Determinants of Corticotropin Releasing Factor. Receptor Selectivity of Corticotropin Releasing Factor Related Peptides

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The corticotropion-releasing factor (CRF) peptide family is an important target in pharmaceutical research. The CRF system consists of two receptors, corticotropin releasing factor receptor 1 (CRF1R) and corticotropin releasing factor receptor 2 (CRF2R), a nonreceptor binding protein, and the peptide agonists of these receptors. The recent discovery of the CRF2R selective peptide agonists, UCN2, UCN3 and URP, prompted investigations into the structural source of CRF1R versus CRF2R selectivity of CRF peptide family members. Data from chimeric peptides demonstrated that amino acids in the N-terminus and C-terminus of CRF, UCN1, UCN2 and Sauvagine peptide families influence CRFR selectivity. Analysis of specific amino acid residues in the N-terminus and C-terminus demonstrated that the presence of a proline at position 11 and alanine at positions 35 and 39 (hCRF numbering) decreases CRF1R activity and increases CRF2R selectivity in CRF, UCN1 and sauvagine peptides. The availability of a large group of selective and nonselective CRF receptor peptide agonists will facilitate the development of CRF receptor selective drugs.

Introduction

The corticotropin-releasing factor peptide family consists of a large number of natural mammalian, amphibian, and fish peptides.[1−5] The most prominent representatives of this group include corticotropin releasing factor (CRF) also known as corticotropin releasing hormone (CRH), urocortins I, II, and III (UCN1, UCN2, UCN3), the amphibian hormone sauvagine (Svg), and the fish peptide urotensin 1. Many physiological functions such as coordination of the endocrine, autonomic, behavioral, and immune responses to stress have been demonstrated for these peptides.[2−5] Consequently, there has been a growing interest in the research on both agonists and antagonists of CRF receptors for potential therapeutic applications.[1−5]

An extensive characterization of the structure−activity relationship of CRF peptide family members has revealed the existence of three main functional domains.[6−9] The first domain, consisting of residues 1−16, is responsible for both binding and agonist activation. The second domain, consisting of residues 17−31, is considered a linker providing the appropriate spatial and conformational support for the two binding regions located in domains 1 and 3 and contains the CRF binding protein binding site. Finally, the third domain, consisting of residues 32−41, is important for receptor binding.

Our recent discovery that CRF2R agonists may be useful in the treatment of skeletal muscle atrophy[10] stimulated an interest in finding molecular characteristics of CRF peptides responsible for CRFR selectivity. Therefore, we have investigated how variations of amino acid residues in CRF peptides influence their CRFR selectivity.

Peptide Designs

The sequence alignment of all the peptides discussed in this paper is based on hCRF as shown in Table 1. Inspection of the peptide sequences listed in Table 1 reveals a number of differences between CRF2R selective agonists of UCN2 and UCN3 peptide families and the CRFR nonselective agonists of the CRF, UCN1, Utn1, and Svg peptide families.[11] One parameter that has not been fully delineated in CRF-related peptides is the role of three domains in determining CRFR selectivity. To investigate this, we designed a series of chimeric peptides focusing on four CRF peptide family members: the CRF2R selective peptide UCN2 and the nonselective CRFR peptides CRF, UCN1, and sauvagine. These peptides were chosen for several reasons including pharmacological properties, commercial interest, and availability of structure−activity relationship information.

Once the CRFR selectivity role of specific domains in CRF-related peptides was determined, specific amino acids were evaluated for their role in conferring CRFR selectivity. On the basis of a homology analysis of CRFR selective and nonselective peptides, several amino acids attracted our attention. One residue in the first domain, proline at position 11, is of interest in that it is found only in CRF2R selective peptides (see Table 1). A proline residue in this region is of interest also because it may alter the α-helix structure of the first domain in CRF-related peptides. Among natural amino acids, proline is the strongest modifier of α-helix structure and a promoter of turn motifs. Consequently, the presence of proline at position 11 in the CRF2R selective peptides UCN2, UCN3, and URP may indicate that the α-helix

