A Single-Point Slight Alteration Set as a Tool for Structure−Activity Relationship Studies of Ovine Corticotropin Releasing Factor†

Michael Beyermann,* Klaus Fechner, Jens Furkert, Eberhard Krause, and Michael Bienert

Institute of Molecular Pharmacology, Alfred-Kowalke-Strasse 4, 10315 Berlin, Germany

Received February 5, 1996[⊗]

In order to determine which amino acid side chains of ovine corticotropin releasing factor (oCRF) are most sensitive to alterations with respect to receptor binding and activation, we synthesized a single-point replacement set by replacing each residue by a similar, preferably proteinogenic amino acid, maintaining a minimal change of character at each position (Ser by Thr, Gln by Asn, Glu by Asp, Arg by Lys, and vice versa, Pro by N-MeAla, Ile by Leu, Leu by Nle, Phe by Trp, His by Ala, Val by Leu, Met by Nle, Ala by Leu). In general, any loss in the biological potency by a single-point substitution in oCRF parallels a decrease in receptor binding, indicating that, in contrast to previous suggestions,[1] there is no specific side chain in the peptide that is more responsible for receptor activation than for receptor binding. In addition to Arg(16), Ala(31), and Arg(35), amino acid residues in the N-terminal sequence (5−14) were found to be sensitive to alteration, demonstrating their particular importance for the receptor interaction of CRF agonists. Most of the analogs tested exhibited agonistic potencies in an in vitro pituitary cell culture assay at a concentration of 0.3 nM, and all analogs showed full agonistic potency at 1 μM. In contrast to the results of an alanine replacement study,[2] the strongest decrease in receptor binding and biological potency was observed for analogs with substitutions of hydrophilic amino acids Ser(7), Arg(16), Glu(17), or Asn(34). In the case of Ser(7) and Arg(16), side chain specific interactions with the receptor may be required for high affinity. Alanine replacements at positions 17 or 34 resulted in analogs that were as potent as oCRF, while replacement of Glu(17) by Asp or Asn(34) by Gln caused a dramatic loss in potency, thereby suggesting an important effect at sterically or conformationally sensitive positions. In contrast to corresponding alanine analogs which exhibited a significant loss in biological potency,[2] slight alterations of lipophilic side chains at positions 6, 12, or 38 did not cause a significant reduction of receptor binding and activation, indicating that it is not specific side chains but rather lipophilicity which is essential at these positions. Indeed, replacement of Phe(12) by Trp provides an agonist with significantly increased receptor binding and biological potency.

Introduction

Corticotropin releasing factor (CRF), a 41 amino acid peptide originally isolated from the ovine hypothalamus based on its ability to stimulate adrenocorticotropin (ACTH) and β-endorphin release from cultured anterior pituitary cells,[3] is the principal neuroregulator of the basal and stress-induced secretion of ACTH, β-endorphin, and other proopiomelanocortin-related peptides from the anterior pituitary.[4] In addition to its endocrine role in the regulation of the hypothalamic−pituitary−adrenal axis, CRF seems to be implicated in a variety of other central and peripheral functions including food intake,[5] thermoregulation,[6] reproduction,[7] inflammation,[8] and cardiovascular function.[9] CRF exerts its effects by binding to high-affinity membrane receptors which are coupled to Gs-protein, resulting in increased intracellular cAMP levels.[10,11] Recently, cDNAs encoding CRF receptors were identified.[12−20] Two CRF receptor subtypes were characterized pharmacologically by their different rank order of dose-dependent intracellular cAMP responses induced by frog sauvagine, urotensin I, and CRF,[17−19] all of which are members of a family of peptides from different species that activate CRF receptors. While cells expressing the CRF1 receptor responded to sauvagine, urotensin, rat CRF, and ovine CRF in a dose-dependent manner with almost the same half-maximal concentration (EC_{50}) of 3 nM, 4 nM, 4 nM, and 10 nM, respectively, cells expressing the CRF2 receptor responded at (EC_{50}) 0.5 nM, 2 nM, 20 nM, and 80 nM, respectively.[17] Therefore, for structure−activity relationship studies, one has to take into consideration which receptor subtype is involved. In rat, the mRNA for the CRF1 receptor is predominantly expressed in the brain (cerebellum, cerebral cortex, olfactory bulb) and in the pituitary. CRF2 receptor mRNA was detected in brain, lung, and heart, but unremarkably in pituitary. Since radiolabeled Tyr(0)-oCRF did not show a high level of specific binding to the CRF2 receptor compared to the CRF1 receptor, and since the CRF2 receptors may be expressed to a minor extent in rat brain,[17] application of [^{125}I]-Tyr(0)-oCRF as the ligand in a displacement assay (rat brain membranes) guarantees that binding will mainly occur at the CRF1 receptor. This provides the possibility of discussing binding results relative to the potency of ACTH release from the pituitary and of comparing our results with those of previous structure−activity relationship investigations.

† **Abbreviations**: The abbreviations for the amino acids are in accord with the recommendations of the IUPAC−IUB Joint Commission on Biochemical nomenclature (*Eur. J. Biochem.* **1984**, *138*, 9−37). The symbols represent the L-isomer. In addition: Nle, norleucine; MeA, *N*-methylalanine; Aib, α-aminoisobutyric acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; oCRF, ovine corticotropin releasing factor; ACTH, adrenocorticotropin hormone; BSA, bovine serum albumin; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; Fmoc, 9-fluorenylmethyloxycarbonyl.

⊗ Abstract published in *Advance ACS Abstracts,* July 1, 1996.

S0022-2623(96)00116-1 CCC: $12.00

Concerning structure−activity relationship studies of CRF,[1,2,21,23−29] the major contribution was the finding that deletion of the N-terminal sequence 1−8 led to CRF antagonists.[1] Recently, a more potent CRF antagonist, D-Phe(12),-Nle(21,38),-C^{α}-MeLeu(37)-rCRF(12−41), was described.[21,22] Therefore, it was suggested that the N-terminus, particularly residues 4−8, is most important for receptor activation. On the other hand, the fact that N-terminally deleted analogs, such as D-Phe(12),-Nle(21,38),-Aib(25)-rCRF(12−41), show relatively high intrinsic activity[21] makes it clear that the structural basis for agonistic activity is not yet fully understood.

