Discovery of Corticotropin Releasing Factor 2 Receptor Selective Sauvagine Analogues for Treatment of Skeletal Muscle Atrophy

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Received June 26, 2004

The corticotropin release factor 2 receptor (CRF2R) has many biological activities including modulation of the stress response. Recently, we have demonstrated that CRF2R activation functions to prevent skeletal muscle wasting resulting from a variety of physiological stimuli. Thus we are interested in identifying CRF2R selective agonists with optimal pharmacological properties for use in treating muscle wasting diseases. Several CRF2R agonists are known including the frog peptide sauvagine (Svg), which display superior pharmacological properties compared to other CRF2R agonists. Unfortunately sauvagine is a nonselective CRFR agonist, thus making it of less utility due to side effects resulting from corticotropin release factor 1 receptor (CRF1R) activation. Because our initial modifications of Svg at position 11 improved CRF2R selectivity, we investigated the role of amino acids at positions 12 and 13 in Svg. We observed that phenylalanine, leucine, isoleucine, threonine, glutamine, histidine, and tyrosine at the 12th position were the strongest promoters of CRF2R selectivity whereas phenylalanine, glutamine, trytophane, tyrosine, valine, isoleucine, leucine, and 2-naphthylalanine were the preferred residues at the 13th position. Selective sauvagine peptides demonstrated improved antiatrophy effects in a mouse-casting model when compared to sauvagine itself. Thus, we demonstrate that the CRF2R selectivity can be improved by optimizing amino acids at positions 12 and 13 (all with proline at position 11) and that the selective sauvagine analogues demonstrate better in vivo efficacy than sauvagine itself.

Introduction

Skeletal muscle atrophy or wasting is a clinical condition that affects millions of people and can lead to disability, quality of life deprivation, and even increased mortality. Skeletal muscle atrophy results from a variety of conditions including starvation,[1] disuse,[2] denervation/nerve damage,[3] high levels of glucocorticoids,[4] sepsis,[5] cachexia,[6] chronic pulmonary obstructive disease,[7] congestive heart failure,[8] neurodegenerative disease,[9] and muscular dystrophy.[10] An important role of corticotropin releasing factor receptor 2 (CRF2R) in modulating skeletal muscle mass was recently demonstrated.[11] Treatment of corticotropin release factor 1 receptor (CRF1R) deficient mice expressing CRF2R, but not CRF2R knockout mice expressing the CRF1R, with the nonselective CRFR agonist, sauvagine, blocked nerve-damage-induced muscle atrophy. Also, the CRF2R selective agonist urocortin 2 blocked casting-induced atrophy. These studies also demonstrated that CRF1R activation resulted in muscle wasting and loss of muscle mass, probably through the production of corticosteroids, but this phenomenon is related only to a pharmacological activation of CRF1R because it was shown that the physiological mechanisms of skeletal muscle atrophy did not involve CRF1R.[12] Therefore, a concomitant pharmacological activation of CRF1R and CRF2R has no net effect on skeletal muscle mass because the increase in muscle mass observed following activation of the CRF2R is canceled by the loss of muscle mass resulting from activation of the CRF1R. Consequently, CRF2R selectivity of potential antiatrophy medicaments is critical for increasing skeletal muscle mass.

The corticotrophin-releasing factor system consists of two classes of CRF binding proteins, the signaling receptors (CRF1R and CRF2R) and a nonsignaling CRF binding protein (CRFBP).[13] The CRF1R and CRF2R are G-protein coupled receptors that are positively coupled to Gαs, which, upon agonist binding, activates adenylyl cyclase catalyzing the formation of cAMP.[14−18] In addition, coupling to Gαq, resulting in an increase in inositol trisphosphate upon receptor stimulation, has also been described for CRF receptors.[18] The specificity of coupling is apparently dependent on the particular tissue investigated. Both the rodent and the human CRF1R and CRF2R (with multiple splice variants for each receptor) have been cloned with unique distribution patterns observed for each receptor.[14,19−21] Thus, CRF1R is present mainly in the brain whereas CRF2R is widely expressed in peripheral tissues in addition to the brain.[22−25] The use of receptor selective agonists and antagonists, along with the CRF receptor knockout mice, have been useful in determining which CRF receptor mediates specific biological responses.[19,21,26−28] The corticotropin-releasing factor peptide family consists of a relatively large number of natural mammalian, amphibian, and fish peptides with the sizes ranging between 38 and 41 amino acid residues. The most prominent representatives of the CRF peptide family

