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NOVEL Y_2-SELECTIVE, REDUCED-SIZE AGONISTS OF NEUROPEPTIDE Y

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Abstract

The prerequisite for Y_2-mediated biological activity was elaborated by synthesizing analogs of neuropeptide Y (NPY) 1-4-Ahx-25-36. The finding, that a pronounced hydrophobic segment is required in the C-terminal segment for signal transduction led to the development of a Y_2-selective agonist, which contains cyclohexylalanine at position 30 and/or 31.

Introduction

Neuropeptide Y (NPY) is a 36 amino acid peptide amide of the pancreatic polypeptide hormone family [1], which is found in both the central and peripheral nervous system of mammals, including man. In the periphery, NPY mediates increase in blood pressure, both *per se* and by potentiation of neurotransmitters. Furthermore, on presynaptic membranes it acts to inhibit the release of noradrenaline. In the central nervous system NPY evokes the increase in food intake, the enhancement of memory and the modulation of LHRH. The activity of NPY has been shown to be mediated through at least two subtypes of NPY specific receptors, the so-called Y_1- and Y_2-receptors. The neuroanatomy and physiology of NPY has recently been reviewed in a symposium volume [2].

C-terminal segments have been found to bind to the rabbit kidney receptor, which probably belongs to the Y_2-subtype of NPY receptors [3]. However, binding of Ac-25-36 was reduced to an IC_{50} of 160 nM compared to 0.5 nM for NPY [3]. Following molecular dynamics investigations on NPY, we previously developed a new type of reduced-size analogs, containing only 17 amino acid residues: NPY 1-4-Ahx-25-36 [4] (Fig. 1).

This type of analogs characteristically comprises a short N-terminal NPY segment, C-terminally linked to the N-terminus of C-terminal NPY fragments via ω-amino-alkanoic acids. NPY 1-4-Ahx-25-36 shows much higher receptor binding [4] and higher agonistic properties [3] on Y_2-receptor related systems when compared to simple C-terminal NPY segments such as NPY 25-36 or 18-36. Binding of the C-terminal as well as of the discontinuous analogs to Y_1-receptors is reduced by at least three orders of magnitude [5]. Single substitutions of NPY 1-4-Ahx-25-36 with L-alanine, glycine or the corresponding D-amino acid revealed that binding to the Y_2-receptor is mediated by residues 33 to 36 [6]. However, the elongation by the residues 1-4-Ahx-25-32 contributes to higher receptor binding, since H-Arg^{33}-Gln^{34}-Arg^{35}-Tyr^{36}-NH_2 is, *per se*, inactive. Here we report on the biological activity of these discontinuous analogs of NPY in the vas deferens assay, which is an assay for testing signal transduction of Y_2-receptors [7]. Single substitution of the natural sequence revealed the amino acids essential for signal transduction and lead to the development of prerequisites for agonistic activity.

Results

The peptides were synthesized by solid phase peptide synthesis using Fmoc/tBu strategy and semiautomated tea-bag synthesis [8] or the robot system SMPS 352 (Zinsser Analytics, Frankfurt, Germany). 5-(4'-Aminomethyl-3',5'dimethoxyphenoxy)pentanoyl acid was linked to alanyl-polystyrene-1%-divinylbenzene in