Concerning the role of individual amino acid side chains for receptor activation, an alanine replacement set of oCRF was investigated.[2] According to ACTH release from anterior pituitary cells induced by the alanine analogs, it was found that substitutions in the N-terminus (5−19) were always detrimental to biological activity, while substitution in the C-terminal part (20−41) had little effect on ACTH release. Although alanine scans have often been used for elucidation of the role of individual side chain functionalities in biologically active peptides,[30−34] the approach has the drawback that in the case of decreased biological activity it cannot be concluded whether the effect is caused by involvement of the parent amino acid side chain in a highly specific interaction with the receptor or by a change in the general character of the side chains (hydrophilic, lipophilic, charged, aromatic). In order to answer the question, we synthesized a single-point slight alteration set of oCRF by replacing each amino acid residue by a similar, preferably proteinogenic one, whereby a minimal change of lipophilicity, size, and of charge at each position was intentional (Ser by Thr, Gln by Asn, Glu by Asp, Arg by Lys, and vice versa, Pro by N-MeAla, Ile by Leu, Leu by Nle, Phe by Trp, His by Ala, Val by Leu, Met by Nle, Ala by Leu). Because rat tissues were used for both binding and functional assays, it would have been appropriate to study such a replacement set of the native ligand rat CRF. But, in order to make a comparison possible between results of a slight alteration set with those of both alanine and D-amino acid scans, which have been done using oCRF and rat tissues,[2,23] corresponding analogs of oCRF were investigated.

Results

Peptide Synthesis, Purification, and Characterization. CRF and its analogs were synthesized automatically on a MilliGen 9050 peptide synthesizer by the solid-phase method using standard Fmoc chemistry in the continuous flow mode. Syntheses were carried out on a 4-[(2′,4′-dimethoxyphenyl)aminomethyl]phenoxyacetamido resin (0.22 mmol/g) using Nα-Fmoc-protected amino acid derivatives (0.3 M) and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (0.3 M) as coupling reagent in the presence of 2 equiv of diisopropylethylamine in dimethylformamide (DMF). Single couplings for 20 min were allowed to proceed, N-terminal deblocking was carried out with 25% piperidine in DMF for 10 min, and all washes were made with DMF. Final cleavage from the resin and deprotection of side chain functionalities were achieved by a mixture of 88% TFA/5% phenol/5% H_2O/2% triisopropylsilane for 3 h. Purification of 100-mg samples was carried out by preparative HPLC on PolyEncap A300, 10 μm, 250 × 20 mm i.d., using an acetonitrile/water−0.1% TFA solvent system (linear gradient 25−45% acetonitrile in 70 min), to give final products >95% pure by HPLC analysis.

HPLC analyses were carried out on a PolyEncap A300 using two solvent systems (0.1% TFA/water−acetonitrile or 0.07 M phosphate−acetonitrile). Peptides were characterized by matrix-assisted laser desorption/ionization mass spectrometry which gave the expected $[M + H]^+$ mass peaks (Table 1). Amino acid analyses of purified peptides gave results which were consistent with the expected structures (data not shown).

Receptor Binding. Receptor binding of the oCRF analogs was estimated in a displacement assay by use of a rat brain membrane fraction (P2) and [^{125}I]-Tyr(0)-oCRF as radioligand. With respect to their receptor affinity, the oCRF analogs may be divided into three groups (Table 1): (i) analogs with decreased affinity (<60% of oCRF) substituted at positions 5, 6, 7, 8, 9, 10, 11, 13, 14, 16, 17, 20, 30, 31, 34, 35, or 41; (ii) analogs as potent as oCRF (60−150%) with substitution at positions 1, 2, 3, 4, 15, 19, 21, 22, 23, 24, 26, 27, 28, 29, 32, 36, 37, and 39; (iii) analogs with increased affinity (>150%) substituted at positions 12, 18, 25, 33, 38, or 40 (Table 1). The results show that amino acid residues in the N-terminal region of CRF (positions 5−17) are most sensitive to alteration.

Substitution of completely conserved amino acid residues within the CRF family (Table 2) is expected to result in decreased receptor binding. In fact, substitution of Pro(5), Ile(6), Ser(7), Asp(9), Leu(10), Leu(14), Arg(16), Glu(20), Gln(30), Ala(31), Asn(34), or Arg(35) led to analogs with decreased affinity. Only substitution of Leu(15) by Nle or Pro(4) by *N*-MeAla, respectively, was possible with little or no loss in affinity. In principle, substitutions in oCRF at functionally conserved positions (12, 18, 19, 23, 25, 37, 38, and 39), with amino acid side chains of the same character, yielded analogs without loss in receptor binding, but in the case of substitution at positions 8, 11, or 41 a significant loss in affinity was observed. The expectation that substitutions at nonconserved positions (1, 2, 3, 13, 17, 21, 22, 24, 26, 27, 28, 29, 32, 33, 36, 40) should have no influence on receptor affinity was generally met except for two exceptions. Strongly decreased binding was observed on replacing His(13) by Ala or Glu(17) by Asp.

According to very low receptor affinity of their substituted analogs (≤10%) at Ser(7), Arg(16), Glu(17), and Asn(34), these positions were shown to be of particular importance for the receptor binding of oCRF.

ACTH Release. The biological potency of the analogs studied here to induce ACTH release from anterior pituitary cells was determined at approximately the half-maximal concentration of oCRF (0.3 nM). Of greatest interest to us were those analogs that exhibited the same or even increased receptor affinity compared with oCRF, since these derivatives may be better agonists, or in the case of those lacking receptor activation, competitive antagonists. Actually, only single-point substitution of Phe(12) by Trp led to an analog with significantly increased ACTH release. All other analogs with high receptor affinities exhibited agonistic activities comparable to that of oCRF (Table 1). Those analogs showing decreased receptor affinity also exhibited decreased biological potency with a direct correlation being observed between receptor binding and activation.

Table 1. Characterization of oCRF and Its Analogs: Ser-Gln-Glu-Pro-Pro-Ile-Ser-Leu-Asp-Leu-Thr-Phe-His-Leu-Leu-Arg-Glu-Val-Leu-Glu-Met-Thr-Lys-Ala-Asp-Gln-Leu-Ala-Gln-Gln-Ala-His-Ser-Asn-Arg-Lys-Leu-Leu-Asp-Ile-Ala-NH_2 (oCRF)