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10.1021/jm049490m CCC: $30.25
Published on Web 12/10/2004

Table 1. Examples of Key CRF1R and CRF2R Agonists[a]

peptide	sequence 1	11	21	31	41	CRF2R EC_{50} (nM)	CRF2R E_{max} (%)	CRF1R EC_{50} (nM)	CRF1R E_{max} (%)
hCRF	SEEPPISLDL	TFHLLREVLE	MARAEQLAQQ	AHSNRKLMEI	I	49.2	82	20.0	87
hUCN2	- - -IVLSLDV	PIGLLQILLE	QARARAAREQ	ATTNARILAR	V	4.3	96	>1000	8
Sauvagine(Svg)	-ZGPPISIDL	SLELLRKMIE	IEKQEKEKQQ	AANNRLLLDT	I	6.0	95	17.6	99

[a] The sequence alignment in all peptides discussed in this paper is based on hCRF as shown in Table 1. hCRF = human corticotropin releasing factor. hUCN2 = human urocortin 2.

include corticotropin-releasing factor (CRF), also known as corticotropin releasing hormone (CRH), urocortins 1, 2, and 3 (UCN 1, UCN 2, UCN 3), the frog peptide sauvagine (Svg), and the fish peptide urotensin 1 (Utn1). Many physiological functions such as coordination of the endocrine, autonomic, behavioral, and immune responses to stress have been demonstrated for these peptides.[20,29−31]

Our initial investigations of CRFR agonists useful for treating skeletal muscle atrophy have demonstrated that although the CRF2R selective compound urocortin 2 demonstrates good in vivo efficacy for preventing skeletal muscle atrophy[11] it has relatively low in vivo potency. In contrast, the nonselective CRFR agonist sauvagine demonstrates both good in vivo efficacy and potency in preventing skeletal muscle atrophy in CRF1R knockout mice; however, sauvagine, lacking CRF2R selectivity, was not effective in preventing skeletal muscle atrophy in normal mice.[11] Thus, we are interested in improving the CRF2R selectivity of sauvagine in order to improve its in vivo efficacy without altering its in vivo potency.

Previously,[32] we reported modifications of CRF peptides designed to better understand how changes in amino acid composition of various domains influence CRF2R selectivity. When we introduced proline at position 11 of Svg, CRF1R activity decreased, thereby increasing CRF2R selectivity. However CRF2R selectivity was improved only about 3-fold relative to sauvagine, indicating that additional amino acid changes are required to achieve selectivity similar to urocortin 2. Analysis of the sequences at positions 11−13 of CRF2R selective and nonselective CRF peptide family members revealed a strong conservation of amino acids at positions 12 and 13 within each group of peptides.[32] Consequently, we decided to investigate the CRF2R selectivity of [$P^{11}X^{12}X^{13}$]Svg variants by substituting the amino acids at position 12 and 13 with the majority of the natural amino acids and an unnatural amino acid 2-naphthylalanine (using B as the single letter notation symbol for 2-naphthylalanine).

Results and Discussion

Chemistry. Peptides were synthesized by standard methods using Fmoc solid-supported chemistry and were purified on a reverse phase chromatography column to at least 95% purity as determined by analytical high-performance liquid chromatography (HPLC). All peptide sequences were confirmed using mass spectrometry.

