Novel, Potent ORL-1 Receptor Agonist Peptides Containing α-Helix-Promoting Conformational Constraints

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The ORL-1 receptor has recently been cloned and is implicated in a wide variety of physiological and pathophysiological processes. Toward the goal of elucidating important features of the receptor-bound conformation of the endogenous ligand, nociceptin (NC), several conformationally constrained analogues were prepared. Either α-aminoisobutyric acid (Aib) or *N*-methylalanine (MeAla) were inserted as replacement(s) for Ala7, Ala11, or Ala15 in the native NC sequence (FGGFTGARKSARKLANQ). In vitro assays measuring human ORL-1 receptor affinity (competition binding against [^{3}H] NC), functional potency ([^{35}S]GTPγS), and efficacy (as compared to NC) were performed for each new peptide. The receptor affinities of the Aib-containing peptides generally matched NC, showing K_i's in the range of 0.1−0.5 nM. By comparison, the receptor affinities of the MeAla-containing peptides were significantly diminished. Peptide **14** (FGGFTG[Aib]RKS[Aib]RKLANQ-NH_2), which contains two constrained alanine residues (positions 7 and 11) and a C-terminal amide modification, was found to be a very potent agonist with $K_i = 0.05$ nM and $EC_{50} = 0.08$ nM in the human ORL-1 assays. The data support a hypothesis that the receptor-bound form of NC might adopt an amphipathic helix in the "address" segment of the sequence.

Introduction

The opioid receptor-like 1 (ORL-1) receptor is the most recently identified member of the opioid receptor family joining the better known, μ, δ, and κ, opioid receptors. The original homology cloning of this G protein-coupled receptor was published almost simultaneously by several groups.[1−8]

A number of studies have demonstrated a broad spectrum of physiological functions of the ORL-1 receptor in both the central and peripheral nervous systems and in nonneuronal tissues. These functions include modulation of nociception,[9−11] locomotor activity,[10] reversal of stress-induced analgesia,[12] attenuation of stress responses,[13] modulation of learning and memory,[14−16] regulation of neurotransmitter and hormone release,[17,18] modulation of kidney function,[19] and a potential role in neuronal differentiation.[20−22] The endogenous agonist of this receptor is the heptadecapeptide (H_2N-FGGFTGARKSARKLANQ-COOH) known either as nociceptin (NC)[9] or orphanin FQ.[10]

It has been suggested that the N-terminal FGGF segment of NC (the "message") is primarily responsible for triggering a stimulation of the ORL-1 receptor, while the TGARKSARKLANQ segment (the "address") is primarily involved in binding and receptor specificity.[23] Throughout this manuscript, we have adopted these previously reported subsequence definitions for the NC peptide.

A molecular level understanding of how NC binds and activates the ORL-1 receptor would be valuable toward the goal of designing novel molecules with high ORL-1 affinity and specificity. Molecules with this profile may have utility as therapeutic agents or as mechanistic tools in various pharmacological experiments. In the absence of an experimentally derived, three-dimensional structure of this ligand−receptor complex, alternative (indirect) approaches toward this type of structural information have been pursued. These approaches include molecular modeling of the NC-ORL-1 complex,[24,25] elucidation of the NC solution conformation by NMR,[26] and synthesis of conformationally constrained NC analogues.[27]

The rationale for the latter approach is based on the premise that conformational degrees of freedom in the native, linear NC sequence might be significantly reduced via the introduction of conformational constraints. Constraint(s) that can reduce the peptide's flexibility to a very small number of predictable/experimentally solvable conformations typically yield the most insights into the so-called bioactive form. The experimentally determined affinities and/or functional potencies of such constrained peptides, when considered together with the imposed three-dimensional conformation, can provide tangible clues regarding the bioactive structure of the peptide ligand.