substituted oCRF analog	HPLC purity(%)[a]	mw calcd	mw found	rel binding [$K_i \pm$ SE (nM)]	ACTH release related to max response (%) (SD)
oCRF	99.5	4670.4	4671	1.00 (4.31 ± 0.99)	52.2 (7.6)
Thr^{1}-oCRF	99.5	4684.4	4684	0.84 (5.13 ± 0.87)	
Asn^{2}-oCRF	99.1	4656.4	4657	1.12 (3.84 ± 0.46)	
Asp^{3}-oCRF	98.5	4656.4	4655	1.21 (3.56 ± 0.53)	
MeA^{4}-oCRF	98.3	4659.4	4658	1.17 (3.68 ± 0.96)	42.6 (4.1)
MeA^{5}-oCRF	99.7	4659.4	4657	0.37 (11.6 ± 2.44)	33.9 (1.7)
Trp^{6}-oCRF	98.3	4743.5	4743	0.45 (9.49 ± 1.23)	
Leu^{6}-oCRF	100	4670.4	4672	0.28 (15.5 ± 3.73)	36.0 (7.0)
Thr^{7}-oCRF	100	4684.4	4683	0.05 (82.7 ± 24.0)	4.10 (3.6)
Nle^{8}-oCRF	98.7	4670.4	4671	0.20 (21.8 ± 7.20)	34.3 (5.7)
Glu^{9}-oCRF	99.4	4684.4	4686	0.17 (26.1 ± 4.17)	22.2 (4.2)
Nle^{10}-oCRF	98.4	4670.4	4671	0.13 (32.6 ± 9.13)	25.3 (4.3)
Ser^{11}-oCRF	99.5	4656.4	4656	0.11 (41.0 ± 16.0)	19.3 (5.0)
Leu^{12}-oCRF	98.8	4636.4	4635	0.34 (12.7 ± 3.30)	
Trp^{12}-oCRF	97.8	4709.4	4708	5.03 (0.86 ± 0.14)	73.3 (5.8)
Ala^{13}-oCRF	95.8	4604.3	4604	0.13 (32.9 ± 4.94)	16.3 (3.9)
Glu^{13}-oCRF	91.8	4662.4	4662	0.26 (16.3 ± 2.12)	
Nle^{14}-oCRF	98.8	4670.4	4671	0.24 (18.1 ± 6.32)	25.5 (5.4)
Nle^{15}-oCRF	99.7	4670.4	4673	0.69 (6.29 ± 2.20)	39.1 (13.2)
Lys^{16}-oCRF	99.6	4642.4	4643	0.02 (180.9 ± 38)	7.1 (2.6)
Asp^{17}-oCRF	99.6	4656.4	4655	0.06 (72.5 ± 13.8)	9.2 (1.9)
Ala^{17}-oCRF	96.4	4669.5	4670	1.21 (3.56 ± 0.68)	
Leu^{18}-oCRF	98.8	4684.4	4685	2.85 (1.51 ± 0.36)	49.2 (9.6)
Nle^{19}-oCRF	98.5	4670.4	4671	0.68 (6.33 ± 1.46)	36.1 (13.1)
Asp^{20}-oCRF	100	4656.4	4655	0.36 (12.0 ± 3.00)	29.4 (11.9)
Nle^{21}-oCRF	99.6	4652.4	4653	1.30 (3.31 ± 0.63)	59.0 (4.3)
Ser^{22}-oCRF	99.5	4656.4	4657	0.75 (5.71 ± 1.03)	58.0 (3.2)
Arg^{23}-oCRF	97.9	4698.4	4698	1.00 (4.30 ± 0.90)	45.0 (8.1)
Leu^{24}-oCRF	95.8	4712.5	4711	0.91 (4.73 ± 0.95)	41.8 (7.1)
Glu^{25}-oCRF	99.3	4684.4	4685	1.94 (2.22 ± 0.44)	63.8 (7.4)
Asn^{26}-oCRF	98.5	4656.4	4656	1.02 (4.24 ± 1.36)	51.6 (6.7)
Nle^{27}-oCRF	99.4	4670.4	4671	0.62 (6.98 ± 1.12)	45.0 (3.5)
Leu^{28}-oCRF	97.9	4712.5	4714	1.00 (4.30 ± 0.90)	43.4 (5.8)
Asn^{29}-oCRF	99.4	4656.4	4657	0.69 (6.28 ± 1.13)	47.4 (3.0)
Asn^{30}-oCRF	98.7	4656.4	4657	0.19 (22.4 ± 4.49)	28.0 (4.2)
Leu^{31}-oCRF	98.7	4712.5	4714	0.12 (36.6 ± 6.96)	18.6 (3.4)
Ala^{32}-oCRF	99.0	4604.3	4606	0.82 (5.24 ± 1.47)	54.1 (15.8)
Thr^{33}-oCRF	98.5	4684.4	4685	2.47 (1.75 ± 0.37)	65.9 (2.9)
Gln^{34}-oCRF	99.0	4684.4	4685	0.01 (383.7 ± 103.6)	3.0 (2.1)
Lys^{35}-oCRF	99.5	4642.4	4644	0.14 (30.7 ± 4.91)	25.7 (7.2)
Arg^{36}-oCRF	98.2	4698.4	4699	0.87 (4.93 ± 1.38)	57.2 (3.2)
Nle^{37}-oCRF	98.9	4670.4	4672	0.71 (6.06 ± 1.09)	59.0 (2.2)
Nle^{38}-oCRF	97.5	4670.4	4671	3.57 (1.21 ± 0.31)	60.3 (4.8)
Glu^{39}-oCRF	98.2	4684.4	4684	0.88 (4.90 ± 0.88)	63.2 (1.4)
Leu^{40}-oCRF	100	4670.4	4672	1.63 (2.64 ± 0.63)	62.6 (2.9)
Leu^{41}-oCRF	99.4	4712.4	4714	0.38 (11.3 ± 2.03)	50.6 (3.9)

[a] HPLC analyses were carried out on a PolyEncap A300 column, 250 × 4.6 mm i.d., 5 μm, in 0.1% TFA/water (mobile phase A), 0.1% TFA in 70% acetonitrile/30% water (v/v) (mobile phase B), and with a linear gradient of 20−80% B in 40 min.

Table 2. The CRF Family

	1				5					10					15					20											
Ovine/Caprine CRF	S	Q	E	P	P	I	S	L	D	L	T	F	H	L	L	R	E	V	L	E										
Bovine CRF	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-										
Human/Rat CRF	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-										
Porcine CRF	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-										
Sucker CRF	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-										
Frog Sauvagine	-	-	G	-	-	-	-	-	-	S	L	E	-	-	-	K	M	I	-											
Carp Urotensin	N	D	D	-	-	-	-	I	-	-	-	-	-	-	-	N	M	I	-											
										25					30					35					40					
Ovine/Caprine CRF	M	T	K	A	D	Q	L	A	Q	Q	A	H	S	N	R	K	L	L	D	I	A	NH_2								
Bovine CRF	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	NH_2								
Human/Rat CRF	-	A	R	-	E	-	-	-	-	-	-	-	-	-	-	-	-	M	E	-	I	NH_2								
Porcine CRF	-	A	R	-	E	-	-	-	-	-	-	-	-	-	-	-	-	M	E	N	F	NH_2								
Sucker CRF	-	A	R	-	E	-	-	-	-	-	-	-	-	-	-	-	M	M	E	-	F	NH_2								
Frog Sauvagine	I	E	-	Q	E	K	E	K	-	-	-	A	N	-	-	L	-	-	-	T	I	NH_2								
Carp Urotensin	-	A	R	N	E	N	Q	R	E	-	-	G	L	-	-	-	Y	-	-	E	V	NH_2								

In addition, the maximal ACTH response of the single-point replacement set was investigated at a peptide concentration of 1 μM. All oCRF analogs exhibited full agonistic activity at this concentration (data not shown here).