In Vitro Investigations. Table 2 contains the most potent Svg-based CRF2R agonists with EC_{50} < 30 nM and a CRF2R/CRF1R selectivity of >10. (The complete list of all investigated variations at these positions is provided in Supporting Information.) Compound **1** is an analogue of sauvagine, derived by a substitution of serine 11 with proline that shows an improvement of the CRF2R/CRF1R selectivity by approximately 3-fold

Table 2. Sauvagine $P^{11}X^{12}X^{13}$ Analogues with CRF2R EC_{50} < 30 nM and CRF2R/CRF1R Selectivity >10[a]

	X^{12}	X^{13}	CRF2R EC_{50} (nM)	(±)	CRF2R E_{max} (%)	CRF1R EC_{50} (nM)	(±)	CRF1R E_{max} (%)	selectivity
1	L	E	10.9	0.8	95	96.0	4.5	99	9
2	L	Q	6.5	2.2	88	423.0	138.0	100	65
3	L	V	5.5	0.3	94	265.5	68.0	95	48
4	L	H	1.9	0.9	81	52.2	11.0	96	28
5	L	L	7.4	0.5	100	162.5	52.0	96	22
6	L	I	5.0	1.6	96	106.0	1.0	94	21
7	L	W	4.8	0.7	96	98.4	10.0	100	20
8	L	B	3.3	0.1	73	63.4	7.0	100	19
9	L	Y	4.3	0.4	73	67.9	16.0	79	16
10	L	G	21.6	7.8	82	311.5	48.5	99	14
11	L	F	10.0	0.3	88	103.0	1.0	93	10
12	I	I	14.8	1.1	73	>1000	NA	4	>68
13	I	L	15.6	3.3	88	>1000	NA	72	>64
14	I	F	12.8	1.6	100	756.0	244.0	83	59
15	I	Y	22.3	1.4	100	>1000	NA	8	>45
16	I	Q	24.6	3.4	78	>1000	NA	6	>41
17	I	W	23.4	1.0	94	562.0	118.0	99	24
18	I	B	16.1	1.1	100	608.0	61.0	98	38
19	A	F	9.4	3.8	100	384.5	106.5	95	41
20	A	Y	20.2	1.2	98	606.0	84.0	95	30
21	A	B	13.3	0.3	100	581.5	194.5	100	44
22	F	Q	1.8	0.7	100	112.0	3.5	64	62
23	F	W	4.7	1.4	95	94.6	27.4	100	20
24	F	F	3.0	0.2	92	48.1	19.3	81	16
25	F	B	5.8	1.4	97	78.4	23.6	93	14
26	F	V	5.9	1.1	91	85.0	9.8	81	14
27	Y	W	4.0	0.2	100	187.5	66.0	100	47
28	Y	Q	8.6	2.3	99	403.5	48.0	100	47
29	Y	T	8.8	2.3	100	188.5	11.5	99	21
30	Y	V	5.0	0.8	100	100.0	3.0	80	20
31	Y	B	5.6	2.1	100	107.5	3.0	96	19
32	Y	F	4.1	1.2	98	63.6	15.0	81	16
33	Y	L	5.2	1.6	94	76.2	1.0	86	15
34	Y	Y	7.0	1.0	93	102.5	2.0	99	15
35	Y	H	5.6	0.6	88	64.9	4.0	81	13
36	T	W	15.2	3.2	100	>1000	NA	43	>66
37	T	Y	18.2	8.1	82	>1000	NA	48	>55
38	T	I	19.1	1.2	94	>1000	NA	42	>52
39	T	F	19.2	5.6	87	943.5	56.0	64	49
40	T	L	20.6	1.1	100	>1000	NA	48	>49
41	H	Q	21.3	6.1	100	445.0	7.0	100	21
42	H	B	7.2	0.2	93	115.5	6.0	100	16
43	H	W	25.1	9.6	100	408.0	77.0	100	16
44	H	F	7.8	2.2	98	80.9	1.0	100	10
45	Q	I	8.5	1.0	92	706.5	294.0	93	83
46	Q	W	21.7	3.0	99	>1000	NA	78	>46
47	Q	Q	29.9	2.9	100	>1000	NA	50	33
48	Q	Y	10.7	1.5	96	325.0	7.0	100	30
49	Q	F	8.5	0.4	100	236.5	58.0	100	28
50	Q	B	14.1	1.6	100	366.5	98.0	100	26
51	Q	L	27.6	0.6	93	447.0	45.0	96	16
52	N	Y	21.6	4.9	93	220.5	58.0	100	10