Since the original discovery of the NC peptide, there has been only a single report describing the incorporation of conformational constraints into the native NC sequence.[27] In that work, a series of cyclic peptides was made by forming a side chain to side chain disulfide bond between two cysteine residues. Since NC does not contain cysteine in the native form, the authors altered the native sequence with replacement by, and/or insertion of, cysteine (Cys) at various positions. Moreover, the authors truncated the native sequence by eliminating certain residues from the C-terminus. It was reported that cyclizations in the N-terminal or middle portion of the sequence significantly diminished binding and functional potency at the ORL-1 receptor, while

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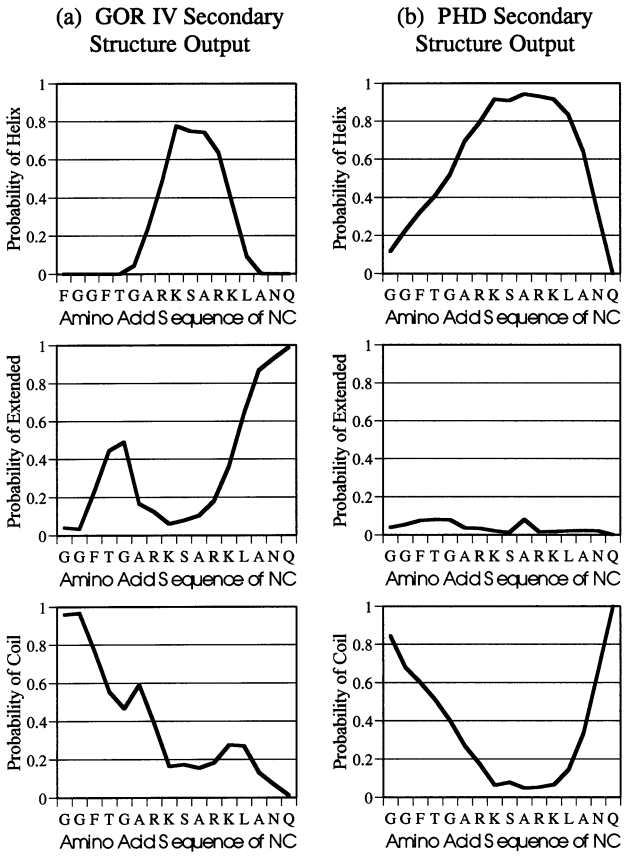

Figure 1. Secondary structure prediction of the primary NC sequence (FGGFGARKSARKLANQ) using either (a) GOR IV or (b) PHD. Probability predictions of helix, extended, and random coil conformation, respectively, are plotted vertically as a function of NC peptide sequence.

cyclization at the C-terminus afforded agonists with potencies comparable to NC itself. One main conclusion from that work was that the bioactive form of NC is likely not cyclic or pseudo-cyclic at the N-terminus or in the middle portion of the sequence. However the truncations and the insertion/replacement of certain residues in the native NC sequence add caveats to the ultimate interpretation of the data.

The work presented here is based on our observation that in the "address" segment of NC (TGARKSARKLANQ) there are three regularly spaced alanine (Ala) residues, Ala7, Ala11, Ala15 that are arranged in the ordered pattern of, ARKxARKxA. This symmetrical repeat of Ala, separated by a regular repeat of the highly basic R-K (Arg-Lys) pair, suggested the possibility of an amphipathic helix within this segment of the peptide. This concept was supported by the secondary structure predictions that we obtained when the primary sequence of NC was input into either a hierarchical neural network or an information theory-based prediction algorithm. For the former, we used the program PHD[28] which predicted a helical conformation spanning the sequence from Gly6 to Leu14. For the latter, we used GOR IV[29] which predicted a helical conformation spanning Arg8 to Lys13. The program prediction outputs from each are presented in Figure 1.

