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Highly potent and small neuropeptide Y agonist obtained by linking NPY 1-4 via spacer to α-helical NPY 25-36

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Analogues of neuropeptide Y (NPY) containing small N- and C-terminal segments linked via flexible spacer arms were found to exhibit receptor binding affinity constants almost as high as NPY as well as post- and presynaptic NPY-agonistic activities. One of the most active analogues contains N-terminal NPY segment 1–4 linked via ε-aminocaproic acid (Aca) to the C-terminal partially α-helical peptide amide segment 25–36. NPY 1–4-Aca-25–36 is the first highly potent NPY agonist, which is of considerably reduced size in comparison to the native hormone. The analogues are accessible by solid-phase synthesis using Fmoc strategy.

Neuropeptide Y; Receptor binding; Agonist; Conformation

1. INTRODUCTION

Neuropeptide Y (NPY) [1,2], a linear 36-amino-acid peptide amide, is one of the most potent endogenous vasoconstrictors. NPY is reported to be an important neurotransmitter/neuromodulator in the regulation of cardiovascular functions [3] and may be involved in the pathophysiology of hypertension [4,5] which needs clarification by specific NPY antagonists. Therefore, we wished to evaluate structure-activity relationships on NPY. Earlier binding studies [6–11] using synthetic segments of NPY like NPY 19–36 revealed that the C-terminal part including the tyrosine amide is most relevant for its biological activity. Further truncation of this sequence leads to reduced receptor binding and biological activity [6,7,10,11].

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Abbreviations: Aca, ε-aminocaproic acid; Aoa, ω-aminooctanoic acid; APP, avian pancreatic polypeptide; Aua, ω-aminoundecanoic acid; Boc, butyloxycarbonyl; tBu, *tert*-butyl; FAB-MS, fast-atom-bombardment mass spectrometry; Fmoc, fluorenylmethoxycarbonyl; Mtr, 4-methoxy-2,3,6-trimethylbenzenesulfonyl; NPY, neuropeptide Y; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid; Trt, trityl

2. MATERIALS AND METHODS

2.1. *Synthesis of NPY analogue 1–4-Aca-25–36*

The 17-peptide amide was synthesized via the Fmoc strategy starting with 4-(Fmoc-aminomethyl)-3,5-dimethoxyphenoxy-valeric acid (Fmoc-ADPV) as amide anchor [12] bound to alanyl-benzhydrilamine-polystyrene-1% divinylbenzene (0.5 mmol/g). The protecting groups for side-chain functions were Arg(Mtr), His(Trt), Tyr(tBu), Thr(tBu), Ser(tBu) and Lys(Boc). Double couplings using firstly 1-hydroxybenzotriazol (HOBt) and diisopropylcarbodiimide in dimethylformamide and secondly symmetric anhydrides in *N*-methylpyrrolidone were carried out on a 430A peptide synthesizer (Applied Biosystems): activation time 30 min, coupling time 60 min each, for Thr, Ile, Tyr and Aca 90 min. Fmoc-Asn-OH was coupled twice as the HOBt ester in dimethylformamide and Fmoc-Gln-OH as *p*-nitrophenyl ester in *N*-methylpyrrolidone (activation time 10 min). Between the two couplings a wash step with *N*-ethylmorpholine in *N*-methylpyrrolidone was carried out. Cap-

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ping was performed with acetic anhydride and *N*-ethylmorpholine. The resin was washed with *N*-methylpyrrolidone, dichloromethane, methanol and 2-propanol. Fmoc groups were removed in piperidine/dimethylformamide (1:3) within 2 × 10 min. The final peptide amide was split off in trifluoroacetic acid containing thioanisole and 2-methylthiophenol within 4 h in 90–95% yield. Complete removal of the Mtr blocking group required an additional acidic treatment of the peptide amide at 50°C for 30 min [13].

The peptide was purified on Sephadex G-25 in 5% acetic acid followed by preparative RP-HPLC. Amino acid analyses and the racemization test of total hydrolyzates were carried out by ion-exchange chromatography and by gas chromatography of pentafluoropropionylamino acid methyl esters on the chiral stationary phase Chirasil-Val. The peptide amide was tested for resistance against carboxypeptidase Y. Furthermore, FAB-MS, ^{13}C NMR, CD and sequencing positions 1–4 on the peptide sequencer 477 (Applied Biosystems) were used for characterising the NPY agonist.