[a] Entries ordered by X^{12} and by selectivity within groups. Selectivity is expressed as a ratio of EC_{50} values CRF1R/CRF2R. All assays were performed in triplicate with two independent experimental analyses of each peptide at both CRF receptors. B designates 2-naphthylalanine. NA = not applicable.

compared to sauvagine itself. However, this selectivity is not close to the selectivity of UCN2 or UCN3, thus additional amino acid modifications are necessary. Changing the amino acids at positions 12 and 13 to those found in UCN2, P11, I12, or G13, greatly reduced the CRF2R potency of this sauvagine analogue (CRF2R potency >100 nM, see Supporting Information); changing the amino acids at position 12 and 13 to those found in UCN3, P11, T12, or N13, also greatly reduced the CRF2R potency of this sauvagine analogue (CRF2R potency > 100 nM, see Supporting Information). Thus, sauvagine requires an alternative combination of amino acids at positions 11, 12, and 13.

Substitutions at position 12 (Table 2) demonstrated that leucine (**1**−**11**), isoleucine (**12**−**18**), phenylalanine (**22**−**26**), tyrosine (**27**−**35**), threonine (**36**−**40**), histidine (**41**−**44**), and glutamine (**45**−**51**) provided the most consistent improvement in CRF2R/CRF1R selectivity. The best combination of CRF2R potency and CRF2R/CRF1R selectivity was observed with leucine and tyrosine at position 12 although excellent selectivity was observed with isoleucine and threonine at position 12 albeit with a loss of CRF2R potency.

Substitutions at position 13 demonstrated that leucine, isoleucine, valine, phenylalanine, tyrosine, glutamine, tryptophane, and the unnatural amino acid, 2-naphthylalanine, provided the most consistent improvement in CRF2R/CRF1R selectivity. A converted Table 2 in which the entries are ordered according to the identity of X^{13} substituent is provided for the reader's convenience in the Supporting Information. The best combination of CRF2R potency and CRF2R/CRF1R selectivity was observed with phenylalanine and glutamine at position 13 although most position 13 substitutions demonstrated good potency and selectivity with an appropriate position 12 substitution. Interestingly, although valine, tryptophan, and 2-naphthylalanine substitutions at position 13 provided good selectivity, these substitutions did not improve selectivity when they were placed in position 12. Conversely, threonine and histidine substitutions at position 12 provided good selectivity, but threonine and histidine were not effective at increasing selectivity when substituted at position 13. In contrast, leucine, isoleucine, phenylalanine, tyrosine, and glutamine when substituted at either position 12 or position 13 provided good CRF2R/CRF1R selectivity.

The most potent analogues at CRF2R have been found among peptides substituted at position 12 (Table 2) with leucine, phenylalanine, and tyrosine. It should be noted that phenylalanine is a highly conserved residue at this position in CRF, UCN1, and Utn1[32] peptide family members, with the exception of Svg having leucine at the position 12. Lower activities were observed with isoleucine and threonine, even though isoleucine and threonine are also present in the CRF2R selective peptides of the UCN2/UCN3 family.[32]

Charged amino acid and glycine at positions 12 and 13 did not improve CRF2R/CRF1R selectivity due to large decreases in CRF2R potency (Supporting Information).

In Vivo Investigations. Next, we wanted to evaluate the antiatrophy effectiveness of several of the CRF2R selective sauvagine analogues. The compounds listed in Table 3 were administered for 14 days by continuous infusion dosing using a subcutaneously implanted ALZA osmotic minipump to mice with their lower right leg casted. The mass of the tibialis anterior from the casted leg was analyzed and compared to the appropriate vehicle treated control to arrive at the percent inhibition of atrophy. Although sauvagine provided complete inhibition of muscle atrophy in CRF1R knockout mice,[11] it has practically no effect (Table 4) on preventing skeletal muscle atrophy in normal mice, similar to what has been previously reported. As can be seen in Table 4, the CRF2R selective sauvagine analogues were considerably more efficacious and potent than sauvagine in preventing the loss of casting-induced skeletal muscle mass. Differences in in vivo potency were observed with different sauvagine analogues, difference that did not appear to correlate with CRF2R selectivity but did correlate reasonably well with CRF2R potency. Finally, in vivo dose responsiveness was not always observed for the most potent compounds, probably because the doses tested, which were chosen to compare efficacy of the sauvagine analogues with sauvagine, were above the linear portion of the dose response curve.

