Structure−Activity Studies on Nociceptin Analogues: ORL1 Receptor Binding and Biological Activity of Cyclic Disulfide-Containing Analogues of Nociceptin Peptides

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Nociceptin/Orphanin FQ is an endogenous peptide ligand for the opioid receptor-like 1 (ORL1) receptor. To investigate the structural and conformational requirements of the nociceptin (NC)−receptor interaction, six cyclic analogues containing Cys disulfide linkages were designed and synthesized. Analogues cyclized at the N-terminal part, cyclo[Cys^0, Cys^7]NC(1−13)-NH_2 (**2**) and cyclo[Cys^0, Cys^{11}]NC(1−13)-NH_2 (**4**), and their corresponding linear peptides had very low activities in both the receptor binding and the GTPγS functional assays using human ORL1 transfected cell membranes. On the contrary, analogues cyclized at the C-terminal parts by the disulfide linkages at positions 6−10, 7−11, 7−14, and 10−14 sustained relatively high potencies in both assays. Notably, cyclo[Cys^{10}, Cys^{14}]NC(1−14)-NH_2 (**12**) was found to be a potent NC agonist nearly as active as the parent peptide or NC. The maximum efficacy (Emax) of the C-terminally cyclized analogues and their linear counterparts in the GTPγS functional assay showed more than 94% (vs NC as 100%), suggesting that these analogues are full agonists. Analogue **12** is the first conformationally constrained NC analogue with almost full activity, and thus may serve to analyze the bioactive conformations of NC at the receptor site as well as serving as a template for more potent NC agonists.

Introduction[1]

Nociceptin (NC)[2] or Orphanin FQ[3] (Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln) is an endogenous ligand for the opioid receptor-like 1 (ORL1) receptor and a member of the G protein-coupled receptor family, and has a structure similar to that of an opioid peptide dynorphin A (Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln). The most striking similarities between NC and dynorphin A seem to be the N-terminal X-Gly-Gly-Phe sequences (X = Phe in NC and Tyr in dynorphin A), and the existence of basic residues, although with different distributions, at the C-terminal part. Despite the sequence homology, NC binds to opioid receptors with very low affinities,[4] and the pharmacological properties of NC differ from those of the opioids.[2,3] Recent structure−activity relationship studies of NC and dynorphin A have revealed that the two Phe residues at positions 1 and 4 are important structural requirements for ORL1 receptor binding and biological activity, and for discriminating NC activity from opioid activities.[5−14] The number and distribution of basic residues at the C-terminal part in the two peptides are also important and critical factors for either NC or opioid activity.[8, 9, 11, 13]

The conversion of linear peptides to their cyclic analogues not only generates conformational constraints within the peptide backbone and substantially reduces the degree of freedom of the side chain orientations, but also often creates structures with enhanced metabolic stability. The cyclic peptide analogues are also useful in defining a preferred conformation for the receptor interaction. In fact, a number of cyclic dynorphin A analogues have been reported, containing a i−(i + 4) lactam linkage[15−17] which is generally utilized to stabilize an α-helical structure.[18] Other analogues containing a Cys disulfide linkage were designed based on a potential conformation with a reverse turn.[19−21]

To investigate the structural and conformational requirements of NC for the receptor interaction, in this study, we synthesized six Cys-containing cyclic disulfide analogues. These analogues were based on NC(1−13)-NH_2 or NC(1−14)-NH_2 which have been shown to possess full biological activity,[6,8] and we evaluated their binding affinities for the ORL1 receptor and in vitro biological activities in comparison with those of the corresponding linear peptides. These analogues were designed to cyclize either the N-terminal or the C-terminal part of NC. For the cyclization of the N-terminal part, an extra Cys was added at the 0 position. The other positions replaced by Cys were Gly^6, Ala^7, Ser^{10}, Ala^{11}, and Leu^{14}, given that most of these residues have been found to be tolerant to substitution with other amino acids.[5,6,11,13] Thus, the cyclic analogues prepared in this study have a disulfide linkage at positions 0 and 7, 0 and 11, 6 and 10, 7 and 11, and 11 and 14.