To more fully explore this structural hypothesis, we have prepared a series of full-length NC analogues containing either *N*-methyl alanine (MeAla) or C^{α}-methyl alanine (Aib) as a replacement for Ala7, Ala11, or Ala15. *N*-Methyl substitution on an amino acid in a given peptide sequence is known to disfavor the adoption of local ϕ, ψ angles that would correspond to a helical secondary structure (ϕ, ψ approximately $-60°$, $-60°$), while favoring an extended backbone (ϕ, ψ approximately 180°, 180°). The complimentary, although contrasting, C^{α}-methyl modification has been shown to favor a helical, rather than extended conformation.[30,31] These conformational preferences apply only to the backbone ϕ, ψ angles (where ϕ_i and ψ_i correspond to backbone dihedral angles for residue i defined by the four adjacent amino acid backbone atoms $C_{i-1}-N_i-C^{\alpha}_i-C_i$ and $N_i-C^{\alpha}_i-C_i-N_{i+1}$, respectively) of the same amino acid bearing the additional methyl group. It was anticipated that, if the bioactive form of NC adopts an amphipathic helix within the address portion of the sequence, the Aib-containing peptides would have binding affinities and potencies similar, or superior, to NC. Likewise, the MeAla-containing peptides were expected to lose affinity and be less potent.

We report herein for the first time the results of human ORL-1 receptor binding and functional assays using these novel, conformationally constrained, analogues of NC. Our results provide evidence for the adoption of an amphipathic helix within the "address" segment of NC in the receptor-bound state. Moreover, several of the peptides reported here have binding affinities and potencies that are superior to the native ligand, NC, making them among the most potent ORL-1 receptor agonists published to date.

Methods

Abbreviations. DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene]-*N*-methylmethan-aminium hexafluorophosphate *N*-oxide; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid. Amino acids are of L-configuration unless otherwise noted.

Peptide Synthesis. All reagents were obtained commercially from Applied Biosystems, SynPep, or Bachem. Peptides were prepared by manual solid-phase synthesis using standard Fmoc chemistry.[32,33] Briefly, either Tentagel Rink amide resin or Tentagel HMP loaded resin was used to prepare C-terminal amide-containing peptides or C-terminal acid-containing peptides, respectively. For each Fmoc deblocking step, the growing resin-bound peptides were treated with a solution comprised of 20% piperidine/DMF for 7 min. Prior to coupling, each amino acid was activated as follows: In DMF solution, 4 equiv of amino acid was incubated for 3−5 min with 4 equiv of HATU and 8 equiv of DIEA. Following the addition of the coupling solution to the resin-bound, growing peptide, the mixture was stirred briefly, then allowed to stand, for 30 min unless otherwise noted. Aib and MeAla residue couplings were repeated 2 × 1 h and 2 × 3 h, respectively. Full length peptides were deprotected and cleaved from the resin by stirring in a solution of TFA:phenol:H_2O:thioanisole:1,2-ethanedithiol; 33:2:2:2:1 for 2 h at room temperature.[34] The crude peptides were purified by Waters system Prep LC 4000. Purity was determined by HPLC to be >95%, and peptide composition was determined by mass spectral analysis (MS, electrospray). A summary of the analytical results for each peptide described in this report is located in Table 1.

Human ORL-1 Receptor Binding. All reagents were from Sigma (St. Loius, MO) unless noted otherwise. Membranes from recombinant HEK-293 cells expressing the human opioid receptor-like (ORL-1) receptor (Perkin-Elmer, Boston, MA) were prepared by lysing confluent cell monolayer in ice-cold

Table 1. Analytical Results Corresponding to All Synthesized Peptides (**2**−**14**)

peptide	analytical HPLC t_R (min) method 1	analytical HPLC t_R (min) method 2	formula weight calcd	[M + H] found
2	24.65[ast]	20.42[c]	1823.09	1822.6
3	15.49[b]	21.08[c]	1823.09	1823.2
4	23.99[a]	19.64[c]	1823.09	1823.0
5	23.15[a]	17.48[c]	1823.09	1824.0
6	12.48[b]	16.53[c]	1823.09	1823.0
7	12.44[b]	17.87[c]	1823.09	1823.0
8	14.47[b]	20.52[c]	1822.10	1823.2
9	25.45[a]	21.86[c]	1822.10	1822.0
10	13.89[b]	19.89[c]	1822.10	1822.0
11	11.24[b]	17.76[c]	1822.10	1822.0
12	11.68[b]	16.73[c]	1822.10	1822.0
13	23.83[a]	17.46[c]	1822.10	1822.2
14	16.13[b]	24.13[c]	1836.11	1837.0