Discussion

For biologically active peptides that exert their activity via highly specific binding to proteins such as receptors, single-point amino acid replacement sets are valuable tools for the systematic elucidation of the peptide−receptor interaction. Most frequently, alanine replacement sets have been used in order to investigate the role of individual amino acid side chains in receptor interaction, and D-amino acid replacement sets were applied to gain insight into bioactive conformations. More recently, He et al.[35] have proposed another interesting approach by comparing the influence of peptide main chain (Cα) with side chain methylation. However, this method requires considerable effort in the preparation of the corresponding analogs of the proteinogenic amino acids. As a simple approach which retains the general character of a substituted amino

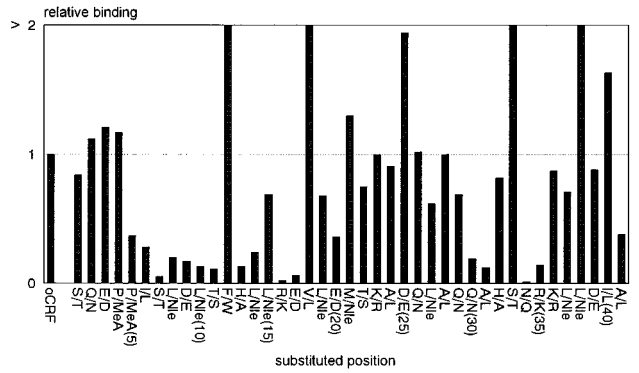

Figure 1. Single-point slight alteration set of oCRF: relative receptor binding (see Table 1).

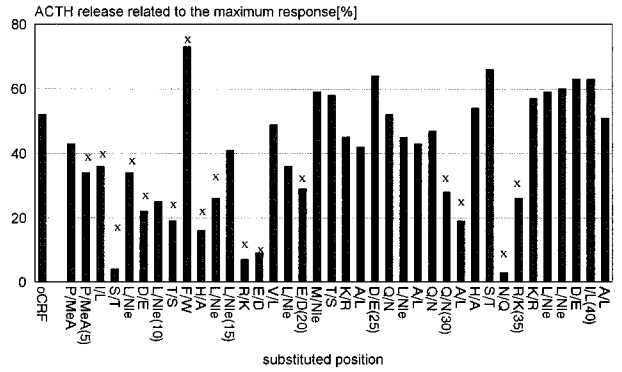

Figure 2. Single-point alteration set of oCRF: induced ACTH release at 0.3 nM related to the maximal response at 1 μM [x, statistically significant different from oCRF ($p < 0.005$)] (see Table 1).

acid, we synthesized a single-point slight alteration set of oCRF by replacing each residue with a similar, preferably proteinogenic amino acid. The receptor affinities and the biological potencies of the various peptide analogs were examined in order to determine whether any amino acid side chains might be more important for receptor binding than for activation, and which residues are most important for interaction with the receptor.

With respect to receptor binding, the results of the slight alteration set show that amino acid residues in the nonconserved regions within the CRF family (21−29, 32−33, and 36−41) as well as in the N-terminus (1−4) can be replaced without significant loss in receptor affinity. Amino acid side chains in the N-terminal region 5−17 are particularly sensitive to slight alteration, much more so than amino acids in the C-terminal region 18−41 (Figure 1), indicating that the N-terminus represents the most sensitive binding site for the receptor.

Thus, deletion of the entire sensitive N-terminal sequence 5−11 must result in a dramatic loss in affinity to the receptor binding site. The fact that the N-terminally shortened antagonist cyclo(30−33)[D-Phe(12),Nle(21,38),Glu(30),Lys(33)]-hCRF(12−41) showed an increased receptor affinity compared to CRF itself, and that incorporation of the lactam bridge had a strong effect on receptor binding of the antagonist but no effect at all on the binding of an agonist,[36] suggests that the receptor binding sites of CRF and the N-terminally shortened CRF antagonists are not identical. Therefore, conclusions from results of structure−activity relationship studies of N-terminally shortened antagonists might be of limited value in the case of agonists.

Replacements of leucine by norleucine at positions 8, 10, and 14 resulted in decreased receptor binding (20%, 13%, and 24% of oCRF, respectively), and substitutions of isoleucine at position 6 by leucine or tryptophan had moderate effects on receptor binding (28% or 45% of oCRF, see Table 1). Replacements of Phe(12) by Trp and Leu(38) by Nle resulted in analogs with increased affinities, showing that these residues are essentially lipophilic but not individually important for high affinity to the receptor. An aromatic side chain at position 12 seems to be advantageous, since in the case of Leu(12)-oCRF decreased receptor binding was observed (34% of oCRF, see Table 1). The hydrophilic residues Asp(9), Thr(11), His(13), Gln(30), and Arg(35) responded significantly to slight alteration, showing that their side chains seem to be important, but the remarkable loss in affinity which occurred upon replacing Ser(7), Arg(16), Glu(17), and Asn(34) indicated the exceptional role of these amino acids. Interestingly, residues at positions 7, 16, and 34 are highly conserved within the CRF family, but Glu(17) goes to Asn or Lys, a fact that is in contrast to the idea of the highly specific involvement of Glu(17) in receptor binding. Additionally, Ala(17)-oCRF exhibited receptor binding comparable to that of oCRF (Table 1). Therefore, a minimal change of an amino acid side chain, such as that made here, does not necessarily imply a slight alteration with respect to steric and conformational changes.

In order to gain insight into the role that individual amino acid residues may play for receptor activation, we investigated the replacement set in an in vitro anterior pituitary cell assay (Figure 2).

Most interestingly, the biological potencies correlated well with the corresponding receptor affinities of the CRF analogs. Loss in the biological potency of analogs always followed decreased receptor affinity. These results explain the fact that alanine-substituted oCRF analogs without agonistic potency also exhibited no antagonistic potency.[2] With the exception of Leu(15), all amino acid residues in the N-terminal region 5−17 were found to be sensitive to slight alteration, indicating their remarkable importance for receptor binding and activation, while in the region 18−41 alterations at only five positions significantly affected biological potency. Taking into consideration that at three of these five positions a single-point alanine replacement led to analogs that were approximately as potent as oCRF,[2] only Ala(31) and Arg(35) in the region 18−41 are markedly sensitive to alteration. Arg(35) replacement by lysine resulted in partially retained potency, while an alanine replacement led to a dramatic loss in potency, suggesting that the positive charge at position 35 is essential for high receptor affinity. The observation that substitutions of Ala(31) by leucine or D-alanine[23] were detrimental to the biological potency will be the subject of further investigation.