Results and Discussion

Chemistry. All peptide analogues were synthesized by a Fmoc-based solid-phase method using either a manual apparatus with a standard synthetic schedule

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Table 1. Physicochemical Properties of Synthetic Nociceptin Analogues

analogue no.	peptide	$[\alpha]_D{}^a$ (°)	TLC^b (Rf)	HPLC^b (t$_R$, min)	FABMS (M+H$^+$)	amino acid analysis^c							
						Thr	Ser	Gly	Ala	Phe	Lys	Arg	Cys^c
1	[Cys^{0,7}]NC(1−13)-NH$_2$	−29.7	0.83	18.01	1516	0.94	0.97	3.11	1.10	2.00	2.05	2.07	0.37
2	c.[Cys^0, Cys^7]NC(1−13)-NH$_2$	−14.6	0.78	16.28	1514	0.95	0.91	3.13	1.05	2.00	2.05	2.09	0.82
3	[Cys^{0,11}]NC(1−13)-NH$_2$	−29.2	0.81	15.72	1516	0.93	0.57	3.03	1.06	2.00	2.01	2.01	0.69
4	c.[Cys^0, Cys^{11}]NC(1−13)-NH$_2$	−25.0	0.80	15.69	1514	0.98	0.95	3.09	1.06	2.00	1.98	2.06	0.83
5	[Cys^{6,10}]NC(1−13)-NH$_2$	−40.0	0.82	16.17	1443	1.01	-	2.10	2.16	2.00	2.12	2.02	0.52
6	c.[Cys^6, Cys^{10}]NC(1−13)-NH$_2$	−31.0	0.85	14.73	1441	1.03	-	2.06	2.13	2.00	2.00	2.06	0.89
7	[Cys^{7,11}]NC(1−13)-NH$_2$	−25.5	0.44	15.13	1445	0.88	0.61	2.63	-	2.00	1.86	2.04	0.76
8	c.[Cys^7, Cys^{11}]NC(1−13)-NH$_2$	−26.6	0.43	13.62	1443	1.00	0.69	3.10	-	2.00	2.02	2.02	0.67
9	[Cys^{7,14}]NC(1−14)-NH$_2$	−30.9	0.48	15.89	1517	0.90	0.86	2.77	0.96	2.00	1.86	1.95	0.47
10	c.[Cys^7, Cys^{14}]NC(1−14)-NH$_2$	−23.1	0.47	14.99	1515	0.83	0.82	2.72	0.73	2.00	1.93	1.93	0.76
11	[Cys^{10,14}]NC(1−14)-NH$_2$	−39.8	0.82	16.76	1500	0.81	-	3.11	2.10	2.00	1.96	2.07	0.51
12	c.[Cys^{10}, Cys^{14}]NC(1−14)-NH$_2$	−43.2	0.82	15.02	1499	0.90	-	2.93	2.06	2.00	1.96	2.06	0.85

^{a} Optical rotations were measured in 1% AcOH ($c = 1$) at 20 °C. ^{b} See Experiental Section. ^{c} More than half of Cys usually decomposed under the present hydrolysis conditions.

Table 2. ORL1 Receptor Binding Assay and [35S]GTPγS Functional Assay of NC Analogues

analogue no.	peptide	ORL1 affinity IC$_{50}$ (nM)^a	[^{35}S]GTPγS assay	
			EC$_{50}$ (nM)^b	Emax%^{c}
	NC	0.286 ± 0.100	1.93 ± 0.11	100
	NC(1−13)-NH$_2$	0.353 ± 0.155	1.12 ± 0.09	106
	NC(1−14)-NH$_2$	0.520 ± 0.129	1.97 ± 0.21	105
1	[Cys^{0,7}]NC(1−13)-NH$_2$	2710 ± 800	>10000	12
2	c.[Cys^0, Cys^7]NC(1−13)-NH$_2$	874 ± 130	>10000	18
3	[Cys^{0,11}]NC(1−13)-NH$_2$	1290 ± 200	286 ± 78	59
4	c.[Cys^0, Cys^{11}]NC(1−13)-NH$_2$	387 ± 114	439 ± 38	81
5	[Cys^{6,10}]NC(1−13)-NH$_2$	3.03 ± 1.46	29.7 ± 2.3	94
6	c.[Cys^6, Cys^{10}]NC(1−13)-NH$_2$	0.833 ± 0.266	31.3 ± 7.7	99
7	[Cys^{7,11}]NC(1−13)-NH$_2$	8.70 ± 0.43	26.3 ± 4.8	99
8	c.[Cys^7, Cys^{11}]NC(1−13)-NH$_2$	3.04 ± 0.88	156 ± 89	120
9	[Cys^{7,14}]NC(1−14)-NH$_2$	37.2 ± 10.6	22.3 ± 5.2	101
10	c.[Cys^7, Cys^{14}]NC(1−14)-NH$_2$	4.97 ± 1.56	25.0 ± 9.8	95
11	[Cys^{10,14}]NC(1−14)-NH$_2$	0.471 ± 0.261	3.90 ± 1.46	105
12	c.[Cys^{10}, Cys^{14}]NC(1−14)-NH$_2$	0.122 ± 0.051	4.37 ± 1.44	99