[a] 0−30% acetonitrile (0.075% TFA) (30 min), 1 mL/min, 220 nm, Vydac C_{18}, 5 μm, 250 × 4.6 mm. [b] 10−40% acetonitrile (0.075% TFA) (30 min), 1 mL/min, 220 nm, Vydac C_{18}, 5 μm, 250 × 4.6 mm. [c] 0−35% acetonitrile (0.1% TFA) (40 min), 1 mL/min, 220 nm, Delta Pak C_{18}, 5 μm, 150 × 3.9 mm.

hypotonic buffer (2.5 mM $MgCl_2$, 50 mM HEPES, pH 7.4) (10 mL/10 cm dish) followed by homogenization with a tissue grinder/Teflon pestle. Membranes were collected by centrifugation at 30000*g* for 15 min at 4 °C and pellets resuspended in hypotonic buffer to a final concentration of 1−3 mg/mL. Protein concentrations were determined using the BioRad (Hercules, CA) protein assay reagent with bovine serum albumen as standard. Aliquots of the ORL-1 receptor membranes were stored at −80 °C.

Radioligand dose-displacement binding assays used 0.1 nM [^{3}H]-nociceptin (NEN, Boston, MA) (87.7 Ci/mmol) with 20 μg membrane protein/well in a final volume of 500 μL of binding buffer (10 mM $MgCl_2$, 1 mM EDTA, 5% DMSO, 50 mM HEPES, pH 7.4). Reactions were carried out in the absence and presence of increasing concentrations of unlabled nociceptin (Sigma) or its modified analogues. All reactions were conducted in 96-deep well polypropylene plates for 2 h at room temperature. Binding reactions were terminated by rapid filtration onto 96-well Unifilter GF/C filter plate presoaked in 0.5% polyethylenamine using a 96-well tissue harvester (Brandel, Gaithersburg, MD) followed by three filtration washes with 500 μL of ice-cold binding buffer. Filter plates were subsequently dried at 50 °C for 2−3 h. BetaScint scintillation cocktail (Perkin-Elmer) was added (50 μL/well), and plates were counted on a Packard Top-Count for 1 min/well. The data were analyzed using the one-site competition curve fitting functions in GraphPad PRISM, v. 3.0 (San Diego, CA).

[^{35}S]GTPγS Functional Assay. [^{35}S]GTPγS functional assays were conducted using freshly thawed ORL-1 receptor membranes (see above). Assay reactions were prepared by sequentially adding the following reagents (final concentrations indicated): ORL-1 membrane protein (0.066 μg/μL), saponin (10 μg/mL), GDP (3 μM) and [^{35}S]GTPγS (0.20 nM; Perkin-Elmer) to binding buffer (100 mM NaCl, 10 mM $MgCl_2$, 20 mM HEPES, pH 7.4) on ice. The prepared membrane solution (190 μL/well) was transferred to 96-shallow well polypropylene plates containing 10 μL of 20× concentrated stock solutions of agonist (nociceptin or nociceptin analogues) prepared in DMSO. Plates were incubated for 30 min at room temperature with shaking. Reactions were terminated by rapid filtration onto 96-well Unifilter GF/B filter plates using a 96-well tissue harvester followed by three filtration washes with 200 μL of ice-cold binding buffer (10 mM NaH_2PO_4, 10 mM Na_2HPO_4, pH 7.4). Filter plates were subsequently dried at 50 °C for 2−3 h. BetaScint scintillation cocktail (Perkin-Elmer) was added (50 μL/well) and plates counted in a Packard Top-Count for 1 min/well. Data were analyzed using the sigmoidal dose−response curve fitting functions in GraphPad PRISM, v. 3.0.

