Pergamon

Bioorganic & Medicinal Chemistry Letters 11 (2001) 737–740

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Structure–Activity Analysis of Truncated Orexin-A Analogues at the Orexin-1 Receptor

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Received 23 August 2000; revised 21 December 2000; accepted 16 January 2001

Abstract—Truncated peptide analogues of orexin-A were prepared and their biological activity assesed at the orexin-1 receptor. Progressive N-terminal deletions identified the minimum C-terminal sequence required for maintaining a significant agonist effect, whilst an alanine scan and other pertinent substitutions identified key side-chain and stereochemical requirements for receptor activation.

Introduction

Human orexin-A (**1a**) is a 33 residue peptide incorporating an N-terminal pyroglutamyl residue and C-terminal amidation. It has a mass of 3562 Da and contains two intramolecular disulphide bonds of topology Cys^6-Cys^{12} and Cys^7-Cys^{14}.

<Glu[1]-Pro-Leu-Pro-Asp-Cys-Cys-Arg-Gln-Lys-Thr-Cys-
-Ser-Cys-Arg[15]-Leu-Tyr-Glu[18]-Leu-Leu-His-Gly-Ala-
-Gly-Asn-His[26]-Ala-Ala-Gly-Ile-Leu-Thr-Leu[33]-NH_2

1a

H-Arg[1]-Ser-Gly-Pro-Pro-Gly-Leu-Gln-Gly-Arg-Leu-
Gln-Arg-Leu-Leu-Gln-Ala-Ser-Gly-Asn-His-Ala-Ala-
Gly-Ile-Leu-Thr-Met[28]-NH_2

1b

Human orexin-B (**1b**) is a C-terminally amidated 28 residue linear peptide. Both peptides are derived from a 130 amino acid precursor protein, prepro-orexin which is encoded by a gene localised to human chromosome 17q21.[1] These peptides are located predominantly in the hypothalamus[1] and locus coeruleus,[2] but are also found elsewhere in the brain,[3] and the spinal cord.[4] Orexin-A binds with high affinity to G-protein coupled receptors orexin-1 (OX_1) and orexin-2 (OX_2) causing an increase in intracellular calcium concentration ($[Ca^{2+}]_i$).[1]

Monitoring of $[Ca^{2+}]_i$ levels in Chinese hamster ovary (CHO) cells expressing OX_1 or OX_2 confirms that orexin-A is a potent agonist at both receptors ($pEC_{50} = 8.03 \pm 0.08$ at OX_1 and 8.18 ± 0.10 at OX_2), whilst orexin-B displays a moderate selectivity for OX_2 ($pEC_{50} = 7.30 \pm 0.08$ at OX_1 and 8.43 ± 0.09 at OX_2).[5] In vivo studies with orexin-A suggest the peptide is involved in a range of physiological functions[6] including a role in feeding[1,7] and in the orchestration of the sleep–wake cycle.[2]

A key part of the structure–activity study of a peptide is the elucidation of the contribution of each amino acid to the stability of the overall structure and to biological functions. The interaction of a peptide ligand with its receptor(s) will be more clearly understood through the resolution of these contributions, which may help in the design of biologically selective analogues.

We report here the synthesis and biological evaluation of C-terminal analogues of orexin-A, in order to identify amino acid side chains that are essential for functional potency at OX_1. The major part of this study was an alanine scan,[8] in which each (non-alanine) residue of orexin-A(15-33) amide (**2**) was systematically replaced with L-alanine. The decision to omit the N-terminus

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0960-894X/01/$ - see front matter
PII: S0960-894X(01)00043-9

from the alanine scan was based on the fact that orexin-B, which lacks the N-terminal disulphide bridge arrangement of orexin-A and is highly conserved (68%) over the C-terminal 19 residues of orexin-A, is only 5-fold less potent than orexin-A at OX_1, suggesting a key role for the C-terminus in receptor activation at OX_1. Similarly, the negligible difference in potency between orexin-A and orexin-B in OX_2 activation again suggests that the C-terminus plays a major role in receptor interaction. In addition, the C-terminal leucine residue and adjacent threonine were each separately replaced with their corresponding D-enantiomer, and the results of these substitutions are also reported. Peptide activity was determined by recording functional potency, as reflected by changes in $[Ca^{2+}]_i$ measured using fluorometric imaging technology. In addition, the binding affinity of some sequences was investigated using laser scanning cytometry.

