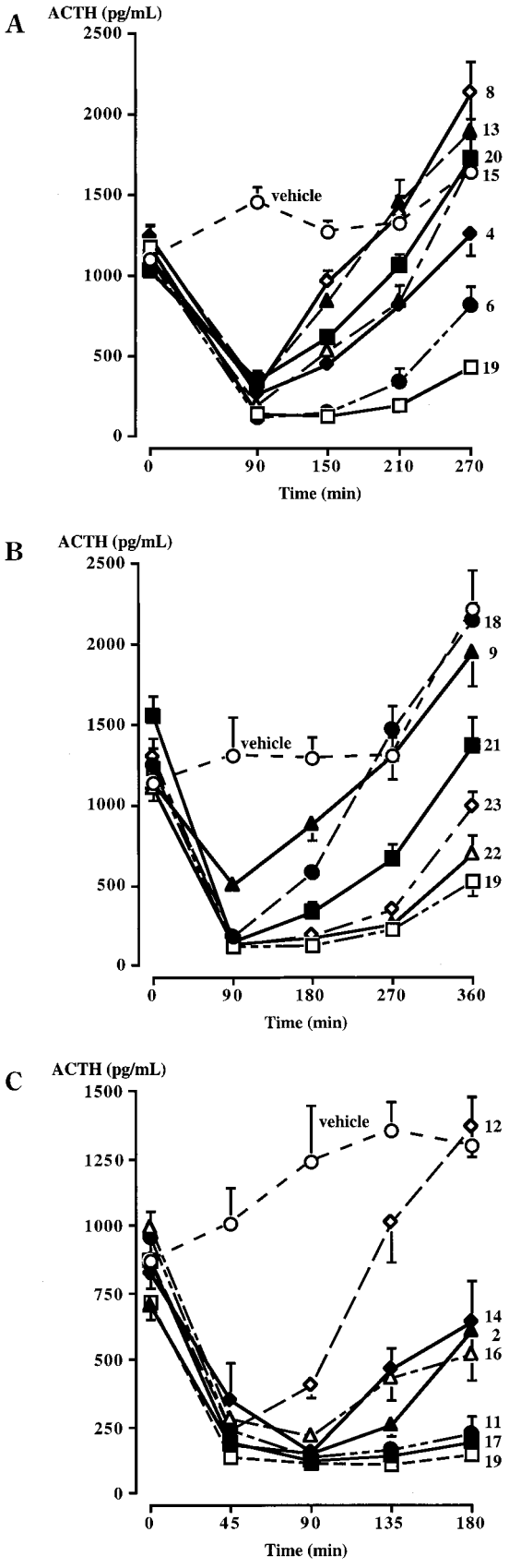

Figure 1. (A−C) Effect of CRF antagonists (25 μg/rat iv) on ACTH secretion in adx rats. Each point represents the mean ± SEM of 4−7 animals. When not visible, SEM are encompassed within the symbols.

less scattered and only vary from 51% (**17**) to 110% (**19**). This emphasizes the importance of in vivo data in selecting those modifications more likely to improve the desirable properties (potency and duration of action) of CRF antagonists. Finally, it should be noted that while the first significant decrease in plasma ACTH levels was measured at 90 min in Figure 1A,B, earlier experiments as well as results shown in Figure 1C (first time point at 45 min) show that the onset of action of CRF antagonists is almost immediate and profound.

Next, we investigated the effect of more than two CαMeLeu substitutions on in vitro potencies and duration of action of these CRF antagonists. As indicated by the in vivo data shown for astressin B (**19**) (Figure 1A−C), this analogue has been consistently the longest acting, and its inhibition curve could be used as a reference point. As can be seen, the duration of action of and extent of inhibition of ACTH by **19** is consistent from assay to assay. We therefore asked whether additional CαMeLeu substitutions would further increase duration of action. In the few analogues synthesized and tested (**21**−**23**), this was not the case; introduction of CαMeLeu at position 14 (**21**) was detrimental or without additional improvement at positions 21 and 37 (**22**, **23**). Finally, two CαMeLeu substitutions in addition to those at positions 27 and 40 had either no additional positive effect (**24**) or was deleterious (**25**). In vivo data for analogues **3**, **5**, **7**, **10**, **22**, and **25** are not shown as they are similar to those shown in Figures 1A−C.

Reinvestigation of the role of residues at positions 9 and 10 with respect to duration of action confirmed earlier results that these residues were important for sustained duration of action.[1] Antagonists **7**, **12**, and **10** (analogues that lack these residues) are either as short acting or shorter acting than their parent analogues (**8**, **13**, and **11**, respectively).

Figure 1B shows that the duration of action of **19** extends beyond 6 h at the dose of ca. 25 μg/rat. We hypothesized that such duration of action could be modulated by the way the peptide was delivered and by the excipient in which it was delivered.