Results

Individual peptides, prepared as part of an initial systematic series, differed from NC only in that they contained *N*- or C^{α}-methylalanine (MeAla or Aib, respectively) as a replacement for the native Ala, either at position 7, 11, or 15. Like native NC, each of these peptides also contained a free C-terminal carboxyl and N-terminal amino functionality. Table 2 (peptides **2**−**7**) lists the amino acid sequences for the peptides in this initial series. The corresponding human ORL-1 receptor affinities, EC_{50} and E_{max} (as measured in the [^{35}S]-GTPγS functional assay) for these peptides are also presented in Table 2.

On the basis of the assay results summarized in Table 2, it is apparent that the Aib substitution, and therefore the imposed local helical conformation, is readily tolerated by the receptor regardless of the substitution being located at position 7, 11, or 15. The measured binding affinities for these three peptides (**2**−**4**) range from 0.1 to 0.48 nM. These values are highly similar to the K_i measured for NC (0.3 nM). Similarly, these three peptides (**2**−**4**) are full ORL-1 agonists having potencies and efficacies comparable to NC.

The MeAla containing peptides from this series (**5**−**7**), although agonists, bind with significantly lower affinities and are less potent than NC or the corresponding Aib-containing peptides. In particular, the MeAla7 peptide (**5**) binds to the ORL-1 receptor with 150-fold less affinity than the corresponding Aib7 peptide (**2**) and is approximately 400-fold less potent in the functional assay. A similar difference in potency is observed when comparing the MeAla11 peptide (**6**) to the Aib11 peptide (**3**). Only a slight difference in binding

Table 2. Amino Acid Sequences and in Vitro Human ORL-1 Receptor Pharmacological Results for NC and All Aib- or MeAla-Containing Peptide Analogs

peptide	amino acid sequence	ORL-1 K_i (nM)	ORL-1 EC_{50} (nM)	ORL-1 E_{max}, %
1	NC	0.3 ± 0.02	0.45 ± 0.06	100
2	[Aib^7]NC-COOH	0.1 ± 0.02	0.27 ± 0.08	97 ± 2
3	[Aib^{11}]NC-COOH	0.48 ± 0.18	1.0 ± 0.1	96 ± 2
4	[Aib^{15}]NC-COOH	0.15 ± 0.02	0.47 ± 0.14	91 ± 4
5	[MeAla^7]NC-COOH	15 ± 4	96 ± 2	95 ± 4
6	[MeAla^{11}]NC-COOH	20 ± 8	407 ± 97	97 ± 3
7	[MeAla^{15}]NC-COOH	1.1 ± 0.4	5.3 ± 1.1	93 ± 5
8	[Aib^7]NC-$CONH_2$	0.05 ± 0.01	0.06 ± 0.03	82 ± 8
9	[Aib^{11}]NC-$CONH_2$	0.08 ± 0.01	0.14 ± 0.08	82 ± 7
10	[Aib^{15}]NC-$CONH_2$	0.02 ± 0.01	0.2 ± 0.01	97 ± 4
11	[MeAla^7]NC-$CONH_2$	2.2 ± 0.7	10.5 ± 1.5	94 ± 3
12	[MeAla^{11}]NC-$CONH_2$	6.7 ± 2	52 ± 8	91 ± 1
13	[MeAla^{15}]NC-$CONH_2$	0.06 ± 0.02	0.73 ± 0.11	87 ± 5
14	[Aib^7,Aib^{11}]NC-$CONH_2$	0.05 ± 0.01	0.08 ± 0.03	90 ± 9

and functional activity (approximately 10-fold) was noted between the MeAla15 and Aib15 peptides (**7** and **4**, respectively). These data support the hypothesis that local helical conformation, particularly about Ala7 and Ala11, is preferred over extended conformation in the receptor-bound state. Conformational preference may be less critical about Ala15, although on the basis of our results, helix is still favored over extended ϕ,ψ.

As a follow-on study, we prepared an analogous series of six peptides (Table 2, **8**−**13**) which, rather than containing a C-terminal carboxylate moiety, contained a C-terminal amide. The rationale behind this second series was based on published results that support the hypothesis that a significant contribution to the NC-ORL-1 binding energy is the result of electrostatic interactions between acidic residues (particularly in EL2) of the ORL-1 receptor and basic residues in the address segment of NC.[24,25,35,36] We presumed that masking the acidic character of the C-terminal carboxylate as an amide might enhance the receptor interaction by presenting a more complementary electrostatic environment.