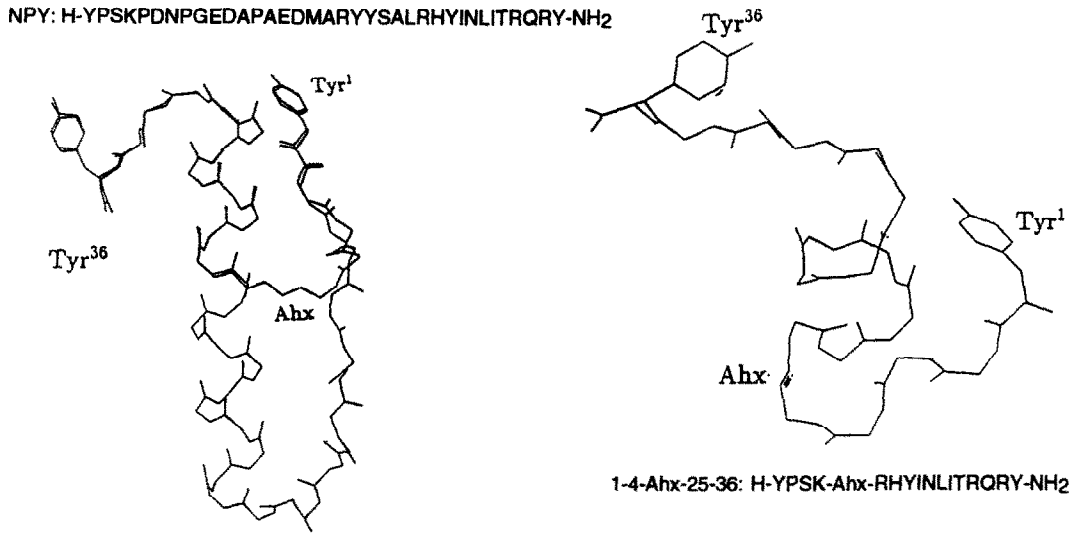

Fig. 1. Sequences and 3D models according to molecular dynamics simulations [3] of neuropeptide Y and NPY 1-4-Ahx-25-36

order to obtain peptide amides. The peptides were purified and characterized by HPLC, amino acid analysis and electrospray mass spectrometry. Receptor binding of the analogs was tested according to [3] using rabbit kidney cortex membrane preparations and according to [5] on LN319 (human Y_2) or SK-N-MC (human Y_1) cells. Biological activity was investigated in the vas deferens assay as described previously [3]. Data are means of 2 to 3 independent experiments and IC_{50} values are presented.

Neuropeptide Y 1-4-Ahx-25-36 (H-YPSK-Ahx-RHYINLITRQRY-NH_2) and analogs with the replacement of one amino acid of the natural sequence by L-alanine, glycine or a special amino acids were investigated. Previous results [6,9] concerning the receptor binding of these peptides are summarized in Fig. 2. The biological activity was investigated by the capacity of the analogs in inhibiting the electrically evoked rat vas deferens contractions, which probably is due to the presynaptic inhibition of noradrenaline release [7]. 50% inhibition was found for NPY at 19 nM. NPY 1-4-Ahx-25-36, which binds to Y_2 subtypes of receptors as well as NPY [5] has a reduced capacity of signal transduction (IC_{50} 180 nM). Analogs containing L-alanine or glycine at position 27, 28, 30 or 31 as well as analogs with several L-alanine replacements within this segment exhibit a strongly reduced activity or were inactive (IC_{50} > 10 000 nM). However, receptor binding of these peptides was not affected (Fig. 2). In contrast, analogs containing space filling amino acids such as lysine,