Experimental

Measurement of $[Ca^{2+}]_i$

OX_1 receptor-mediated changes in $[Ca^{2+}]_i$ were measured as described previously.[5] In brief, CHO cells stably expressing OX_1 receptors (CHO-OX_1), were seeded (20,000 cells/well) into black walled clear-base 96-well plates (Costar, UK) in MEM-Alpha medium and cultured overnight. Cells were loaded with the cytoplasmic calcium indicator, Fluo-3AM (4 μM; Teflabs, Austin, Texas) in the presence of 2.5 mM probenecid, and then washed four times with, and finally resuspended in, Tyrode's medium containing 2.5 mM probenecid and 1% gelatine, before being incubated for 30 min at 37 °C with either buffer alone (control) or buffer containing test peptide (10 pM–10 μM). The fluorescence ($\lambda_{ex} = 488$ nm, $\lambda_{EM} = 540$ nm) was then monitored using a fluorometric imaging plate reader (FLIPR; Molecular Devices, UK) before and after the addition of orexin-A or test peptide (10 pM–10 μM).

Data analysis

Responses were measured as peak fluorescence intensity (FI) minus basal FI, and curve-fitting and parameter estimation were carried out according to a four parameter logistic model. For peptides which showed agonist-like activity, but for which it was not possible to fully define the concentration response curve, the % response at 10 μM, relative to the maximal orexin-A induced response, is quoted. Data are expressed as mean ± SEM unless otherwise stated.

Binding assay

CHO-OX_1 cells (17,000 cells/well) were seeded onto 16-well chambers (Lab-Tek, Nalge Nunc International). Cells were grown in MEM-Alpha medium for 24 h and subsequently incubated (37 °C, 30 min) with 28 nM Rhodamine Green™ tagged orexin-A ($N^{6,10}$-(Rhodamine Green™)orexin-A) and different concentrations of competitor peptide in Hepes buffered saline containing 2.5 mM $MgCl_2$, 1.5 mM $CaCl_2$ and 0.5% BSA. Cells were then washed in the same buffer without BSA and fixed with 4% paraformaldehyde. Prior to the fluorescence reading, cells were stained with 0.6 μM Syto 62 (Molecular Probes) for 10 min at 20 °C.

Fluorescence measurements and data analysis

Cells were analysed using a laser scanning cytometer (Compu Cyte). Cells were selected based on their red fluorescence, by exciting the Syto probe with a 5 mW HeNe laser and collecting the emitted fluorescence with a 650 nm longpath filter. The green fluorescence from the selected cells was also measured by scanning the cells with a 20 mW argon ion laser and collecting the emitted fluorescence with a 530 nm/30 filter. Displacement of $N^{6,10}$-RG-orexin-A from OX_1 was measured by monitoring green fluorescence maximal pixel intensity and the data was fitted to a four parameter logistic model using sigmaplot (SPSS Inc.). Data are expressed as mean ± SEM ($n = 5$ for orexin-A; $n = 4$ for orexin-A(15–33) amide; $n = 3$ for orexin-A(18-33) amide).

Synthesis

Peptides were synthesised by conventional solid-phase techniques using Fmoc (9-fluorenylmethoxycarbonyl) chemistry[9] and purification was conducted by preparative reverse phase HPLC.[10] Preparation of $N^{6,10}$-(Rhodamine Green™)orexin-A was accomplished through the reaction of the side-chain amino group of orexin-A with the *N*-hydroxy succinimide ester of Rhodamine Green™ carboxylic acid (5,6-mixed isomers; Molecular Probes).