The results of ORL-1 receptor binding and functional assays are presented in Table 2 for the C-terminal amide-containing peptides in this follow-on series. Generally, the structure−activity relationship observed within this series parallels what was observed in the initial C-terminal carboxylate-containing series of peptides. One notable difference is that, while the trends are the same, the addition of the C-terminal amide always enhances the affinity (3−10-fold improvement) and potency (5−10-fold improvement) with respect to the C-terminal carboxylate-containing peptides. Each of these Aib-containing peptides is more potent and binds the ORL-1 receptor with higher affinity than the native ligand, NC.

Due to the high receptor affinities, we conclude that the Aib substitutions in this second series are conformationally acceptable replacements for Ala7, Ala11, or Ala15. In contrast, significant loss in relative affinity and potency was observed for the corresponding MeAla substituted peptides with the one exception of peptide **13**, which contains the MeAla at position 15. As was the case for series one, these data also support the hypothesis that local helical conformation, particularly about Ala7 and Ala11, is preferred over extended conformation in the receptor-bound form. About position 15, conformational preference is less important. Moreover, masking the acidic character of the native C-terminus as an amide appears to enhance binding affinity and potency.

As a final test of the hypothesis, and based on the results shown in Table 2, we prepared and assayed peptide **14**, which contains two Aib replacements in a single peptide, one at Ala7, the other at Ala11. In addition, this peptide contains a C-terminal amide. This double backbone constraint is likely to induce significant helical character into the secondary structure of the "address" segment of this peptide. Assay results on peptide **14** show that it is a highly potent (EC_{50} = 0.08 nM) and efficacious agonist with remarkable affinity (K_i = 0.05 nM) to the human ORL-1 receptor. It has significantly higher affinity and is more potent than NC (K_i = 0.3 nM; EC_{50} = 0.45 nM).

Discussion

Being a linear, highly polar heptadecapeptide, one would anticipate that in an aqueous environment, NC would be highly solvated by water and would rapidly interconvert between many conformational states. At least one publication has demonstrated this by describing attempts to solve the solution conformation of NC using NMR techniques.[26] The authors reported that NC adopts no preferential secondary structure in water because, on the NMR time scale, the rapid conformational interconversion appears random. However, it is likely that upon interaction with the ORL-1 receptor during, or just prior to, the binding event, the NC peptide adopts a discreet, ordered structure that is highly complementary to the receptor binding site such that high affinity binding, and ultimately signaling, can occur readily.

Thoughtful inspection of the primary sequence of NC, together with results obtained from two methodologically differing secondary structure prediction algorithms, led to a hypothesis that the address segment of the NC peptide (TGARKSARKLANQ) may adopt an α helical conformation in the receptor-bound state. Results taken from secondary structure prediction algorithms, PHD and GOR IV, further supported the concept (Figure 1). Both methods predicted a helical core, flanked by either random coil or extended conformation.

The hypothesis was experimentally tested by incorporating backbone constraints known to impose, either extended (ϕ, ψ approximately 180°, 180°) or helical (ϕ, ψ approximately −60°, −60°), structure into the sequence. Specifically we replaced either Ala7, Ala11, or Ala15 within the full length NC sequence with MeAla, to impose a local extended structure, or with Aib, to impose a local helical structure.

All of the Aib-containing peptides reported herein have higher ORL-1 receptor affinity than the native ligand, NC. We interpret this to support the hypothesis that, in the receptor-bound state, NC adopts a helix (or helix-like structure) in the "address" segment. Due to the distribution of adjacent basic residues in this part of the sequence, we propose that the helix has significant amphipathic character.