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Table 1. CRF1R and CRF2R Natural Agonists[a]

peptide	sequence 1	10	20	30	40
		CRF Family			
human CRF	SEEPPISLDL	TFHLLREVLE	MARAEQLAQQ	AHSNRKLMEI	I
ovine CRF	SQEPPISLDL	TFHLLREVLE	MTKADQLAQQ	AHSNRKLLDI	A
bovine CRF	SQEPPISLDL	TFHLLREVLE	MTKADQLAQQ	AHNNRKLLDI	A
porcine CRF	SEEPPISLDL	TFHLLREVLE	MARAEQLAQQ	AHSNRKLMEI	F
rat CRF	SQEPPISLDL	TFHLLREVLE	MARAENLAQQ	AHSNRKLMEI	I
xenopus CRF	AQEPPISLDL	TFHLLREVLE	MARAEQIAQQ	AHSNRKLMDI	I
salmon CRF	SDDPPISLDL	TFHMLRQMME	MSRAEQIQQQ	AHSNRKMMEI	F
sucker CRF	AQEPPISLDL	TFHLLREVLE	MARAEQIAQQ	AHSNRKMMEI	F
		Urotensin 1 Family			
sucker Utn1	NDDPPISIDL	TFHLLRNMIE	MARIENEREQ	AGLNRKYLDE	V
trout Utn1	NDDPPISIDL	TFHLLRNMIE	MARIESQKEQ	AELNRKYLDE	V
carp Utn1	NDDPPISIDL	TFHLLRNMIE	MARNENQREQ	AGLNRKYLDE	V
sole Utn1	SEEPPMSIDL	TFHMLRNMIH	RAKMEGEREQ	ALINRNLMDE	V
flounder Utn1	SEDPPMSIDL	TFHMLRNMIH	MAKMEGEREQ	AQINRNLLDE	V
shark Utn1	SEEPPMSIDL	TFHMLRNMIH	RAKMEGEREQ	ALINRNLMDE	V
sauvagine (Svg)	-ZGPPISIDL	SLELLRKMIE	IEKQEKEKQQ	AANNRLLLDT	I
		UCN1 Family			
human UCN1	-DNPSLSIDL	TFHLLRTLLE	LARTQSQRER	AEQNRIIFDS	V
hamster UCN1	-EDLPLSIDL	TFHLLRTLLE	LARTQSQRER	AEQNRIILNA	V
rat UCN1	-DDPPLSIDL	TFHLLRTLLE	LARTQSQRER	AEQNRIIFDS	V
ovine UCN1	-DDPPLSIDL	TFHLLRTLLE	LARTQSQRER	AEQNRIIFDS	V
		UCN2/UCN3 Family			
human UCN2	- - -IVLSLDV	PIGLLQILLE	QARARAAREQ	ATTNARILAR	V
mouse UCN2	- - -VILSLDV	PIGLLRILLE	QARYKAARNQ	AATNAQILAH	V
human UCN3	- - -FTLSLDV	PTNIMNLLFN	IAKAKNLRAQ	AAANAHLMAQ	I
mouse UCN3	- - -FTLSLDV	PTNIMNILFN	IDKAKNLRAK	AAANAQLMAQ	I
pufferfish URP1	- - -FALSLDV	PTSILSVLID	LAKNQDMRAK	AARNAELMAR	I
pufferfish URP2	- - -LTLSLDV	PTNIMNVLFD	VAKAKNLRAK	AAENARLLAH	I

[a] Available in NCBI protein and nucleotide databases, www.ncbi.nlm.nih.gov.

present in the first domain of the nonselective CRFR peptides is modified through the introduction of a kink or turn motif, a modification that we hypothesize may be important in determining CRFR selectivity.[12−18] A second region of interest to us was the third domain of the CRF family of peptides. In this region it was observed that CRF2R selective peptides contain alanine residues at positions 35 and 39, while CRFR nonselective peptides contain an invariant arginine at position 35 and an acidic amino acid at position 39. Thus, we hypothesize that CRFR nonselective peptides may gain increased CRF2R selectivity by substituting alanines at positions 35 and 39.

Results and Discussion

Peptides were synthesized using standard methods involving FMOC solid-supported chemistry and purified by reverse-phase chromatography to at least 95% purity as determined by analytical HPLC. All peptide identities were confirmed using MALDI-TOF mass spectrometry. A detailed description of the synthesis and purification methods is provided in the Experimental Section.