A significant loss in biological potency caused by slight alterations was observed for the hydrophilic amino acid residues Glu(17), Gln(30), and Asn(34). Surprisingly, these residues cannot be substituted by functionally similar amino acid residues but can be replaced by alanine,[2] suggesting that these residues are more important for the biologically active conformation of CRF than for individual interactions with the receptor.

According to results of secondary structure prediction and circular dichroism studies,[25] the biologically active

conformation of CRF was suggested to be predominantly α-helical. Recently, NMR studies were carried out in trifluoroethanol/water, showing that CRF adopts under these conditions an α-helical conformation comprising residues 6−36[37] or 6−30,[38] respectively. According to the retention behavior of D-amino acid analogs of oCRF in reversed-phase HPLC, an amphipathic helical structure was found comprising residues 7−22.[39] A single-point D-amino acid replacement set of oCRF could not confirm the biological relevance of the conformation found in trifluoroethanol/water, because of the lack of correlation between helical content and biological potency. Interestingly, D-amino acid incorporation, which has been suggested to disturb helical structures,[40] if made within the putative helical motif at positions 12 and 20, led to analogs with even greater biological potencies.[23]

Providing that Glu(17), Gln(30), and Asn(34) are located in a helical bioconformation, replacement of Glu(17) by alanine without significant loss in potency might be due to the high helix propensities of both of these amino acids.[41] The loss in biological potency in the case of Asp(17)-oCRF could be explained by the lower helix propensity of aspartic acid, but asparagine at position 17 in the more potent urotensin is expected to show a comparable effect. The local conformational disturbance observed by exchanging Asn for Gln(30) can be explained on the basis of the lower helix propensity of asparagine compared with glutamine, and the increasing biological acceptance of D-amino acid replacements[23] agrees with the increasing biological potencies of Gln/Asn replacement analogs at positions 30, 29, or 26. Considering their helix propensities, the fact that asparagine at position 34 can be replaced by alanine but not glutamine without loss in biological potency remains inexplicable. Interesting results were recently described on the helix- stabilizing effects of amino acid residues located at helix termini. Despite its relatively low helix propensity, asparagine at the N-terminal position stabilizes a model helix markedly better than alanine or glutamine,[42,43] suggesting a helical bioconformation in the C-terminal region 34−41. In agreement with this interpretation, D-amino acid replacements in this region caused a dramatic loss in biological potency.[23] On the other hand, the side chain of glutamine at position 34 might simply be too bulky in comparison with asparagine or alanine. More basic work is required to understand the conformational influence of a single amino acid exchange in a peptide sequence. Nevertheless, the observed strong effects of such alterations at residues 17, 30, and 34 in comparison with a moderate or lack of effect of analogous replacements at other positions in CRF or alanine replacements at those positions suggested an exceptionally high conformational sensitivity at these positions. This conclusion is supported by the fact that the incorporation of the corresponding D-amino acid at positions 17, 30, and 34 has also caused decreased biological potencies.

The results show that nearly the entire N-terminal region 5−16 is sensitive to slight alteration, marking this section of the peptide chain as the most important site with respect to receptor binding as well as activation. With the exception of Phe(12) and Leu(15), substitution here yielded analogs with significantly decreased biological potencies. Replacement of Phe(12) by Trp provided an analog that exhibited a significantly increased biological potency, and Leu(15) replaced by norleucine showed no effect. In the case of histidine, no other proteinogenic amino acid fulfills our requirement that the replaced residue exhibits only a slight alteration of the character of the side chain. Replacement by alanine led in the case of His(32) to an analog that was approximately as potent as oCRF, but alanine replacement of His(13) resulted in a significant loss in biological potency. His(13) is conserved within CRFs from different species and in urotensins but not in sauvagine (His goes to Glu). Interestingly, Glu(13)-oCRF exhibited in comparison with Ala(13)-oCRF significantly increased receptor affinity (26% of oCRF, Table 1), showing that histidine at position 13 in oCRF may be substituted preferentially by hydrophilic amino acids.

A previous investigation of the biological potencies of alanine analogs gave similar results with regard to the sensitivity of the N-terminal region 6−19,[2] although some differences were observed. While alanine replacement affected the biological potency to a much greater effect at lipophilic residues 6, 8, 10, 12, and 14, than at hydrophilic residues 7, 9, 11, and 13, such a differentiation was not seen in the case of the slight alteration set, suggesting that moderate side chain variation leads to comparable effects, whereas elimination of the lipophilic behavior causes a more dramatic loss in biological potency. Thus, alanine replacement of Phe(12) as well as the corresponding replacements of Ile(6), Leu(8), Leu(10), or Leu(14) caused a complete loss in potency. Surprisingly, alanine replacement of Thr(11) resulted in an analog with moderate potency, although substitution by serine affected the potency significantly, thus suggesting a conformational influence rather than a side chain effect. Most important for the interaction of oCRF with the receptor are the side chains of residues Ser(7), Leu(8), Asp(9), Leu(10), Leu(14), and Arg(16) in the N-terminal region, where slight alterations as well as alanine substitutions caused significantly decreased biological potency. Looking at the entire peptide sequence, the strong effects of slight alteration at Ser(7) and Arg(16) suggest that their side chains may play an exceptional role for receptor binding and activation.

In summary, loss in biological potency by slight alteration at any position in oCRF always correlated with decreased receptor affinities, and none of the analogs synthesized showed intact receptor binding accompanied by a loss in receptor activation. In conclusion, there is no evidence for a differentiation of amino acid side chains in CRF being responsible either for receptor activation or for receptor binding. Thus, a rational approach for the design of fully competitive CRF antagonists on the basis of changing single amino acid side chains of the entire peptide seems to be unlikely. Consistently, analogs of the slight alteration set of oCRF, with the exception of Thr(7)- and Gln(34)-oCRF, exhibited significant agonistic potencies in an in vitro anterior pituitary cell assay at a concentration of 0.3 nM, and all analogs were fully potent at a concentration of 1 μM, showing that no individual side chain is essential for full intrinsic potency. Peptidic CRF antagonists so far described may be partially competitive,[44] and investigations are in progress to find the requirements needed for competitively binding CRF antagonists by incorporation of conformational con-

straints which do not affect receptor binding but prevent the receptor−ligand complex from assuming the conformation necessary for transduction.[45]

Most interestingly, two amino acid residues in oCRF, serine at position 7 and arginine at position 16, were found to be exceptionally sensitive to alteration, suggesting their involvement in more specific interactions with the receptor. Glu(17) and Asn(34) were also shown to be very sensitive to slight alteration but not to a corresponding alanine replacement, suggesting high conformational or steric sensitivity at these positions. Thus, investigation of a single-point slight alteration set uncovered interesting results regarding the importance of individual side chains for interaction with the receptor, but also indicated that steric or conformational changes caused by such alterations have to be considered carefully. A complementary combination of a slight alteration set and an alanine replacement set, the latter of which incorporates the chiral amino acid having the smallest side chain, turned out to be a powerful tool for structure−activity relationship studies of peptides.