^{a} Each value represents mean ± SE of 4−8 independent determinations. ^{b} Each value represents mean ± SE of 5 independent determinations. ^{c} The maximal response calculated as percentage from that produced by NC.

or a PE Biosystems synthesizer with the manufacturer's recommended synthetic protocol. Peptides were liberated from the resin by treatment with Reagent K[22] and purified by medium-pressure HPLC as previously reported.[23] Disulfide bond formation was performed by air oxidation of the corresponding linear peptide. All synthetic peptides gave satisfactory amino acid analytical and FABMS data as shown in Table 1.

ORL1 Receptor Binding Activity. The receptor binding affinities of peptides were determined by displacement experiments with [^{3}H]NC using cell membranes expressing the human ORL1 receptor in HEK-293 cells. To examine the effects of cyclization of the N-terminal part on the receptor interaction, two cyclic peptides containing a Cys disulfide linkage at positions 0 and 7 (**2**), or 0 and 11 (**4**), and their corresponding linear peptides (**1** and **3**) were prepared, as shown in Table 2. The linear peptides (**1** and **3**) exhibited drastically reduced binding affinities for the ORL1 receptor. Their cyclic disulfide analogues, **2** and **4**, also showed markedly reduced affinities, although the affinities were somewhat improved as compared to those of the corresponding linear peptides. These results may be the consequence of the N$^\alpha$-acylation of Phe^1 residue because the N-terminal acetylated or dialkylated analogues of NC(1−13)-NH$_2$ have significantly decreased activity or no activity.[13] Next, to examine the effect of Cys substitution and cyclization at the C-terminal part, linear peptides (**5**, **7**, **9**, and **11**) and their corresponding cyclic analogues (**6**, **8**, **10**, and **12**) were prepared. Most of the

linear peptides showed lower affinities as compared to those of the parent peptides. The potency, however, depended on the Cys substitution sites with IC$_{50}$ values from 0.471 to 37.2 nM. The Cys^{7,14}-analogue (**9**) showed the lowest affinity among the analogues, whereas the Cys^{10,14}-analogue (**11**) had the highest affinity, which was comparable to that of the parent peptide NC(1−14)-NH$_2$. These results suggest that positions 10 and 14 are the most ideal places to substitute Cys for the cyclization, and they coincided well with the results from the Ala-substitution studies of NC.[5,6] On the other hand, cyclization by the disulfide linkage resulted in analogues with 3−7-fold higher affinities than those of the corresponding linear peptides, suggesting that the cyclization is an effective modification for NC to improve receptor affinity. Notably, analogue **12**, which is cyclized via the 10 and 14 positions, possessed the highest receptor affinity and is significantly higher than NC(1−14)-NH$_2$ or comparable to NC. It should also be noted that the influences of cyclization at the C-terminal part on the receptor interaction are relatively small such that the reduction in binding affinities of the C-terminally cyclized analogues vs NC are less than 10-fold. These data seem to support the idea that the positively charged basic residues, which remained intact in this series of analogues, are more important for receptor interaction than the conformational factors at the C-terminal part.[5,6,8,12,13]