Our initial efforts to delineate the CRFR selectivity domains of CRF-related peptide family members involved the generation of a series of peptide chimeras created by mixing domains of hUCN2 with either sauvagine or CRF (Table 2). The first sauvagine/hUCN2 chimera **3** combines the N-terminus of sauvagine with the C-terminus of hUCN2, while the second sauvagine/hUCN2 chimera **4** combines the N-terminus of hUCN2 with the C-terminus of sauvagine. Both chimera showed CRF2R selectivity that was better than sauvagine **1** but not as good as hUCN2 **2** (Table 2). The next chimera contained the N-terminus of sauvagine, the middle domain of hUCN2, and the C-terminus of sauvagine **5** demonstrated approximately the same level of selectivity as sauvagine itself. Chimera **6**, which contained the N-terminus of hUCN2, the middle domain of sauvagine, and the C-terminus of hUCN2, had a similar selectivity as hUCN2 itself. A chimera composed of N-terminus 2/3 of sauvagine and the C-terminus of hUCN2 **7** demonstrated CRF2R selectivity that was only slightly better than sauvagine **1** itself. Substitution of the serine at position 11 with proline in this chimera **8** resulted in increased CRF2R selectivity. The next chimera series involved combinations of hUCN2 and oCRF segments. The combination of oCRF N-terminus and hUCN2 C-terminus resulted in a peptide **10** that demonstrated increased CRF2R selectivity compared to oCRF **9.** The combination of hUCN2 N-terminus and oCRF C-terminus resulted in a peptide that was more CRF2R selective than oCRF **9** but less selective than hUCN2 **2**. A chimera consisting of the N-terminus of hUCN2 and the C-terminus of hCRF **13** demonstrated CRF2R selectivity similar to that of hUCN2 and was similar in selectivity to a chimera consisting of the N-terminus 2/3 of hUCN2 and the C-terminus 1/3 of hCRF **14**. Finally, a chimera composed of the N-terminus 2/3 of hCRF and the C-terminus 1/3 of hUCN2 **15** showed only marginal activity at both receptors. Together, the chimera data demonstrate that both the N-terminus and C-terminus of hUCN2 contain CRF2R selectivity domains while the middle 1/3 of hUCN2 does not appear to contain domains that have a major influence on CRF2R selectivity.

In an effort to identify specific amino acids that determine CRF2R selectivity, amino acid substitutions in the N-terminus and C-terminus of sauvagine and

Table 2. Activity of Chimeras of CRF, Svg, and hUCN2[a]

compd	sequence	CRF2R EC_{50} (nM)	CRF2R $\pm EC_{50}$ (nM)	CRF2R E_{max} (%)	CRF1R EC_{50} (nM)	CRF1R $\pm EC_{50}$ (nM)	CRF1R E_{max} (%)	selectivity CRF2R/CRF1R
1	Svg	6.0	0.8	95	17.6	7.5	99	3
2	hUCN2	4.3	0.7	96	>1000	NA	8	>233
3	$Svg_{(2-19)}hUCN2_{(20-41)}$	9.3	2.6	79	705.0	296.0	100	76
4	$hUCN2_{(4-20)}Svg_{(21-41)}$	6.4	1.9	89	301.0	67.0	100	47
5	$Svg_{(2-16)}hUCN2_{(17-29)}Svg_{(30-41)}$	17.6	8.5	100	47.9	0.1	93	3
6	$hUCN2_{(4-16)}Svg_{(17-31)}hUCN2_{(32-41)}$	9.2	1.0	96	>1000	NA	11	>110
7	$Svg_{(2-31)}hUCN2_{(32-41)}$	9.1	0.2	77	43.4	0.2	85	5
8	$[P^{11}]Svg_{(2-31)}hUCN2_{(32-41)}$	11.1	0.8	86	232.0	13.0	100	21
9	oCRF	>100	NA	33	27.0	0.1	98	<0.3
10	$oCRF_{(1-20)}hUCN2_{(21-41)}$	42.0	3.4	61	393.0	144.0	100	10
11	$hUCN2_{(4-20)}oCRF_{(21-41)}$	20.6	3.8	94	710.0	290.0	64	34
12	hCRF	49.2	8.6	82	20.0	1.2	87	0.4
13	$hUCN2_{(4-20)}hCRF_{(21-41)}$	2.2	0.8	92	>1000	NA	23	>446
14	$hUCN2_{(4-32)}hCRF_{(33-41)}$	4.5	0.7	96	>1000	NA	17	>223
15	$hCRF_{(1-32)}hUCN2_{(33-41)}$)	>100	NA	88	207.0	100.5	94	<2

[a] The numbering of residues is based on the HCRF sequence. Selectivity is expressed as a ratio of EC_{50} values of CRF1R/CRF2R. All assays were performed in triplicate with two independent experimental analyses of each peptide at both CRF receptors. NA: not applicable.