Experimental Section

HPLC-grade acetonitrile was obtained from J. T. Baker (Phillipsburg, NJ). Water was purified with a Milli-Q system (Millipore, Eschborn, Germany). Eluents were degassed by continuous sparging with helium. All reagents were at least analytical reagent grade. Trifluoroacetic acid (TFA), phenol, and piperidine were supplied by Merck (Darmstadt, Germany). The Fmoc-amino acids and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate used in synthesis were obtained from Novabiochem (Bad Soden, Germany). Dimethylformamide and diisopropylethylamine were obtained from Fluka (Buchs, Switzerland). Triisopropylsilane and sinapinic acid were supplied by Aldrich (Steinheim, Germany).

Peptide Synthesis and Purification. oCRF and its analogs were synthesized automatically by the solid-phase method using standard Fmoc chemistry on 0.5−1.0 g of TentaGel S RAM resin, 0.22 mmol/g (Rapp Polymere, Tübingen). Couplings were carried out with 4−8 equiv of Fmoc-amino acid derivatives at ambient temperature. After final cleavage, crude peptides were purified by preparative HPLC on PolyEncap A300, 10 μm, 250 × 20 mm i.d. (Bischoff Analysentechnik GmbH, Leonberg), using a Shimadzu LC-8A system: mobile phase A, 0.1% TFA/water; mobile phase B, 50% acetonitrile/water−0.1% TFA (v/v/v); linear gradient 50−90% B in 70 min, at a flow rate of 10 mL/min.

Peptide Characterization. Reversed-phase HPLC measurements were performed on a Shimadzu LC-10A gradient HPLC system consisting of two LC-10AD pumps, a SIL-10A autoinjector, a SPD-M10A diode array detector operating at 220 nm, and a CLASS-LC10 software package. Runs were carried out on a PolyEncap A300 column (250 × 4.6 mm i.d., 5 μm; Bischoff Analysentechnik GmbH): mobile phase A, 0.1% TFA in water; B, 0.1% TFA in 50% acetonitrile/50% water (v/v); linear gradient 20−80% B in 40 min. Additionally, peptides were analyzed using a second solvent system using as mobile phase A 0.07 M KH_2PO_4/H_3PO_4, pH 2.2 /5% acetonitrile, mobile phase B 0.07 M KH_2PO_4/H_3PO_4, pH 2.2 /70% acetonitrile, and a linear gradient of 20−80% B in 40 min.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) was performed on a linear time-of-flight mass spectrometer MALDI II (Kratos, Manchester) using the positive detection mode and a sinapinic acid matrix. The spectra were obtained by summing over 50 laser pulses (337 nm). The $[M + H]^+$ peak of bovine insulin (*m*/*z* 5735) was used for internal mass calibration.

Quantitative amino acid analysis was achieved by ion-exchange chromatography and postcolumn derivatization with ninhydrin (Biotronik-Eppendorf LC 3000). Peptides were hydrolyzed in 6 N HCl at 110 °C for 22 h, norleucine being added for internal standardization.

CRF Receptor Assay. Whole brains of male Wistar rats weighing 220−250 g were homogenized with a Teflon−glass homogenizer (10 strokes at 800 rpm) in 0.32 M sucrose, 50 mM Tris/HCl (pH 7.2), 10 mM $MgCl_2$, 2 mM EGTA, and 0.15 mM bacitracin at 50 mg wet weight/mL. After centrifugation at 1000*g* for 5 min, the supernatant was centrifuged at 26000*g* for 20 min. The pellet was resuspended in 50 mM Tris/HCl (pH 7.2), 10 mM $MgCl_2$, 2 mM EGTA, 0.15 mM bacitracin, and 0.0015% aprotinin (assay buffer) and again centrifuged. The resulting pellet was resuspended in assay buffer containing 0.32 M sucrose and stored at −20 °C. All steps were carried out at 4 °C. Protein concentrations were determined by the method of Bradford[46] using BSA as standard.

One hundred micrograms of membrane protein in 300 μL of assay buffer was incubated in quadruplicates with 0.1 nM [^{125}I]Tyr(0)-oCRF in the absence and presence of 12 different concentrations (0.2 nM up to 1 μM) of unlabeled peptides at 25 °C for 2 h. Nonspecific tracer binding was determined in the presence of 1 μM oCRF. At the end of incubation, 3 mL of ice-cold washing buffer (assay buffer without inhibitors containing 0.01% Triton X-100) was added to the assay tube, and the samples were immediately filtered through GF/C filter disks (Whatman), presoaked for 2 h in 0.1% poly(ethylenimine), using a Brandel−Harvester, followed by washing of the incubation tubes and filters with 3 mL of cold washing buffer. Triton X-100 in this buffer strongly reduced the nonspecific tracer peptide binding. Radioactivity retained on the filter was measured by γ-counting.

Receptor affinities (K_{ass}, $K_d = 1/K_{ass}$) and capacities (B_{max}) were estimated using the nonlinear least-squares curve-fitting program RADLIG (BIOSOFT, Cambridge, U.K.) and a K_d of 0.48 nM for the binding of the tracer peptide as determined from tracer saturation assays. Total bound tracer amount was 5%, from which about 30% was nonspecific.

Rat Anterior Pituitary Cell Assay. Pituitary cells were obtained by enzymatic digestion of the anterior pituitary of male Wistar rats weighing 220−250 g following the procedure by Denef.[47] A total of 200 000 cells in DMEM/0.25% BSA per well were seeded in cell culture plates and maintained at 37 °C under 5% CO_2/95% air for 3 days. The culture medium was replaced by 0.5 mL of fresh medium and after 2 h by a culture medium containing one of the peptides studied at different concentrations. After a stimulation period of 3 h, the medium samples were harvested and stored at −70 °C. ACTH in the samples was determined by a radioimmunoassay (HS-ACTH-IRMA from the Nichols Institute Diagnostika GmbH, Bad Nauheim, FRG) using hACTH as standard. This assay uses two antibodies directed against the N- and C-termini which are identical in human and rat ACTH. β-Endorphin was determined by radioimmunoassay using camel β-endorphin as standard. EC_{50} values were calculated from the dose−response curves by a four-parameter logistic curve-fitting program.