[^{35}S]GTPγS Bioassay. The in vitro biological activity was determined by the ability to induce binding of GTP

to the G proteins, which governs the signal transduction of the G-protein-coupled ORL1 receptor and thus is a measure of receptor activations.[12,24−27] The binding of analogues to the cell membrane expressing human ORL1 receptors in HEK-293 cells was examined with the radio-labeled ligand, [^{35}S]GTPγS, and the results, expressed as the effective concentration (EC$_{50}$) of the analogues and the maximum stimulation (Emax) of [^{35}S]-GTPγS binding, are shown in Table 2. On the whole, the results were consistent with those of the receptor binding assay. Analogues **3** and **4** showed a weak activity, but **1** and **2** were almost devoid of activity. Analogues containing Cys at 6 and 10 (**5**), 7 and 11(**7**), 7 and 14 (**9**), and their cyclic counterparts (**6**, **8**, and **10**) showed a moderate potency, except for **8** which possessed a relatively low potency. Consistent with the binding data, analogue **11** and **12** showed a potent activity nearly comparable to that of NC. The Emax values of analogues cyclized at the C-terminal part and their cyclic counterparts were more than 94%, suggesting that these analogues are full agonists. Unlike the receptor affinity, however, no significant difference in potency was observed between the C-terminally modified linear analogues and their cyclic disulfide counterparts in this assay. The reason for the discrepancy is unknown at present. Because the cyclic analogues appear to be full agonists, the possibility of their antagonist actions should be excluded. According to a recent molecular modeling study of the ORL1 receptor and its complex with NC, positively charged NC(8−13) core mainly interacts with acidic second extracellular receptor loop, which is thought to mediate receptor activation.[28] It is, therefore, possible that the introduction of conformational constraint around the positively charged residues, e.g. **6**, **8**, **10**, and **12**, induces the conformational change to exert a limited effect on the receptor activation without hampering the receptor binding ability. To examine the possibility further studies are in progress.

Conclusion

One approach to obtaining information on the bio-active conformations of a peptide at the receptor site is to utilize constrained or conformationally restricted peptide analogues. The present study demonstrated that Ser^{10} and Leu^{14} are the most ideal places to substitute a set of Cys residues at the C-terminal part, and cyclization of the resulting linear peptide formed a constrained cyclic analogue (**12**) without significant reduction of receptor binding and NC agonist activities. To our knowledge, this is the first example of conformationally constrained NC analogues with almost full activity. A comparative NMR study of NC and dynorphin A in solution has recently revealed that the N-terminal part of NC has the same conformational preferences as the N-terminal domain of endogenous opioids, but the C-terminal part of the sequence is more flexible than the corresponding domain of dynorphin A.[29] These observations, however, cannot rule out the possibility of bioactive conformations of the C-terminal part which might be induced by the receptor or lipids at the receptor site. In this regard, analogue **12** may serve to analyze bioactive conformations of NC at the

receptor site as well as serving as a template for more potent NC agonists.

Experimental Section

Optical rotations were measured in 1% AcOH (*c* = 1) at 20 °C using a 10-cm path length cell in a JASCO DIP-40 polarimeter. Analyses by HPLC were performed on a column of Wakosil-II 5C18 AR, 4 × 150 mm, using the following solvent systems: A, 0.06% TFA; B, 0.06% TFA in 80% CH$_3$-CN. A linear gradient elution from 10% B to 50% B over 40 min at a flow rate of 1 mL/min was used, and the eluate was monitored at 220 nm. Amino acid analysis was performed using a HITACHI L-8500 amino acid analyzer after 6 N HCl hydrolysis at 110 °C for 22 h. TLC was carried out on silica gel plates (Merck, Kiesel gel 60F$_{254}$, 5 × 10 cm) with the following solvent system: 1-BuOH/AcOH/pyridine/H$_2$O (15:3:10:12). FABMS was determined on a JEOL JMS-DX303 instrument.

Peptide Synthesis. Solid-phase synthesis using a manual apparatus was carried out by HBTU-mediated Fmoc strategy, starting with Fmoc-NH-SAL-resin (0.47 mmol/g, 100−200 mesh, Watanabe Chem. Ind., Ltd.). The following schedule was employed: (1) DMF wash (×3); (2) 30% piperidine/DMF (×2, 4 and 4 min); (3) DMF wash (×3); (4) Fmoc−amino acid (3 equiv.)/HBTU(3 equiv.)/HOBt (3 equiv.)/DIPEA (6 equiv.) in DMF (×1, 30 min), DMF wash (×3). A mixture of HATU (5 equiv.)/DIPEA (8 equiv.) was employed as a coupling reagent in use of a PE Biosystems apparatus with a manufacturer recommended schedule, starting with Fmoc-PAL-PEG-PS-resin (PerSeptive Biosystems). The side chain protecting groups used were Boc for Lys, Pbf for Arg, *t*Bu for Ser and Thr, and Trt for Cys. For the synthesis of linear peptides, the protected peptide resin was treated with Reagent K (TFA/phenol/H$_2$O/thioanisole/ethanedithiol, 82.5:5:5:5:2.5)[22] at room temperature for 3 h. After being filtered off the resin, the filtrate was concentrated and triturated with abs. ether to yield the crude peptide as a precipitate. The crude product was purified on a Develosil Lop ODS column (24 × 360 mm, Nomura Kagaku) which was eluted with a linear gradient from 10% solvent B to 50% solvent B over 240 min at a flow rate of 3.0 mL/min. The eluate was monitored at 230 nm. Fractions of the main peak were checked by analytical HPLC, and the pure fractions was pooled and lyophilized repeatedly from H$_2$O.

