Studies on the structural requirement for ligand binding to the neuropeptide Y (NPY) receptor from rat cerebral cortex

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Analogues of the NPY model peptide NPY 1-4-Aca-25-36 were synthesized by solid phase peptide synthesis using the tea-bag method for parallel peptide synthesis. The affinity of the peptides as ligands to the NPY receptor in rat cerebral cortex was investigated. The model compound NPY 1-4-Aca-25-36 was a relatively poor ligand to the NPY receptor in rat brain ($IC_{50} = 0.40$ μM). Arg^{35} and Arg^{33} were both important for ligand recognition by the NPY receptor. Substitutions in several positions in the region corresponding to the C-terminal part of NPY resulted in analogues with only minor reduction of the affinity for the NPY receptor.

Cerebral cortex; Neuropeptide Y; Peptide hormone receptor; Synthetic peptide

1. INTRODUCTION

Neuropeptide Y (NPY) is a 36 amino acid long peptide first isolated from porcine brain by Tatemoto et al. [1,2]. NPY is a member of the so-called pancreatic polypeptide family of which also the peptides PYY and pancreatic polypeptide (PP) have been isolated from mammalian sources [3,4]. The PP family is characterised by a stable tertiary structure in water solution [5] rendering the members of this peptide hormone family one of the smallest globular proteins. X-Ray crystallographic studies of avian pancreatic polypeptide (APP) [6] have shown that the structure can be divided into a polyproline helix (residues 1–8), a bend region (9–14), an α-helix (14–30) and a flexible C-terminal hexapeptide. The packing and interdigitating of hydrophobic residues in the polyproline-helix and the α-helix stabilises the tertiary structure. Receptors for NPY have been characterised in the central nervous system [7], in the spleen [8] and in various cell lines [9]. Two subtypes of NPY receptors have been suggested based on different pre- and post-junctional pharmacological actions of the C-terminal fragment NPY13-36 [10].

Attempts have been made to synthesize shorter fragments of NPY which still bind to the NPY receptor with high affinity. Krstenansky et al. [11] designed a 27 amino acid long analogue where amino acids in the bend region had been deleted and the structure was stabilised by a disulfide bridge. This analogue bound to membranes from mouse brain with the same affinity as the native hormone. Another 17 amino acid long analogue NPY 1-4-Aca-25-36 was designed by Beck et al. [12] which bound to rabbit kidney membranes with only slightly reduced affinity as compared to NPY_{1-36}.

Relatively few structure/activity studies of NPY and other members of the pancreatic polypeptide family have been carried out, but it has been shown that the C-terminal amide is of critical importance for binding and biological activity [10,13]. Gln^{34} has been shown to be important for recognition by the Y_2 receptor subtype but not by the Y_1 subtype [9].

In the present study the NPY analogue NPY 1-4-Aca-25-36 and analogues containing substitutions in the region 25–36 were studied as ligands to the NPY receptor in rat cerebral cortex.

2. MATERIALS AND METHODS

2.1. *Reagents*

t-BOC D-Arg(Tos) was purchased from Bachem, Switzerland. All other t-BOC amino acids were purchased from Peninsula, UK. The *p*-methylbenzhydrylamine resin (0.8 mmol of amine/g, 100–200 mesh), dicyclohexylcarbodiimide (DCC) and 1-hydroxy-benzotriazol (HOBt) were from Fluka, Switzerland. ^{3}H-Propionyl NPY (50 Ci/mmol) was purchased from Amersham, UK. NPY was obtained from Bachem. All other chemicals were of reagent grade.

2.2. *Peptide synthesis*

Solid phase peptide synthesis was carried out by the so-called tea-bag method for parallel peptide synthesis developed by Houghten [14]. All amino acids were coupled with DCC in a 1 : 1 molar ratio, ex-

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Abbreviations: Aca, ε-aminocaproic acid; APP, avian pancreatic polypeptide; t-BOC, *tert*-butyloxycarbonyl; BSA, bovine serum albumin; Hepes, (*N*-[2-hydroxyethyl] piperazine-*N*'-[2-ethanesulfonic acid]; PP, pancreatic polypeptide

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Table I

Amino acid substitutions in the sequence of the NPY model peptide NPY 1-4-Aca-25-36 [12] and their affinities as ligands to the NPY receptor in rat cerebral cortex

Analogues of NPY 1-4-Aca-25-36 as ligands to NPY-receptors in rat cerebral cortex.

Peptide	IC_{50} (μM)	Relative affinity
1-4-Aca-25-36	0.4 ± 0.2	1.0
1-4-Aca-25-36(D-Y^{36})	0.6 ± 0.01	0.7
1-4-Aca-25-36(D-R^{35})	1.1 ± 0.7	0.4
1-4-Aca-25-36(K^{35})	>30	<0.01
1-4-Aca-25-36(A^{34})	3.1 ± 0.3	0.1
1-4-Aca-25-36(P^{34})	3.6 ± 2	0.1
1-4-Aca-25-36(H^{34})	0.4 ± 0.2	1.0
1-4-Aca-25-36(D-R^{33})	0.9 ± 0.3	0.4
1-4-Aca-25-36(A^{33})	>30	<0.01
1-4-Aca-25-36(V^{32})	1.6 ± 0.3	0.3
1-4-Aca-25-36(A^{29})	2.0 ± 0.8	0.2
1-4-Aca-25-36(A^{26})	1.2 ± 0.5	0.3
1-4-Aca-25-36(A^{25})	0.7 ± 0.1	0.6

ND, not determined. The IC_{50} values were determined in equilibrium binding experiments with [^{3}H]propionyl NPY_{1-36}. IC_{50} values are the mean of at least 4 different experiments ± SD.

cept for Gln, Arg and His which were coupled as HOBt esters. Side chain protection of amino acids were - arginine: tosyl; histidine: benzyloxymethyl; lysine: 2-chlorobenzoyloxycarbonyl; serine/threonine: benzyl; tyrosine: 2-bromobenzyloxycarbonyl; D-tyrosine: 1,6-dichlorobenzyl. The analogues were cleaved from the resin by hydrogen fluoride.