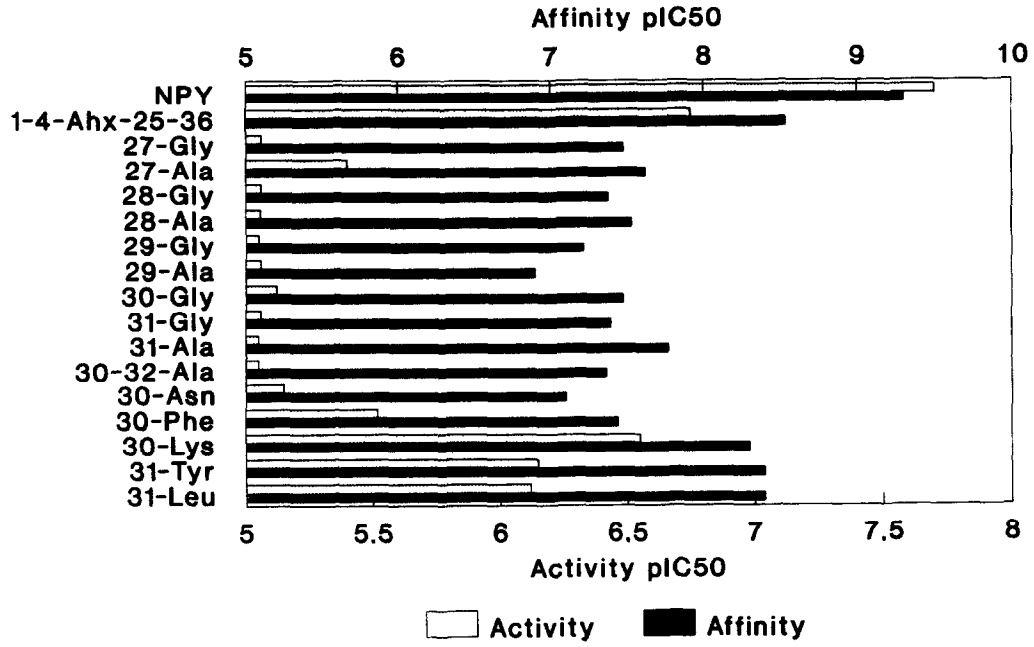

Fig. 2. Receptor binding and biological activity of NPY 1-4-Ahx-25-36 and analogs with amino acid replacements in the segment 27 to 32. Data are given as pIC_{50} .

tyrosine, phenylalanine or leucine instead of Leu^{30} or Ile^{31} retained the receptor binding and the agonistic activity. Asparagine at position 30 was not favourable: the activity of this peptide in the vas deferens assay was reduced, however not completely abolished.

These findings led to the synthesis of further analogs of NPY Ac-25-36. This segment itself exhibits only low capacity in inhibiting the electrically evoked rat vas deferens contractions (IC_{50} 4500 nM). Analogs with one or several replacements by L-alanine, glycine or α-aminoisobutyric acid are inactive [3] (IC_{50} > 10 000). However, the replacement of Ile^{31} and/or Leu^{30} by L-cyclohexylalanine (Cha) increased receptor binding on rabbit kidney membranes to IC_{50} of 16 nM (Ac-25-36 [Cha^{30}]), 18 nM (Ac-25-36 [Cha^{31}]) and 8 nM (Ac-25-36 [Cha^{30},Cha^{31}]) and in human Y_2-expressing LN319 cells to IC_{50} of 8 nM (Ac-25-36 [Cha^{30}]), 5 nM (Ac-25-36 [Cha^{31}]) and 3 nM (Ac-25-36 [Cha^{30},Cha^{31}]). Biological activity (IC_{50}) was found at 1900 nM, 2100 nM and 1600 nM, respectively (Fig. 3). To our knowledge these are the first replacement analogs exhibiting higher affinity and activity than the C-terminal NPY segment Ac-25-36 with the natural sequence. In contrast, IC_{50} of 44 μM (Ac-25-36 [Cha^{30}]), 39 μM (Ac-25-36 [Cha^{31}]) and 26 μM (Ac-25-36 [Cha^{30}, Cha^{31}]) was found in human Y_1-expressing SK-N-MC cells, which is comparable to IC_{50} of the natural sequence Ac-25-36 (35 μM) and which demonstrates the selectivity of these compounds for Y_2-receptors.