Results and Discussion

N-Terminal deletion

Table 1 shows the functional potencies at OX_1 of several analogues of orexin-A truncated at the N-terminus. Truncation to orexin-A(15–33) amide, with concomitant loss of the two intramolecular disulphide bonds, produced a 170-fold drop in functional potency at OX_1. Further progressive N-terminal deletions gave peptides that showed an additional significant drop in potency. Truncation to the C-terminal decapeptide (**6**) or pentapeptide (**7**) led to the loss of activity in each case. These results show that the 14 residues at the N-terminus of orexin-A, which contain the two disulphide loops, are critical to the preservation of full functional potency observed with orexin-A at OX_1. However, a minimum C-terminal sequence of 19 residues can give a significant agonist effect. The binding affinities of two of the truncates, which showed a significant difference in functional potency from each other, were also investigated, along with that of orexin-A itself. The binding data was comparable to the functional data, as shown in Table 1. Truncation of orexin-A to orexin-A(15-33) amide resulted in a 20-fold reduction in affinity at OX_1. Further deletion to the C-terminal hexadecapeptide (**4**) produced an additional significant (>240-fold) drop in affinity.

The importance of the C-terminus for activation at OX_2 was also confirmed through the observed activity ($pEC_{50} = 7.35 \pm 0.04$) of orexin-A(15–33) amide at this receptor sub-type.

Alanine scan

The functional potencies of the peptides comprising the alanine scan of orexin-A(15–33) amide against OX_1 are given in Table 2. Conducting the alanine scan on this 19-residue N-terminal deletion peptide had the added advantage of ease of synthesis relative to orexin-A. The synthesis of peptides containing two or more disulphide bridges requires, for example, either the controlled formation of the disulphide links in turn or careful isolation of the desired component by preparative HPLC from other possible isomers following oxidation of the reduced linear sequence. In all cases where the pEC_{50} value was defined the peptides acted as full agonists. None of the peptides acted as an antagonist.

The data showed that the analogues fell broadly into three groups with respect to their activity relative to orexin-A(15–33) amide.

1. Analogues which showed a significant drop in functional potency. The substituted residues in this group were Leu^{16}, Leu^{19}, Leu^{20}, His^{26}, Gly^{29}, Ile^{30} Leu^{31}, Thr^{32} and Leu^{33} (compounds **9**, **12**, **13** and **18**–**23**).

2. Analogues which exhibited a 2-fold to 4-fold loss in activity. This group comprised the replacement of residues, Tyr^{17}, His^{21}, Gly^{22}, Gly^{24} and Asn^{25} (compounds **10** and **14**–**17**).

3. Sequences which maintained or slightly increased functional potency (subsitution of Arg^{15} and Glu^{18}; compounds **8** and **11**).

The results showed that alanine substitution towards the C-terminus clearly had the most significant effect on functional potency, whereas substitution of the residues at the N-terminus had only a limited effect. It is postulated that residues 26 through 33 are involved in binding at OX_1, whilst the two adjacent leucines at positions 19 and 20, and to a lesser extent the leucine at position 16, may either be involved in receptor interaction or have an important conformational role. It is also possible that replacement of the glycine residue in position 29 with alanine caused significant disruption to the secondary structure resulting in a marked decrease in activity. The data suggest that residues 17, 22, and 25 have at least a minor role in receptor interaction, and further, that the charged residues Arg^{15} and Glu^{18} probably play no role at all. In contrast, the polar

Table 1. Functional potency and binding affinity at OX_1 of C-terminal truncates of orexin-A