2.2. *Analytical data of NPY 1–4-Aca-25–36*

HPLC column: Nucleosil C18, 5 μm; eluant: A, water/TFA (100:0.1); B, acetonitrile/TFA (100:0.1); flow rate, 1 ml/min; gradient, 15–60% B within 30 min; product was eluted after 22.3 min.

Amino acid analysis (calc.) [racemization, % uncorr.]: R 3.0 (3) [1.1], N 1.07 (1) [3.4], T 1.04 (1) [0.1], Q 0.99 (1) [1.8], L 0.93 (1) [2.7], I 1.91 (2) [1.8], H 1.07 (1) [2.4], Y 2.72 (3) [1.5], P 0.82 (1) [2.4], S 0.86 (1) [3.2], K 0.85 (1) [0.98].

FAB-MS (M + 1): 2239 mu. Sequencing of the N-terminus (PTH derivative yields in pmol/cycle): 1, Y (575); 2, P (444); 3, S (157); 4, K (108); 5, cycle no PTH-amino acid, end of Edman degradation due to spacer amino acid Aca.

2.3. *Biological assays*

2.3.1. Postsynaptic activity

The blood pressure increasing potency of NPY or NPY analogues was tested in pithed rats. Male rats (Chbb, THOM; 270–300 g) were anaesthetized with 60 mg sodium pentobarbital i.p., artificially respirated and pithed according to Gillespie and Muir [14]. For blood pressure measurement a carotid artery was cannulated and connected to a Statham pressure transducer coupled to a Watanabe Multicorder. A catheter for injection of test compounds was placed in a jugular vein. Dose-response curves for the pressure responses to NPY or NPY analogues were established and ED_{30} values (dose which increased blood pressure by 30 mmHg) were calculated. At least three animals were used for each test compound.

2.3.2. Presynaptic activity

Presynaptic activity (suppression of noradrenaline release) of NPY or NPY analogues was estimated in rat vas deferens preparations [15]. Seminal ducts were fixed in an organ bath (50 ml modified Krebs solution, 33°C), mounted to force display transducer and contracted repeatedly by means of electrical field stimulation (15 V, 1 ms duration, 0.15 Hz). Concentration-response curves for the inhibitory effects of NPY and NPY analogues were established and IC_{50} values (concentration which inhibits the contraction by 50%) were calculated. At least two vas deferens preparations were used for one compound.

2.3.3. Receptor binding studies

Receptor binding studies were performed according to Chang et al. [16] with minor modification. Adult male white New Zealand rabbits were killed by intravenous injection of pentobarbitone via an ear vein, the kidneys rapidly removed and the cortex dissected. Tissue was homogenized in 50 vols ice-cold Tris-HCl (pH 7.4 at 37°C) with a polytron and centrifuged at 50000 × *g* for 10 min. Pellets were washed in 50 vols buffer and incubated for 40 min at room temperature. The membranes were then centrifuged and washed twice. The resulting membranes were resuspended in 125 vols of the above Tris-HCl buffer containing 5 mM $MgCl_2$, 0.1 mg/ml soybean trypsin inhibitor, 0.1% serum albumin and 0.25 mg/ml of bacitracin. For binding studies 230 μl of membrane solution containing about 100 μg protein were added to 10 μl buffer (for total binding) or 0.3 μM (final concentration) unlabeled NPY (for nonspecific binding) or the displacer each containing 10 μl ^{125}I-NPY (Amersham Buchler, Braunschweig). After incubation at 21°C for 90 min the reaction was terminated by filtration under reduced pressure with Whatman GF-C glass filters (previously soaked for at least 2 h in 1% polyethyleneimine to reduce binding to filters) followed by three 3-ml washes with ice-cold buffer. Specific binding was calculated as the difference in radioactivity bound in the presence and absence of 1.0 μM NPY. The equilibrium-dissociation constant (K_d) of the receptor-^{125}I-NPY complex and the maximal number of specific binding sites (B_{max}) were calculated using the program LIGAND [17]. Half-maximal inhibition of the specific binding of the ^{125}I-NPY is given as the IC_{50} value.

3. RESULTS AND DISCUSSION

A hypothetical 3D structure of NPY [10] has been deduced from the X-ray structure of the homologous avian pancreatic polypeptide APP [18]. NPY was modelled by exchange of side chains of differentiating amino acids followed by minimization using force field calculations and confirmed by molecular dynamics calculations (Discover, Biosym Technologies) (fig.1). Residues 1–8 form a type II polyproline helix and residues 15–32 an amphiphilic α-helix. Experimental proof for the α-helical conformation was obtained from CD data of various C-terminal segments [11]. The amphiphilic α-helix may be stabilized by hydrophobic interactions with the type II polyproline helix.