Table 3. Effect of Substitutions at Positions 11−13, 35, and 39 in CRF-like Peptides[a]

compd	sequence	CRF2R EC_{50} (nM)	CRF2R $\pm EC_{50}$ (nM)	CRF2R E_{max} (%)	CRF1R EC_{50} (nM)	CRF1R $\pm EC_{50}$ (nM)	CRF1R E_{max} (%)	selectivity CRF2R/CRF1R
1	Svg	6.0	0.8	95	17.6	7.5	99	3
16	$[P^{11}]Svg$	10.9	2.6	90	96.0	4.5	85	9
17	$[A^{35}A^{39}]Svg$	18.7	5.7	86	100.0	16.6	100	5
18	$[P^{11}A^{35}A^{39}]Svg$	20.6	4.0	100	434.0	73.0	96	21
19	hUCN1	3.5	0.3	100	9.0	1.0	100	3
20	$[P^{11}]hUCN1$	9.1	2.1	83	135.0	37.0	91	15
21	$[P^{11}A^{35}A^{39}]hUCN1$	8.3	2.5	100	358.0	22.5	96	43
9	oCRF	>100	NA	33	27.0	0.1	98	<0.3
22	$[P^{11}]oCRF$	>100	NA	18	708.0	293.0	98	<7
12	hCRF	49.2	8.6	82	20.0	1.2	87	0.4
23	$[P^{11}]hCRF$	>100	NA	18	>1000	NA	9	NA
24	$[P^{11}A^{35}A^{39}]hCRF$	>100	NA	24	>1000	NA	38	NA
2	hUCN2	4.3	0.7	96	>1000	NA	8	>233
25	$[S^{11}]hUCN2$	51.1	3.8	75	>1000	NA	33	>20

[a] The numbering of residues is based on HCRF sequence. Selectivity is expressed as a ratio of EC_{50} values of CRF1R/CRF2R. All assays were performed in triplicate with two independent experimental analyses of each peptide at both CRF receptors. NA: not applicable.

CRF were made and the resulting peptides were evaluated for CRFR selectivity. The specific amino acids under evaluation were proline at position 11 and alanine at positions 35 and 39 (Table 3). These amino acids were evaluated because they are highly conserved in all CRF2R selective peptides but not the CRFR nonselective peptides (see Table 1). Substitution of the serine at position 11 with proline in this chimera **8** resulted in increased CRF2R selectivity. Substitution of proline for either serine or threonine at position 11 in sauvagine, hUCN1, oCRF, or hCRF had the following effects: substitution of proline at position 11 in sauvagine **16** resulted in an increase in CRF2R selectivity compared to sauvagine **1** mainly through a decrease in CRF1R potency; substitution of proline at position 11 in hUCN1 **20** also resulted in an increase in CRF2R selectivity, also mainly through a loss in CRF1R potency; substitution of proline at position 11 in oCRF **22** and hCRF **23** resulted in a loss of potency at the CRF1R and, in hCRF, a loss of potency at the CRF2R. One could also ask if the inverse is true: Would replacing proline at position 11 in hUCN2 with the corresponding residue from sauvagine change the CRF2R/CRF1R selectivity of hUCN2? As can be seen in Table 3, substitution of proline in hUCN2 with serine **25** resulted in a loss of CRF2R potency and an increase in CRF1R E_{max}, resulting in an overall decrease in CRF2R selectivity when compared to hUCN2 **2**.

The role of alanine at positions 35 and 39 in CRF2R selectivity was also evaluated by substituting alanine in positions 35 and 39 in sauvagine, UCN1, and hCRF. As can be seen in Table 3, alanine substitutions at positions 35 and 39 in peptide **17** resulted in an approximate 2-fold increase in CRF2R selectivity of sauvagine when compared to the parent compound **1**. Modification of sauvagine by introducing proline at position 11 and alanine at positions 35 and 39 resulted in a peptide **18** with more than additive selectivity for each of these modifications separately. The selectivity of peptide **18** was similar to the selectivity of $[P^{11}]Svg_{(2-31)}hUCN2_{(32-41)}$ chimeric peptide **8**, indicating that residues 35 and 39 in hUCN2 provide the increase in selectivity observed with the hUCN2 chimeric addition to $P^{11}Svg$ **16**. A similar large increase in CRF2R selectivity was observed when hUCN1 was modified by substituting proline at position 11 and alanine at positions 35 and 39, **21** (Table 3). In contrast, modification of hCRF by substituting proline at position 11 and alanine at positions 35 and 39 resulted in peptide **24** that was inactive at both CRF1R and CRF2R, demonstrating that unlike sauvagine and hUCN1, CRF re-

quires additional substitutions to achieve improved CRF2R selectivity.

In summary, identification of CRFR selectivity domains was accomplished through the creation of CRFR selective and nonselective peptide chimeras followed by substitution of specific conserved amino acids residues in the N-terminus and C-terminus of CRF-related peptides. Specifically, we demonstrate that substituting proline at position 11 and alanine at positions 35 and 39 results in increased CRF2R selectivity in CRFR nonselective peptides mainly through the loss of CRF1R potency. Interestingly, substitution of proline 11 with serine in hUCN2 decreased CRF2R potency while increasing CRF1R activity. Thus, additional investigations will be required before the complete characterization and identification of all the amino acids that modulate CRFR selectivity in all CRF-related peptide family members are known.