Compound	Peptide	pEC_{50}	% Response (@ 10 μM)	pK_i
1	Orexin-A	8.68 ± 0.02		7.89 ± 0.05
2	Orexin-A(15–33) amide	6.45 ± 0.06		6.57 ± 0.03
3	Orexin-A(17–33) amide		40	
4	Orexin-A(18–33) amide		13	4.18 ± 0.11
5	Orexin-A(19–33) amide		16	
6	Orexin-A(24–33) amide		Inactive (@ 10 μM)	
7	Orexin-A(29–33) amide		Inactive (@ 10 μM)	

Table 2. Functional potency at OX_1 for analogues of orexin-A(15–33) amide

Compound	Peptide	Substituted Residue	pEC_{50}	% Response (@ 10 μM)
8	[Ala^{15}]Orexin-A(15–33) amide	Arg^{15}	6.46 ± 0.03	
9	[Ala^{16}]Orexin-A(15–33) amide	Leu^{16}		71
10	[Ala^{17}]Orexin-A(15–33) amide	Tyr^{17}	6.11 ± 0.03	
11	[Ala^{18}]Orexin-A(15–33) amide	Glu^{18}	6.71 ± 0.04	
12	[Ala^{19}]Orexin-A(15–33) amide	Leu^{19}		60
13	[Ala^{20}]Orexin-A(15–33) amide	Leu^{20}		37
14	[Ala^{21}]Orexin-A(15–33) amide	His^{21}	5.89 ± 0.02	
15	[Ala^{22}]Orexin-A(15–33) amide	Gly^{22}	6.19 ± 0.09	
16	[Ala^{24}]Orexin-A(15–33) amide	Gly^{24}	6.08 ± 0.03	
17	[Ala^{25}]Orexin-A(15–33) amide	Asn^{25}	6.00 ± 0.09	
18	[Ala^{26}]Orexin-A(15–33) amide	His^{26}		61
19	[Ala^{29}]Orexin-A(15–33) amide	Gly^{29}		15
20	[Ala^{30}]Orexin-A(15–33) amide	Ile^{30}		Inactive (@ 10 μM)
21	[Ala^{31}]Orexin-A(15–33) amide	Leu^{31}		12
22	[Ala^{32}]Orexin-A(15–33) amide	Thr^{32}		39
23	[Ala^{33}]Orexin-A(15–33) amide	Leu^{33}		15
24	[D-Thr^{32}]Orexin-A(15–33) amide	Thr^{32}		Inactive (@ 10 μM)
25	[D-Leu^{33}]Orexin-A(15–33) amide	Leu^{33}		18

aromatic histidine residues, which are likely to be charged at physiological pH, appear to be important for full functional activity.

D-Amino acid replacements

In the case of analogues containing a D-amino acid (**24** and **25**), the side chains remain chemically unchanged, and the overall hydrophobicity and dipole moment are only slightly altered, so that the orientation of the side chains may be studied. Replacement of the C-terminal leucine residue with D-leucine led to a significant reduction in potency, and replacement of the adjacent threonine with its D-enantiomer led to a loss of biological activity (see Table 2). Thus, the correct orientation of these side chains appears to be critical for the maintenance of functional potency, emphasising the important role the C-terminus probably plays in receptor binding and activation.

Conclusion

We have shown that the lipophilic C-terminus of orexin-A contains residues that are critical for functional potency at OX_1 and key stereochemical constraints have been identified. A minimum C-terminal sequence of 19 residues is required for a significant agonist effect and it is postulated that residues 15 through 25 may play an important conformational role in receptor interaction. Truncation to the C-terminal hexadecapeptide results in a significant reduction in binding and almost complete loss of functional potency. Removal of the N-terminal region encompassing the two disulphide loops results in a loss of the high functional potency and binding affinity observed for the parent ligand at OX_1, but does not completely eliminate it. A recent communication[11] has also shown that the C-terminal residues of orexin-A are critical for functional potency at OX_1, whilst an agonist response is not lost when modifications are made to the N-terminal region.

Acknowledgements

The authors wish to thank Kellie Darker for her assistance in the preparation of this manuscript.

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