For illustrative purposes, Figure 2 shows a model of the ARKSARKLA segment of the NC "address" sequence created by imposing standard ϕ,ψ angles corresponding to α helix into the backbone. No attempts were made to accurately model the side chain dihedral angles in this model so they appear artificially extended in Figure 2. It is clear, however, that Arg8,12 and Lys9,-13 are situated on the same face of the proposed helix and represent a highly complimentary electrostatic binding partner for the acidic residues in extracellular loop domain(s) of the receptor. Accurate placement of these highly flexible Arg and Lys side chains is not possible using computational means alone. However, it would be an interesting follow-up study to impose various conformational constraints at one or more of these side chain positions.

The introduction of these conformational constraints reduces the overall flexibility of the peptide, favoring a conformation that resembles the bioactive form. This entropic advantage manifests as a higher affinity, as measured by the thermodynamic equilibrium constant,

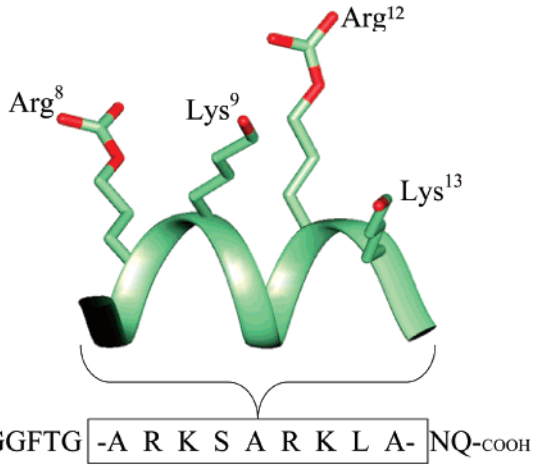

Figure 2. Illustration of the NC sequence where the subsegment, ARKSARKLQ, is shown in an α-helical conformation as a putative bioactive form. The amphipathic nature of the proposed helix is evident by the monofacial distribution of the Arg-Lys pairs of residues in this segment.

K_i. In contrast, MeAla substitutions at either Ala7 or Ala11 result in peptides that are less potent and bind with significantly lower affinity to the ORL-1 receptor. The imposed "extended" local conformation of these peptides must be less similar to the bioactive form of NC.

Our results are in agreement with previous publications that describe modified NC peptides. One such report describes three analogues of NC, each having either Ala7, Ala11, or Ala15 replaced by the more sterically hindered tryptophan (Trp) residue.[37] In all cases, the peptides lost affinity, lost potency, and lost efficacy in human ORL-1 receptor assays that are highly similar to those we have described. The authors interpreted the results to mean that there is restricted space about these alanines at the receptor binding site. It is also possible that the addition of these Trp residues into the sequence might alter the overall amphipathic profile we propose to be important for NC binding since Trp typically positions itself at the peptide/water interface due to it's own local electrostatic environment.

Another interesting result from our series is that peptide affinities and potencies were always improved when the C-terminus of the peptide contained an amide, rather than a free carboxylate group. It is difficult to explain this observation on the basis of any imposed conformational preference associated with the amide. However, if one considers the proposals that have been made regarding the receptor-bound orientation of NC, then some possible electrostatic-based explanations emerge.[24,25,38]

In particular, it is presumed that the N-terminal FGGF, or "message", segment of NC binds to a site of the receptor that is primarily made up of residues from the transmembrane (TM) domains, the hallmark of which is an electrostatic interaction between the N-terminal amino group of NC and the highly conserved aspartic acid residue (Asp130) in TM 3 of the receptor.[38] All computer modeling studies reported thus far on the NC-ORL-1 complex incorporate this interaction as a key anchor point.[24,25] If true, it would mean that the C-terminal segment of NC is likely to be oriented toward, and likely interacting with, one or more of the extracellular loop (EL) domains of the receptor.

Computational and experimental evidence has been published that supports a binding model where one or more of the numerous basic residues in the "address" segment of NC (TGARKSARKLANQ) interact with acidic residues in EL2 of the receptor as part of the binding process.[24,25,35,36] Similar evidence is available from published work (albeit using the mouse form of the ORL-1 receptor) showing that replacement of either Arg8, Arg12, Lys9, or Lys13 with a nonbasic residue eliminates ORL-1 receptor activity in NC (1−13)-NH_2, further supporting the idea that key electrostatic interactions are involved in binding.[23]

If the receptor binding site that accommodates the "address" segment of NC is primarily an acidic environment, then the C-terminal carboxylate of NC may be somewhat repulsive, since it is of the same charge. Transforming the C-terminal acid of NC to an amide, as we have described, may mask the charge such that it is more complementary to the electrostatic environment posed by the corresponding domain of the binding site. This is one explanation for the observed enhancements in potency but additional experimental evidence is required to fully support this possibility.