Experimental Section

Chemistry. The peptides were synthesized using a standard Fmoc chemistry protocol for SPPS (solid-phase peptide synthesis) on Rink amide resin with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) as the amide bond forming reagent. The Fmoc deprotection progress was monitored by the conductivity measurement. The peptide chain is built on a Rink amide resin because the C-terminal amide is needed. The synthesized peptides were unloaded from the synthesizer and briefly air-dried. The cleavage from the resin and simultaneous deprotection of the side chain functionalities (OtBu for Asp, Glu, Tyr, Thr and Ser; Pmc for Arg; Boc for Trp and Lys; Trt for His, Asn, and Gln) were accomplished by the treatment with a solution comprising 95% trifluoroacetic acid, 2.5% 1,2-ethanedithiol, 2.5% thioanisole, and 2.5% phenol (w/v) for 4 h at room temperature. The crude peptide solution was separated from the resin by filtration, precipitated in cold ethyl ether, and washed with ethyl ether four times. The peptide was dissolved in 50% acetic acid, recovered by lyophilization, and purified on a Vydac 1.0 cm i.d., 25 cm length C-8 column with 5 μm particle size and 300 Å pore. Since the crude preparations showed very large solubility variations, to fully standardize the purification conditions, they were injected on the column as 50% acetic acid solutions. Separation was achieved using a linear gradient of 5−95% B/A (solution A is 1% acetonitrile, 99% water, 0.1% trifluoroacetic acid; solution B is 95% acetonitrile, 5% water, 0.1% trifluoroacetic acid) with a run time of 75 min and detection at 220 nm. The purity of individual fractions was analyzed using analytical HPLC, and the peptide identity was confirmed with MALDI-TOF MS (matrix-assisted laser desorption ionization time-of-flight mass spectrometry). Preparative fractions of suitable purity were pooled together and lyophilized. The final purified peptide product was again checked using analytical HPLC, and peptide identity was confirmed using MALDI-TOF MS.

CRF Receptor Activity Assays. The CRF1R-containing but CRF2R-lacking human retinoblastoma Y-79 cell line (ATCC HTB-18) and the CRF2R-containing but CRF1R-lacking rat aortic cell line A7r5 (ATCC CRL1444) were used for cAMP analysis. Y-79 cells were cultured in RPMI 1640/10% fetal bovine serum media, while the A7r5 cells were cultured in DMEM/10% fetal bovine serum medium (Life Technologies Inc., Rockville, MD). IBMX was purchased from Sigma (Sigma-Aldrich, St. Louis, MO). cAMP RIA kits were purchased from Amersham (Amersham Bioscience Corp., Piscataway, NJ).

CRF1R and CRF2R activation following peptide exposure was performed as described previously.[19,20] Briefly, CRF1R-containing Y-79 cells and CRF2R-containing A7r5 cells were plated into 96-well plates at 20 000 cells/well 24 h prior to use. Just prior to assay, the medium was removed, cells were washed with PBS, and cells were incubated in DMEM containing 2.5 mM IBMX. Compound was added at the indicated concentrations, and the cells were incubated at 37 °C for 30 min. Following incubation with the test compound, the media was aspirated off the cells, the cells were lysed in lysis buffer (Amersham), and the cAMP levels were quantitated using an RIA kit (Amersham) and following the manufacturer's recommended procedures. Potencies and EC_{50} calculations were performed using the Graph Pad Prism software. To determine the EC_{50}, we analyzed the data using a nonlinear regression, sigmoidal dose−response (variable slope) equation.

Appendix

Abbreviations. IUPAC rules are used for nomenclature of peptides including one-letter codes for natural amino acids. The letter B is used for 2-naphthylalanine. hCRF, human corticotropin releasing factor; oCRF, ovine corticotropin releasing factor; hUCN1, human urocortin 1; hUCN2, human urocortin 2; hUCN3, human urocortin 3; Svg, sauvagine; CRF1R, corticotropin releasing factor receptor 1; CRF2R, corticotropin releasing factor receptor 2; URP, urocortin-related protein; Utn1, urotensin.

Note Added after Print Publication. The spelling of the name of coauthor Michelle Tscheiner is incorrect in the version that was released ASAP on May 25, 2004, and in the version published in the June 17, 2004 issue (Vol. 47, No. 13, pp 3450−3454). The correct electronic version of the manuscript was published on October 26, 2005, and an Addition and Correction is in the November 17, 2005 issue (Vol. 48, No. 23).

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