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Structure activity studies of mast cell activation and hypotension induced by neuropeptide Y (NPY), centrally truncated and C-terminal NPY analogues

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1 Neuropeptide-induced histamine release is thought to occur via receptor-independent mechanisms, with net charge and lipophilicity being important factors.

2 In this study, the histamine releasing ability of neuropeptide Y (NPY), two C-terminal segments of NPY and 13 centrally truncated NPY analogues was examined. These results were compared with the ability of the peptides to bind to the Y_2 receptor in the rabbit kidney membrane model and with their hypotensive actions in the anaesthetized-rat model.

3 All analogues tested, with the exception of [$Glu^{4,25,33,35}$]-NPY(1-4)-Ahx-(25-36) and [$Asp^{4,25,33,35}$]NPY(1-4)-Ahx-(25-36) which were devoid of histamine releasing activity, evoked a dose-dependent histamine release but there were marked differences between the peptides. The native peptide was the least active.

4 Histamine release was not linked to the ability of the peptides to displace NPY from Y_2 receptors. There was a statistical correlation between the hypotensive effects expressed as ED_{10} values (μmol kg^{-1}, which induced a blood pressure decrease of 10 mmHg) and the EC_{25} for histamine release ($r=0.62$, $P=0.04$), although histamine release may not be the sole determinant of the alterations in blood pressure.

5 There was a strong negative correlation between EC_{25} for histamine release and net positive charge ($r=-0.93$, $P=5.7\times10^{-7}$), i.e. increasing the net positive charge caused greater histamine release. However, there was a 12 fold difference in activity amongst the most positively charged analogues (+5). Helicity did not correlate with histamine releasing ability.

6 In the development of NPY-related drugs the avoidance of compounds with net positive charge is recommended.

Keywords: Mast cells; histamine liberation; structure-activity relationships; neuropeptide Y; anaesthetized rat

Introduction

Neuropeptide Y (NPY) is one of the most abundant peptides in the nervous system. It consists of 36 amino acids, is C-terminally amidated and belongs to the pancreatic polypeptide (PP) family. NPY exerts a number of different actions (for reviews see Wahlestedt & Reis, 1993 and Colmers & Bleakman, 1994) such as a direct postjunctional vasoconstrictor effect, a postjunctional potentiation of noradrenaline and other neurotransmitters and a prejunctional suppression of stimulated noradrenaline release. Specific NYP binding sites are found both in the peripheral and central nervous system. Two NPY receptors have been characterized to date, Y_1 and Y_2, with different NPY analogues acting as selective agonists for each (Beck-Sickinger & Jung, 1995). Whereas [$Leu^{31}Pro^{34}$]NPY binds exclusively to the Y_1 subtype (Fuhlendorff *et al.*, 1990), C-terminal segments such as NPY 13-36 (for review see Grundemar *et al.*, 1993) and centrally truncated segments such as NPY(1-4)-Ahx-(25-36) (where Ahx = 6-aminohexanoic acid) (Beck *et al.*, 1989a; Beck-Sickinger *et al.*, 1990a) show a high selectivity for the Y_2-subtype. In pithed rats, NPY causes a dose-dependent fall in mean arterial blood pressure, which was thought to be due to NPY-induced histamine release (Grundemar *et al.*, 1990). Further, the C-terminal segments 18-36 and 22-36 can also reduce blood pressure and increase heart rate by a non-receptor mediated event, probably via histamine release from mast cells (Shen *et al.*, 1991). Mast cell activation by the 22-36 NPY fragment was found to be equipotent with native NPY by these authors and activation by the 18-36 NPY fragment was subsequently demonstrated by Haworth and co-workers (1992). Further an NPY analogue with the central part substituted with 8-aminooctanoic acid and a cyclic C-terminal hexapeptide of NPY were found to release histamine (Grundemar *et al.*, 1994).