For the synthesis of cyclic analogues, the linear analogue (80 mg) was dissolved in 0.1 M (NH$_4$)HCO$_3$ (80 mL) and air was bubbled into the solution with stirring. Usually, the cyclization reaction proceeded completely within a day and was monitored by analytical HPLC. After evaporation of the solvent, the product was purified on the medium-pressured HPLC as mentioned above to yield the desired cyclic peptide. All synthesized peptides had a purity of more than 95% on analytical HPLC. Physicochemical properties of all analogues are shown in Table 1.

ORL1 Receptor Binding Assay. Cell membranes expressing the human ORL1 receptors in HEK-293 cells were purchased from Receptor Biology Inc. and used for the binding assay. The cell membranes (10.62 μg of protein), 2 nM [^{3}H]-NC (2.78 TBq/mmol), and peptide sample were incubated in a 50 mM HEPES buffer (pH 7.4) containing 1 mM EDTA and 10 mM MgCl$_2$, in a total volume of 100 μL, for 1 h at 25 °C in a siliconized tube. The reaction was terminated by filtration through a Whatman GF/B glass filter, previously soaked in 0.5% polyethyleneimine. The glass filter was washed three times with 2 mL of the HEPES buffer. Nonspecific binding was determined in the presence of 1 μM of unlabeled NC. Filter-bound radioactivity was counted after overnight extraction with 3 mL of Creasol I using a Beckman 9800 liquid scintillation counter. The IC$_{50}$ values were determined from log dose-displacement curves.

[^{35}S]GTPγS Binding Assay. Agonist-stimulated binding of [^{35}S]GTPγS was investigated according to the method of SPA G-protein coupled receptor assay provided by Amersham Pharmacia Biotech with slight modification. The cell membranes (10 μg, Receptor Biology Inc.) were incubated in 20 mM

HEPES buffer (pH 7.4) containing 100 mM NaCl, 10 mM MgCl$_2$, 1 mM EDTA, 5 μM GDP, 400 pM [^{35}S]GTPγS, and 1.5 mg of wheat germ agglutinin-coated scintillation proximity assay (SPA) beads with increasing concentrations of peptides (0.01 nM to 10 μM), in a total volume of 200 μL, for 30 min at 24 °C. Basal binding was assessed in the absence of agonist, and nonspecific binding was measured in the presence of 10 μM unlabeled GTPγS. The activity was estimated as EC$_{50}$, the value of which exhibits the concentration inducing 50% of its own maximal stimulation. The binding data were analyzed by nonlinear regression with the software GraphPad Prism ver. 2.01.

References

(1) Amino acids and peptides are of L-configuration unless otherwise noted. Amino acid and peptide abbreviations used are those recommended by the IUPAC–IUB Commission on Biochemical Nomenclature in *Eur. J. Biochem.* **1984**, *139*, 9–37. Other abbreviations used are as follows: NC, nociceptin or orphanin FQ; Fmoc, N-9-fluorenylmethoxycarbonyl; ORL1, opioid receptor-like 1; Boc, *tert*-butoxycarbonyl; Pbf, ω-2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl; *t*Bu, *tert*-butyl; Trt, trityl; SAL-resin, superacid labile polystyrene resin; PAL-PEG-PS-resin, peptide amide linker-poly-(ethylene glycol)polystyrene resin; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxy-benzotriazole; DIPEA, diisopropylethylamine; DMF, dimethyl-formamide; FABMS, fast atom bombardment mass spectroscopy.
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