The crude peptides were purified by reversed-phase HPLC, on a C_{18} Ultropac column (7.8 × 300 mm, 10 μm particles; TSK ODS 120T, LKB, Sweden). Amino acid analysis was performed on hydrolysed peptides by the gas-phase hydrolysis method [15] followed by phenylisothiocyanate (PITC) derivatization. The phenylthiocarbamyl (PTC) derivatives were eluted with a gradient of 50% acetonitrile in 12.5 mM sodium phosphate buffer, pH 6.4, from a reversed-phase C_{18} Superpac column (4 × 125 mm, 3 μm particle size; Spherisorb ODS 2, Pharmacia, Sweden).

2.3. *Receptor binding studies*

Receptor binding studies were performed according to Undén et al. [7] with slight modifications. Adult male rats (Sprague-Dawley, 180-200 g) were decapitated, the brain rapidly removed and the cerebral cortex dissected. The tissue was homogenized with a loose-fitting glass teflon homogenizer in 10 vols ice-cold 0.32 M sucrose. The homogenate was centrifuged at 1000 × g for 5 min, the supernatant collected and centrifuged at 10000 × g for 40 min. The pellet was resuspended in ice-cold Krebs-Ringer buffer (137 mM NaCl, 2.68 mM KCl, 1.8 mM $CaCl_2$, 1.05 mM $MgCl_2$, 1 g/l glucose; 0.5 g/l BSA, 20 mM Hepes, pH 7.4; bacitracin 0.02%). The homogenate (0.2 mg protein) was incubated at room temperature for 90 min with [^{3}H]NPY (0.4 nM final concentration) and different concentrations of the ligands in a final volume of 1 ml. Nonspecific binding was defined as the binding in the presence of 100 nM unlabeled NPY. After incubation the samples were centrifuged at 10000 × g for 30 s, the supernatant was aspirated and the bottom of the tubes were cut off and counted in a liquid scintillation spectrometer. The IC_{50} values were calculated by nonlinear regression analysis.

3. RESULTS AND DISCUSSION

The analogue NPY 1-4-Aca-25-36 binds to the NPY receptor in membranes from rat cerebral cortex with an IC_{50} value of 0.4 μM (Table I) which has 3 orders of magnitude lower affinity as compared to the original report by Beck et al. on binding of this analogue to NPY receptors from kidney membranes [12].

The relatively low affinity of the parent analogue NPY 1-4-Aca-25-36 for brain NPY receptors is in agreement with results recently reported by McLean et al. [16] where NPY_{20-36} bound to NPY receptors from pig spleen with two orders of magnitude higher affinity as compared with binding to NPY receptors from mouse brain. These data suggest that the NPY receptors in brain membranes have a different structural requirement for ligand recognition than the NPY receptors from spleen and kidney.

The results presented in Table I show that substitutions in several positions of the model peptide NPY 1-4-Aca-25-36 only resulted in minor reduction in affinity.

The arginines in positions 33 and 35 are both important for ligand binding to the NPY receptor. Substitutions such as Lys^{35} and Ala^{33} both resulted in analogues where no displacement could be detected in concentrations up to 30 μM.

Several studies have shown that short C-terminal fragments of NPY are poor ligands to NPY receptors from the mammalian central nervous system. This indicates that residues in the region 1-32 are of critical importance for ligand recognition by brain NPY receptors. Two basic residues Arg^{25} and His^{26} are conserved either as Arg-His or Arg-Arg in all members of the PP family. The result in Table I shows that substitutions in these positions with alanine resulted only in minor reductions of the affinity.

It can be concluded that NPY 1-4-Aca-25-36 is not an ideal model peptide for NPY receptors from rat brain. On the other hand, to carry out structure/activity relation studies in the C-terminal part of the 36 amino acid long peptide is associated with extensive synthesis work. It is also likely that results obtained with shorter analogues such as substituted NPY 1-4-Aca-25-36 analogues reflect the structural requirement of recognition of the C-terminal of the native hormone NPY_{1-36}.

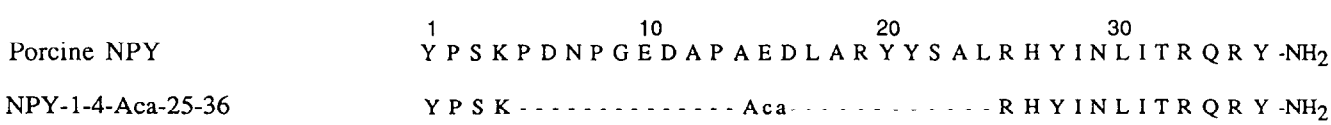

Fig. 1. Primary structure of porcine neuropeptide Y (NPY) and the analogue NPY 1-4-Aca-25-36.

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