Based on the hypothetical 3D structure we designed an NPY analogue consisting of the segments 1–4 and 25–36 covalently linked by a flexible spacer residue ε-aminocaproic acid (fig.1). The resulting NPY 1–4-Aca-25–36 comprises less than half of the amino acid residues of NPY. Surprisingly, this compound exhibits almost the same receptor binding affinity as NPY (table 1). Testing

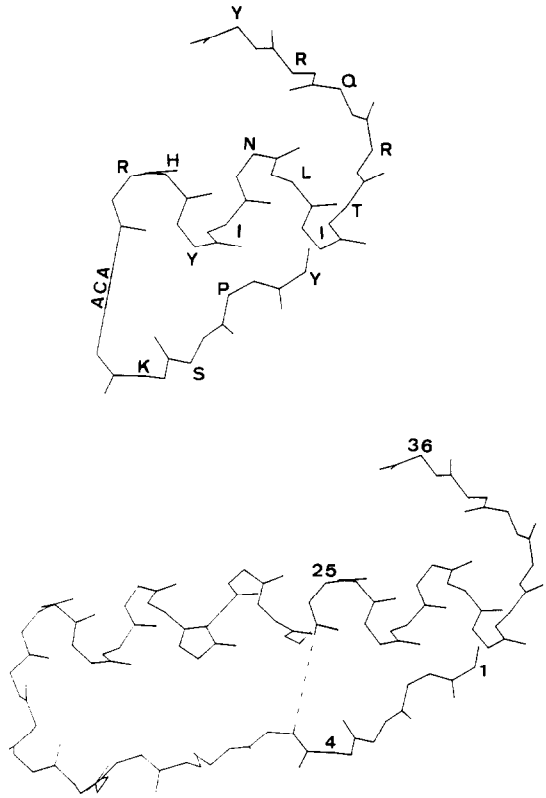

Fig.1. Simulated 3D structure of NPY (lower), and analogue NPY 1–4-Aca-25–36 (H-YPSK-Aca-RHYINLITRQRY-NH_2) (upper). In NPY the N-terminal polyproline type II helix comprises segment 1–8 and the C-terminal α-helix extends from position 14 to 32. The dashed line in the NPY structure indicates the short-cut made for the design of the analogue omitting the icosapeptide segment 5–24.

revealed that NPY 1–4-Aca-25–36 is a highly potent NPY agonist.

Similar receptor affinities were found for the analogues NPY 1–4-Aca-24–36, NPY Ac-1–4-Aca-25–36, NPY 1–2-Aua-25–36 (Aua = 11-aminoundecanoic acid). The binding constants were reduced in case of the analogues NPY 1–4-Aca-25–36 [Phe^1], NPY 1-$(Aca)_2$-25–26 and NPY 1–4-Aca-25–36[Ala^1-Pro^2-Leu^3-Glu^4] containing the bovine pancreatic polypeptide segment 1–4. Results of receptor binding and biological activity are summarized in table 1.

In summary, based on the hypothetical 3D structure of NPY, drastically shortened analogues consisting of N- and C-terminal segments of NPY have been modelled and synthesized. These analogues exhibited high receptor affinity and strong NPY agonistic properties.

Table 1

Receptor binding affinity constants, postsynaptic and presynaptic activities of NPY and short synthetic analogues

Peptides	Receptor binding K_d (nM)	Post-synaptic activity EC_{30} (nM/kg)	Pre-synaptic activity IC_{50} (nM)
NPY	0.05	0.12	19
NPY 1–4-Aca-25–36	0.16	83	180
NPY 1–4-Aca-24–36	0.6	17	180
NPY Ac-1–4-Aca-25–36	0.8	n.d.	n.d.
NPY 1–2-Aua-25–36	0.9	52	280
NPY 1–4-Aca-25–36[Phe^1]	4.8	70	160
NPY 1–4-Aoa-25–36	5.6	460	170
NPY 1–Aca_2-25–36	6.3	72	160
NPY 1–4-Aca-25–36[Ala^1-Pro^2-Leu^3-Glu^4]	10	460	750

n.d., not determined

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