Acknowledgment. The authors thank Annerose Klose, Dagmar Smettan, Barbara Pisarz (synthesis, purification, characterization), Jenny Eichhorst, and Gabriela Vogelreiter (receptor assay, ACTH release) for technical assistance. Research was supported by the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (BMBF Grant 01 ZZ 9508).

References

(1) Rivier, J.; Rivier, C.; Vale, W. Synthetic competitive antagonists of corticotropin-releasing factor: Effect on ACTH secretion in the rat. *Science* **1984**, *224*, 889−891.
(2) Kornreich, W. D.; Galyean, R.; Hernandez, J.-F.; Craig, A. G.; Donaldson, C. J.; Yamamoto, G.; Rivier, C.; Vale, W.; Rivier, J. Alanine series of ovine corticotropin-releasing factor (oCRF): A structure−activity relationship study. *J. Med. Chem.* **1992**, *35*, 1870−1876.
(3) Vale, W.; Spiess, J.; Rivier, C.; Rivier, J. Characterization of a 41 residue ovine hypothalamic peptide that stimulates the secretion of corticotropin and β-endorphin. *Science* **1981**, *213*, 1394−1397.
(4) Owens, M. J.; Nemeroff, C. B. Physiology and pharmacology of corticotropin-releasing factor. *Pharmacol. Rev.* **1991**, *43*, 425−474.