Table 3. In Vivo Results of [P,^{11} X,^{12} X^{13}]Svg Analogues Investigations in the Mouse Casting Model[a]

	compound	CRF2R EC_{50} (nM)	CRF2R selectivity[a]	doses (μg kg^{-1} day^{-1})	% inhibition of casting induced TA mass loss
	Sauvagine (Svg)	6.0	3	30	12
				100	0
				300	17[a]
22	[P^{11}F^{12}Q^{13}]Svg	1.8	46	30	58[a]
				100	46[a]
				300	50[a]
8	[P^{11}L^{12}B^{13}]Svg	3.3	19	10	53[a]
				30	45[a]
				100	73[a]
45	[P^{11}Q^{12}I^{13}]Svg	8.5	83	10	32[a]
				30	73[a]
				100	14[a]
36	[P^{11}T^{12}W^{13}]Svg	15.2	>66	10	30[a]
				30	84[a]
				100	91[a]
13	[P^{11}I^{12}L^{13}]Svg	15.6	>64	10	10
				30	66[a]
				100	91[a]
37	[P^{11}T^{12}Y^{13}]Svg	18.2	>55	30	3
				100	25[a]
				300	59[a]
15	[P^{11}I^{12}Y^{13}]Svg	22.3	>45	30	24[a]
				100	38[a]
				300	58[a]
16	[P^{11}I^{12}Q^{13}]Svg	24.6	>41	30	12
				100	22
				300	31[a]

[a] Effects reported as % inhibition of casting-induced tibialis anterior (TA) muscle mass loss. $p < 0.05$. Selectivity is expressed as a ratio of EC_{50} values CRF1R/CRF2R.

In summary, we have shown that specific amino acid substitutions at positions 12 and 13 in sauvagine, in conjunction with the substitution of proline at position 11, can greatly improve the CRF2R selectivity of sauvagine. The increase in CRF2R selectivity provided increased in vivo effectiveness in preventing casting-induced muscle atrophy. Thus, CRF2R selective sauvagine analogues may find utility in treating skeletal muscle wasting diseases.

Experimental Section

Synthesis of peptides and biological assays of CRF1R and CRF2R activity in cell lines were described in our previous publication[32] and are also available as Supporting Information.

In Vivo Mouse Casting Atrophy Model. Eight mice per treatment group were anesthetized with isoflurane, and the lower right leg was casted from the knee to the toes with heat activated casting material (Vet Lite, Kruuse Inc., Marslev, Denmark). The test materials were administered by implantation of 14 day osmotic minipumps (Alza, Palo Alto, CA) in the midscapular region. Fourteen days after casting, animals were euthanized by carbon dioxide asphyxiation followed by cervical dislocation. The cast was removed, the tibialis anterior muscle was dissected from both legs, cleaned of tendons and connective tissue, and weighed, and the mass was recorded. Statistical analysis of the data was performed using an ANCOVA model with treatment effect and starting weight as the covariates. Pairwise comparisons for all end points were generated using least-squares means (SAS, Cary, NC), adjusted for unequal sample sizes and starting weight.

Supporting Information Available: Tables containing a complete list of X^{12} and X^{13} substitutions in [$P^{11}X^{12}X^{13}$]Svg with activities at CRF1R and CRF2R, a converted Table 2 with entries sorted according to X^{13} identity, as well as experimental details for the peptide syntheses and in vitro assays of CRF1R and CRF2R acitivity. This information is available free of charge via the Internet at http://pubs.acs.org.

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JM049490M