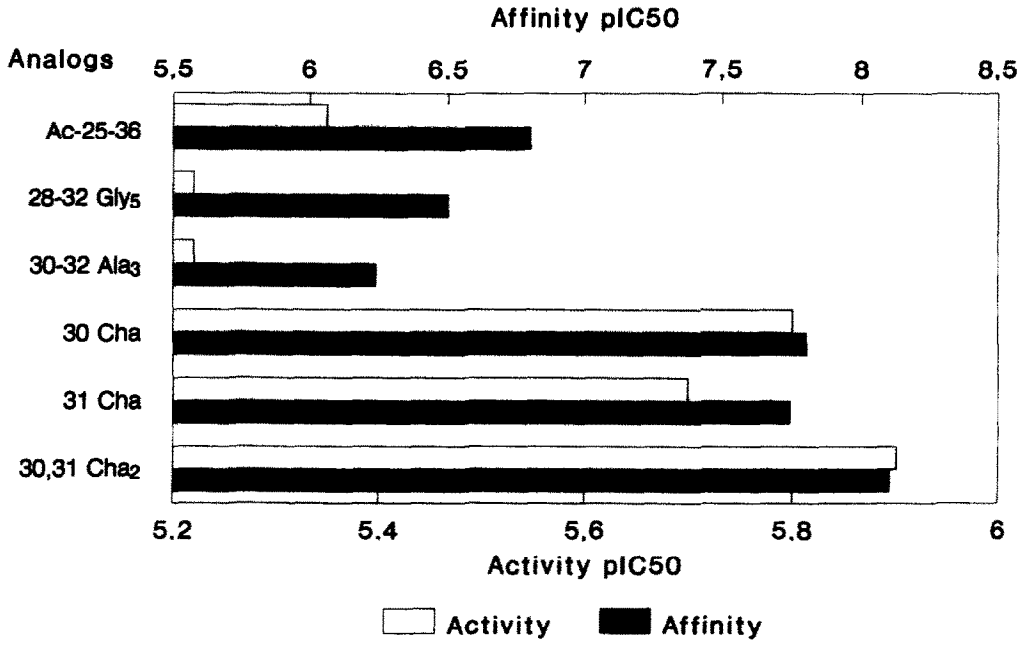

Fig. 3. Receptor binding and biological activity of Ac-25-36 and analogs with single or multiple amino acid replacements in the segment 28 to 32. Data are given as -lgIC_{50}.

Discussion

Here we report on our investigations on the agonistic properties of series of analogs of NPY 1-4-Ahx-25-36 and Ac-25-36. Whereas for receptor binding mainly the residues 33-35 are most important and specific exchanges at positions 29, 32 and 36 indicate some relevance of these amino acids [10], additional residues are required for signal transduction. Replacement of the hydrophobic amino acids in the segment 28 to 31 by glycine or L-alanine led to reduced or abolished agonistic activity. Analogs, containing space filling residues at these positions are still active, which suggests that not the specific side-chain, but the hydrophobicity of the segment 28-31 in general is important. This led to the development of even smaller compounds, containing cyclohexylalanine instead of leucine or isoleucine at positions 31 and/or 30. Receptor binding of these peptides on Y_2-systems, compared to the natural sequence is 10-fold increased and agonistic properties 2- to 3-fold, whereas on Y_1-cells, binding is strongly reduced for the natural sequence and the analogs. But these are the first analogs of segments of NPY, which exhibit a higher activity than the natural segment itself. Up to now, exchanges of single or multiple amino acids always led only to equal or reduced affinity [7,9,11-15], compared to the corresponding segment of NPY. Whether the increase is due to the increased size of the hydrophobic segment or due to an increased amphiphilicity, which has been suggested to play an important role in the interaction [9,16], can not be distinguished. However, using different types of cyclic analogs, it has been shown, that the presence of a large hydrophobic segment situated before the binding site 33-36 in the C-terminal part of NPY is a prerequisite for good receptor affinity [17].

Although these Cha-containing analogs of the C-terminal segment of NPY exhibit a higher activity compared to the natural Ac-25-36, the high activity of NPY itself is not obtained. Even NPY 1-4-Ahx-25-36 is 10-fold more active than Ac-25-36 [Cha^{30},Cha^{31}]. Therefore, we conclude, that either parts of the N-terminal four amino acids or the non-peptidic spacer contribute to the biological activity. However, revealing the essential residues of neuropeptide Y, by using rationally designed peptides and non-peptide segments or non-proteinic amino acids might be promising for the development of non-peptidic drugs.

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