It is generally accepted that sc administration of peptides extends duration of action as compared to iv administration and that the excipient used may also influence release of the peptide from the injection site. We investigated the effect, over 24 h, of sc administration of graded doses of **19** in an aqueous buffer (Figure 2B) and peanut oil (Figure 2C) compared to iv administration of an equivalent dose (Figure 2A). Intravenous administration of **19** at a dose of 100 μg/rat inhibited ACTH secretion at 6 but not 12 h, suggesting possible degradation and elimination. A similar profile of inhibition was obtained with 30 μg/rat ($^1/_3$ the dose administered iv) when administered sc while 100 and 300 μg/rat significantly inhibited ACTH secretion for more than 24 h (Figure 2B). The effect of **19** on ACTH secretion in adx rats was even more dramatic after sc administration in peanut oil, and inhibition was observed for more than 24 h after administration of only 30 μg of **19** per rat (Figure 2C). For the sake of simplicity, as we expect **19** to be widely used for biological studies, and because of its homology with astressin, we decided to call it astressin B.

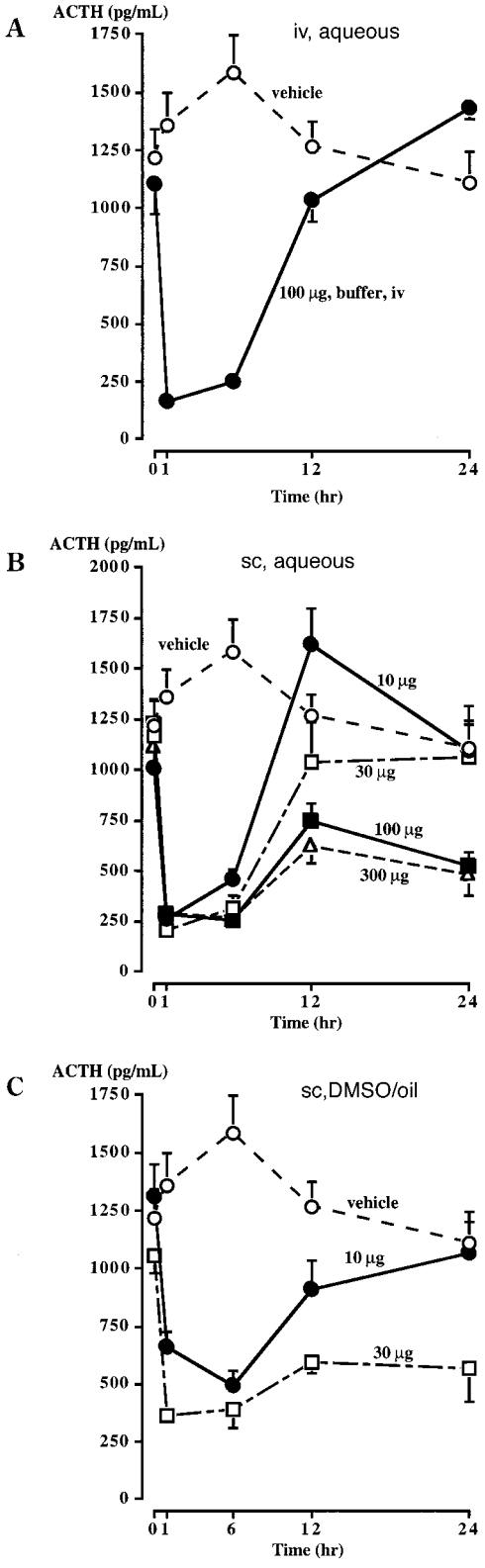

Figure 2. (A) Effect of astressin B (**19**, 100 μg/adx rat iv) on ACTH secretion. Each point represents the mean ± SEM of 6 animals. When not visible, SEM are encompassed within the symbol. (B) Effect of astressin B (**19**, graded doses/adx rat, sc in aqueous buffer) on ACTH secretion. Each point represents the mean ± SEM of 5−8 animals. When not visible, SEM are encompassed within the symbol. (C) Effect of astressin B (**19**, graded doses/adx rat, sc in DMSO/peanut oil) on ACTH secretion. Each point represents the mean ± SEM of 6 or 7 animals. When not visible, SEM are encompassed within the symbol.

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,[52] and the Boc strategy with orthogonal protection (Fmoc and OFm) of the side chains of residues to be cyclized.[51] 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-CαMe-L-Leu,[64] Boc-Glu(OFm), and Boc-Lys(Fmoc)[65] were synthesized 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 carrying out the synthesis 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.[56] 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.[52] 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).[57,58]