In both series of peptides we report the most modest contrast in results between the incorporation of MeAla versus Aib occurred at position 15, toward the C-terminal end of NC. Specifically, in C-terminal acid-bearing series one (Table 2) the difference in binding and functional potency was 10-fold, favoring the Aib-containing peptide (**4**) over the MeAla-containing peptide (***7***). The difference was even less in the corresponding series two peptides (**10** and **13**). In that case, there was no apparent loss in binding affinity and the potency loss was only 3-fold. We interpret this to mean that the C-terminal part of NC does not make a substantial energetic contribution to binding and conformational preference is minimal. Indeed, our results provide evidence that, in the vicinity of Ala15, there is considerable tolerance for different ligand conformation since both the Aib15 and MeAla15 peptides bind similarly.

Further evidence for this proposal is found in a recent publication demonstrating that a disulfide-containing cyclic peptide analogue of NC containing a Ser→Cys replacement at position 10, and a single Cys extension at the C-terminus is a full agonist, only slightly less potent than NC.[27] Together with our constrained peptides, this cyclic peptide represents an additional conformational type that can bind, and activate, the receptor with potency similar to the native ligand. Moreover, it contains a large, hindered disulfide bond in the C-terminal domain, which is apparently accommodated by the receptor, again suggesting significant steric and conformational tolerance within the last four residues at the NC C-terminus.

The recent report that NC (1−13)-$CONH_2$ is also a full agonist with affinity similar to NC[39] provides additional evidence. This peptide is effectively a NC C-terminal truncation peptide, missing four residues from the end of the native sequence (LANQ). This peptide further exemplifies the lack of conformational stringency, or required binding energy contribution, associated with the C-terminal portion of NC because

the last four residues are eliminated. It is of further interest that analogues of this truncated peptide, when cyclized in a fashion that stabilizes a helical conformation, also shows potent in vitro ORL-1 pharmacolgy.[40]

Conclusions

We have presented a series of conformationally constrained analogues of NC, several of which are among the most potent agonists yet reported for the ORL-1 receptor.[41] Due to the predictable nature of the preferential ϕ,ψ values associated with the constraints used, we propose a high probability of α helical structure in the "address" segment (TGARKSARKLANQ) of NC in the bioactive form. The importance of a helical conformation about the C-terminal segment LANQ is significantly less than in the preceding segment TGARKSARK in that the ORL-1 affinities of the MeAla15- and Aib15-substituted peptides were highly similar despite differing preferential local conformations. The high affinity and functional potency of the doubly constrained [Aib7,-Aib11]NC-NH_2 peptide (**14**) provides additional strong evidence for an α-helical bioactive secondary conformation adopted within the "address" segment of NC. Finally, the C-terminal amide-containing peptides described herein provide evidence that NC-like peptides can be made more complementary to the receptor binding site than the native C-terminal acid-containing analogues. This is likely to be the result of improved hydrogen bond capabilities, a different solvation pattern at the C-terminus, or some related electrostatically based phenomenon. The interpretation of our results is consistent with other data that has been recently published for different NC analogues as discussed previously but experimentally determined evidence, perhaps NMR, CD, or X-ray crystallography remains to be done for ultimate confirmation of the hypothesis.

The data we have presented, as it relates to a putative receptor-bound form of NC, may be useful toward the goal of conducting complex molecular modeling simulations aimed toward a more detailed understanding of the receptor binding/signaling process. It may also form the basis for the design and use of peptide structural mimetics that could facilitate the discovery of novel ligands, and possibly new therapeutic agents, whose actions are mediated by the ORL-1 receptor.

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