(5) Uehara, A.; Sekiya, C.; Takasugi, Y.; Namiki, M.; Arimura, A. Anorexia induced by interleukin-1: Involvement of corticotropin-releasing factor. *Am. J. Physiol.* **1989**, *257*, R613−R617.
(6) Strijbos, P. J.; Hardwick, A. J.; Relton, J. K.; Carey, F.; Rothwell, N. J. Inhibition of central action of cytokines on fever and thermogenesis by lipocortin-1 involves CRF. *Am. J. Physiol.* **1992**, *263*, E632−E636.
(7) Rivier, C.; Vale, W. Influence of corticotropin-releasing factor on reproductive functions in the rat. *Endocrinology* **1984**, *114*, 914−928.
(8) Vamvakopoulous, N. C.; Chrousus, G. P. Hormonal regulation of human corticotropin-releasing hormone gene expression: Implications for the stress response and immune/inflammatory reaction. *Endocr. Rev.* **1994**, *15*, 409−420.
(9) Fisher, L. A.; Rivier, J.; Rivier, C.; Spiess, J.; Vale, W. W.; Brown, M. R. Corticotropin-releasing factor (CRF): Central effects on mean arterial pressure and heart rate. *Endocrinology* **1982**, *110*, 2222−2224.
(10) Giguere, V.; Labrie, F.; Cote, J.; Coy, D. H.; Sueiras-Diaz, J.; Schally, A. V. Stimulation of cyclic AMP accumulation and corticotropin release by synthetic ovine corticotropin-releasing factor in rat anterior pituitary cells: site of glucocorticoid action. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 3467−3469.
(11) Bilezikjian, L. M.; Vale, W. W. Glucocorticoids inhibit corticotropin-releasing factor-induced production of adenosine 3′,5′-monophosphate in cultured anterior pituitary cells. *Endocrinology* **1983**, *113*, 657−662.
(12) Chang, C. P.; Pearse, R. V., II; O'Connel, S.; Rosenfeld, M. G. Identification of a seven transmembrane helix receptor for corticotropin-releasing factor and sauvagine in mammalian brain. *Neuron* **1993**, *11*, 1187−1195.
(13) Chen, R.; Lewis, K. A.; Perrin, M. H.; Vale, W. W. Expression cloning of a human corticotropin-releasing factor receptor. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 8967−8971.
(14) Perrin, M. H.; Donaldson, C. J.; Chen, R.; Lewis, K. A.; Vale, W. W. Cloning and functional expression of a rat brain corticotropin-releasing factor (CRF) receptor. *Endocrinology* **1993**, *133*, 3058−3061.
(15) Vita, N.; Laurent, P.; Lefort, S.; Chalon, P.; Lelias, J.-M.; Kaghad, M.; Le Fur, G.; Caput, D.; Ferrara, P. Primary structure and functional expression of mouse pituitary and human brain corticotropin-releasing factor receptors. *FEBS Lett.* **1993**, *335*, 1−5.
(16) Ross, P. C.; Kostas, Ch. M.; Ramabhadran, T. V. A variant of the human corticotropin-releasing factor (CRF) receptor: Cloning, expression and pharmacology. *Biochem. Biophys. Res. Commun.* **1994**, *205*, 1836−1842.
(17) Lovenberg, T. W.; Liaw, Ch. W.; Grigoriadis, D. E.; Clevenger, W.; Chalmers, D. T.; DeSouza, E. B.; Oltersdorf, T. Cloning and characterization of a functionally distinct corticotropin-releasing factor receptor subtype from rat brain. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 836−840.
(18) Kishimoto, T.; Pearse, R. V., II; Lin, Ch. R.; Rosenfeld, M. G. A sauvagine/corticotropin-releasing factor receptor expressed in heart and skeletal muscle. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 1108−1112.
(19) Stenzel, P.; Kesterson, R.; Yeung, W.; Cone, R. D.; Rittenberg, M. B.; Stenzel-Poore, M. P. Identification of a novel murine receptor for corticotropin-releasing hormone expressed in the heart. *Mol. Endocrinol.* **1995**, *9*, 637−645.
(20) Xiong, Y.; Xie, L. Y.; Abou-Samra, A.-B. Signaling properties of mouse and human corticotropin-releasing factor (CRF) receptors: Decreased coupling efficiency of human type. *Endocrinology* **1995**, *136*, 1828−1834.
(21) Hernandez, J.-F.; Kornreich, W.; Rivier, C.; Miranda, A.; Yamamoto, G.; Andrews, J.; Tache, Y.; Vale, W.; Rivier, J. Synthesis and relative potencies of new constrained CRF antagonists. *J. Med. Chem.* **1993**, *36*, 2860−2867.
(22) Menzhagi, F.; Howard, R. L.; Heinrichs, St. C.; Vale, W.; Rivier, J.; Koob, G. F. Characterization of a novel and potent corticotropin-releasing factor antagonist in rats. *J. Pharmacol. Exp. Ther.* **1994**, *269*, 564−572.
(23) Rivier, J.; Rivier, C.; Galyean, R.; Miranda, A.; Miller, Ch.; Craig, A. G.; Yamamoto, G.; Brown, M.; Vale, W. Single point D-substituted corticotropin-releasing factor analogues: Effects on potency and physicochemical characteristics. *J. Med. Chem.* **1993**, *36*, 2851−2859.
(24) Sawyer, T. K.; Staples, D. J.; Bannow, C. A.; Kinner, J. H.; Maggiora, L. L.; Evans, D.; Prairie, M.; Krueger, W.; Lahti, R. A. Design and structure/conformation-activity studies of a prototypic corticotropin-releasing factor (CRF) antagonist: Multiple alanine substitutions of CRF12−41. In *Growth Factors, Peptides, and Receptors*; Moody, T. W., Ed.; Plenum Press: New York, 1993; pp 21−28.
(25) Pallai, P. V.; Mabilia, M.; Goodman, M.; Vale, W.; Rivier, J. Structural homology of corticotropin-releasing factor, sauvagine, and urotensin I: Circular dichroism and prediction studies. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 6770−6774.
(26) Rivier, J.; Rivier, C.; Galyean, R.; Yamamoto, G.; Vale, W. Corticotropin-releasing factor: Characterization of new analogs. In *Peptide Chemistry*; Shiba, T., Sakakibara, S., Eds.; Protein Research Foundation: Osaka, Japan, 1988; pp 597−600.
(27) Miranda, A.; Koerber, S. C.; Gulyas, J.; Lahrichi, S. L.; Craig, A. G.; Corrigan, A.; Hagler, A.; Rivier, C.; Vale, W.; Rivier, J. Conformationally restricted competitive antagonists of human/rat corticotropin-releasing Factor. *J. Med. Chem.* **1994**, *37*, 1450−1459.
(28) Wei, E. T.; Thomas, H. A. Correlation of neuroendocrine and anti-edema activities of alanine corticotropin-releasing factor analogs. *Eur. J. Pharmacol.* **1994**, *263*, 319−321.
(29) Roloff, B.; Beyermann, M.; Dathe, M.; Fabian, H.; Fechner, K.; Furkert, J.; Hagen, V.; Bienert, M. The effect of dibenzofuran substitutions in corticotropin-releasing factor. *Lett. Pept. Sci.* **1995**, *2*, 209−211.
(30) Beck-Sickinger, A. G.; Wieland, H. A.; Wittneben, H.; Willim, K.; Rudolf, K.; Jung, G. Complete L-alanine scan of neuropeptide Y reveals ligands binding to Y1 and Y2 receptors with distinguished conformations. *Eur. J. Biochem.* **1994**, *225*, 947−958.
(31) Wieczorek, Z.; Zimecki, M.; Pedyczak, A.; Lisowski, M.; Siemion, I. Z. Immunosuppressive activity of alanine analogs of cyclolinopeptide A. *Arch. Immunol. Ther. Exp.* **1993**, *41*, 291−296.
(32) Stagsted, J.; Mapelli, C.; Meyers, C.; Matthews, B. W.; Anfinsen, C. B.; Goldstein, A.; Olsson, L. Amino acid residues essential for biological activity of a peptide derived from a major histocompatibility complex class I antigen. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 7686−7690.
(33) Mimeault, M.; Quirion, R.; Dumont, Y.; St-Pierre, S.; Fournier, A. Structure−activity study of hCGRP8−37, a calcitonin gene-related peptide receptor antagonist. *J. Med. Chem.* **1992**, *35*, 2163−2168.
(34) Kirby, D. A.; Boublik, J. H.; Rivier, J. E. Neuropeptide Y: Y1 and Y2 affinities of the complete series of analogs with single D-residue substitutions. *J. Med. Chem.* **1993**, *36*, 3802−3808.
(35) He, Y. B.; Huang, Z.; Raynor, K.; Reisine, T.; Goodman, M. Synthesis and conformations of somatostatin-related cyclic hexapeptides incorporating specific alpha- and beta-methylated residues. *J. Am. Chem. Soc.* **1993**, *115*, 8066−8072.
(36) Gulyas, J.; Rivier, C.; Perrin, M.; Koerber, S. C.; Sutton, S.; Corrigan, A.; Lahrichi, S. L.; Craig, A. G.; Vale, W.; Rivier, J. Potent structurally constrained agonists and competitive antagonists of corticotropin-releasing factor. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 10575−10579.
(37) Romier, C.; Bernassau, J.-M.; Cambillau, C.; Darbon, H. Solution structure of human corticotropin-releasing factor by ^{1}H NMR and distance geometry with restrained molecular dynamics. *Protein Eng.* **1993**, *6*, 149−156.
(38) Dathe, M.; Fabian, H.; Gast, K.; Zirwer, D.; Winter, R.; Beyermann, M.; Schümann, M.; Bienert, M. Conformational differences of ovine and human corticotropin-releasing hormone: A CD, IR, NMR and dynamic light scattering study. *Int. J. Pept. Protein Res.* **1996**, *47*, 383−393.
(39) Krause, E.; Beyermann, M.; Dathe, M.; Rothemund, S.; Bienert, M. Location of an amphipathic α-helix in peptides using reversed-phase HPLC retention behavior of D-amino acid analogs. *Anal. Chem.* **1995**, *34*, 252−258.
(40) Fairman, R.; Anthony-Cahill, S. J.; DeGrado, W. F. The helix-forming propensity of D-alanine in a right-handed α-helix. *J. Am. Chem. Soc.* **1992**, *114*, 5458−5459.
(41) O'Neil, K. T.; DeGrado, W. F. A thermodynamic scale for the helix-forming tendencies of the commonly occurring amino acids. *Science* **1990**, *250*, 646−651.
(42) Chakrabartty, A.; Kortemme, T.; Baldwin, R. L. Helix propensities of the amino acids measured in alanine-based peptides without helix-stabilizing side-chain interactions. *Protein Sci.* **1994**, *3*, 843−852.
(43) Forood, B.; Feliciano, E. J.; Nambiar, K. P. Stabilization of α-helical structures in short peptides via end capping. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 838−842.
(44) Strader, C. D.; Fong, T. M.; Graziano, M. P.; Tota, M. R. The family of G-protein-coupled receptors. *FASEB J.* **1995**, *9*, 745−754.
(45) Hruby, V. J. Strategies in the development of peptide antagonists. In *Progress in Brain Research*; Joosse, J., Buijs, R. M., Tilders, F. J. H., Eds.; Elsevier Science Publishers B.V.: New York, 1992; Vol. 92, pp 215−224.
(46) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.
(47) Denef, C.; Maertens, P.; Allaerts, W.; Mignon, A.; Robberecht, W.; Swennen, L.; Carmeliet, P. Cell-to-cell communication in peptide target cells of anterior pituitary. *Methods Enzymol.* **1989**, *168*, 47−71.

JM960116Z