Scaled-Up Synthesis and Purification of Cyclo (30−33)[DPhe^{12},CαMeLeu^{27},Glu^{30},Lys^{33},Nle^{21,38},CαMeLeu^{40}]h/rCRF$_{9-41}$ (Astressin B, 19). For most analogues prepared in this investigation, 1.0−2.0 g (0.5 mmol/g) of MBHA resin were deprotected and neutralized according to the protocol described earlier in this section. Here, however, we detail the scale-up of one of the most potent analogues starting with 7.1 g of 0.5 mmol/g substituted MBHA resin, (3.55 mmol). The Boc-CαMeLeu-OH, Boc-Glu(OFm)-OH, and Boc-Lys-(Fmoc)-OH were prepared in-house whereas all other amino acids were commercially available. All coupling reactions were monitored by ninhydrin test[56] and were facilitated by using 0.1 M DIC for 1−3 h with the exception of His(Tos),[32] CαMeLeu,[27] and CαMeLeu[40] which required the use of 3 equiv of BOP/DIPEA to drive the reaction to completion. Because deprotected CαMeLeu does not give a positive ninhydrin test result, the residue following CαMeLeu was always coupled twice and followed by acetylation. In addition, Asn[34] required a double coupling step. After incorporation of the last amino acid, the Nα-Boc protecting group was removed and the N-terminus was acetylated. The OFm/Fmoc groups of Glu[30] and Lys[33], respectively, were removed by two 10 min treatments with 20% piperidine in NMP. The formation of the bridge was accomplished in 12 h (negative ninhydrin test) using BOP/DIEA at room temperature. A total of 15.4 g of protected peptide resin was obtained and was cleaved in three batches (5.3, 5.0, and 4.9 g) by anhydrous HF (50 mL) in the presence of anisole (5 mL) at 0 °C for 90 min. The crude peptide was precipitated and washed with anhydrous diethyl ether, filtered, extracted from the resin with 150 mL of 0.1% TFA in CH_3CN/H_2O (60:40), and lyophilized to give 2.71, 2.13, and 2.10 g of crude product, respectively. Each batch of crude peptide material was independently analyzed by HPLC and determined to be of similar composition so the three batches were combined prior to purification. Preparative purification was performed in three stages as described above. First the peptide (1.5−2.0 g) was dissolved in 300 mL of buffer A (TEAP pH 2.25) and eluted with buffer B (75% CH_3CN in A), with a gradient from 35 to 55% B in 60 min (retention time was ca. 30 min). Approximately 20 fractions containing 50−100 mL were screened under isocratic conditions (76% B, retention time was ca. 4 min); enriched fractions containing the desired product with purity >85% from each of the four separate runs were identified and pooled. Fractions of lower purity (65−85%) were pooled separately and reloaded for an additional TEAP pH 2.25 run from which additional fractions with >85% purity were isolated. In the second system, the pooled fractions (>85% pure) were diluted 1:1 with H_2O and the pH was adjusted to 4 with TEA. This solution was loaded onto the same C_{18} cartridge in two runs. The peptide was eluted using buffer A:TEAP pH 4.3 and buffer B:75% CH_3CN in TEAP pH 4.3, with a gradient from 40 to 60% B in 60 min. A total of 15−20 fractions containing 30−50 mL each were screened; 4 fractions with purities >90% were pooled from each run. In the final step, the pooled fractions (>90% pure in ca. 400 mL) were diluted with H_2O (400 mL) containing 1 mL of TFA. This solution was loaded and eluted by using buffer A:0.1% TFA/H_2O and B:0.1% TFA in CH_3CN/H_2O (75:25), with a gradient from 30 to 80% B in 40 min (retention time was ca. 30 min). A total of 11 fractions containing 30−50 mL each were screened; 3 fractions (150 mL) were pooled that showed purity >95%. Side fractions containing less than 10% hydrophilic and hydrophobic impurities relative to the desired product were reloaded, and an additional amount of highly purified **19** was added to the pool prior to lyophilization. Yield of highly purified **19** was 564 mg (126 μmol), 3.5% of theoretical amount from the original substitution of the MBHA resin.

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.

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.

Capillary Zone Electrophoresis. Capillary zone electrophoresis (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 (15%) was added to the buffers in order to gain sharp elution profiles.[66]

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 52. Calculated values for protonated molecule ions were in agreement with those observed using LSIMS.

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.[61] 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 at five concentrations were applied in the presence of 1 nm hCRF. At the end of a 3 h incubation period, the media were collected and the level of ACTH was determined by radioimmunoassay (Diagnostic Products Corporation).

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. Adrenalectomized rats maintained in a temperature-controlled environment, with

ready access to food and no lurking predators against which to fight (i.e., kept in a nonstressful environment), are quite healthy. They gain weight, have a shiny fur, and are very active. Their life expectancy is normal. They mate and carry and nurse their young with no problems. Such rats were equipped with indwelling jugular cannulae[67] 48 h prior to the iv injection of the vehicle or the antagonists. All protocols were approved by the Salk Institute IACUC. Analogues were 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. The same buffer and dilutions were used for the sc administration (Figure 2B). All peptides remained in solution under these conditions. For the experiment shown in Figure 2C, peptide was dissolved in DMSO (10 mg/mL) and diluted to the desired concentration with peanut oil (Sigma). Blood samples were obtained immediately before treatment as well as at regular intervals. Decanted plasma were frozen until assayed for ACTH concentrations with a commercially available kit (Allegro kit, Nichols Institute, San Juan Capistrano, CA).[67]

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