Blundell and colleagues, who performed X-ray analyses on avian (turkey) pancreatic polypeptide (aPP), have provided data on the secondary structure of PP hormones (Blundell *et al.*, 1981; Blundell & Wood, 1982). Residues 1-8 of the crystalline avian pancreatic polypeptide form a type II proline helix followed by a loop (residues 9-14) and an α-helical segment (residues 15-32). The 4 C-terminal amino acid residues adopt a relatively flexible loop-like conformation. Hydrophobic interactions of the proline helix and the amphilic α-helix lead to the hairpin structure. A model for the 3 dimensional structure of pNYP and hNPY has been deduced based on the X-ray structure of aPP (Allen *et al.*, 1987; MacKerrell, 1988; Beck-Sickinger *et al.*, 1990b). The hairpin structure was present in all molecular dynamics simulations. Circular di-

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chroism investigations confirmed the predominance of the α-helical conformation (Krstenansky & Buck, 1987), which is especially marked in aqueous solution at pH 6. A ^{1}H-NMR study on neuropeptide Y monomers in water confirmed the presence of the hairpin loop based on a series of long range nuclear Overhauser effects (NOEs) between the N- and C-terminal segments (Darbon *et al.*, 1992). The secondary structure obtained by NMR is in agreement with the model of Allen *et al.* (1987), as well as with the results obtained from structure activity relationships, which have suggested a close contact between the N- and C-terminal segments (Beck *et al.*, 1989a, b; Beck-Sickinger *et al.*, 1990a, b; 1992a, b).

Mast cell activation by peptides is thought to occur via receptor-independent mechanisms, possibly by a direct activation of G proteins (Landry *et al.*, 1992), whereby net positive charge and the presence of α-helical structures have been deemed by these authors to be important (Emadi-Khiav *et al.*, 1995). In this study we have therefore examined the histamine releasing ability of NPY analogues, which varied in charge, configuration and conformation and compared this to their *in vivo* hypotensive actions and their receptor-binding properties at the Y_2 receptor.

Methods

Peptide synthesis

The peptides (Table 1) were synthesized by solid phase synthesis using the semi-automated teabag method (Beck-Sickinger *et al.*, 1991) or the automated multiple peptide synthesizer SMPS 350 (Zinsser Analytics, Frankfurt) and Fmoc/tBu strategy. The peptide amides were synthesized on 5-(4'-aminomethyl-3',5'-dimethoxy-phenoxyl)valeric acid anchored via an alanine spacer to aminomethylated polystyrene-(1%)-divinyl-benzene. For activation benzotriazene-tetramethyluronium tetrafluoroborate (1 equivalent) (Nova Biochem, Läufelfingen), 1-hydroxybenzotriazene (1 equivalent) and diisopropylethylamine (1.5 equivalents) were used for the teabag synthesis or 1-hydroxybenzotriazene (1 equivalent) and diisopropylcarbodiimide (1 equivalent) for the automated multiple synthesis. A 10 fold excess of reagents was used for both methods. Deprotection was performed in piperidine/dimethylformamide (1 : 1) within 2 × 15 min. Cleavage was performed in trifluoroacetic acid/thioanisole/thiocresol (90 : 5 : 5) within 4 h at room temperature. The peptides were characterized by amino acid analysis and electrospray mass spectrometry. For amino acid analyses the peptides were automatically hydrolysed in a gas phase (6 M HCl, 160°C, 90 min), subsequently derivatised with phenylisothiocyanate and analysed by microbore h.p.l.c. (Applied Biosystems 420 A). Ion spray mass spectra were recorded on an API III triple quadrupole mass spectrometer equipped with an Ion Spray interface (Sciex, Toronto, Canada). The samples were dissolved in water/acetonitrile/trifluoroacetic acid (50 : 50 : 1) and introduced into the ion spray source at a flow rate of 5 μl min^{-1} using the solvent delivery system ABI 140A. Peptide purity was checked prior to use by h.p.l.c. analysis using a RP-C18 column (Nucleosil, 3 μm, 250 × 2 mm, Macherey-Nagel, Düren). The mobile phases were: (A) 0.1% trifluoroacetic acid (TFA) in water, (B) TFA 0.1% acetonitrile/water (50%/50%, v/v), using a linear gradient 10–70% B in 30 min at a flow rate of 0.3 ml min^{-1}.

Hydrophobicity

In order to estimate the hydrophobicity of the different peptides, a variety of approaches were adopted. The method of Fauchère *et al.* (1988) is based on the partition coefficient of the Nα-acetyl amino acid amides in octanol/water relative to glycine (F). The hydration potential of the amino acid side chains, as calculated from the free energy of transfer of the side chain from the vapour phase to aqueous phase, was used for calculation of the water affinity (W) (Wolfenden *et al.*, 1981). Hydrophobicity was also estimated from the retention time obtained by reversed phase h.p.l.c., short retention times are found for hydrophilic segments, whereas longer times are found for the stronger interactions of more hydrophobic peptides. Since elution was performed at pH 2, peptides with acidic side chains (Glu, Asp) are fully protonated and appear to have an increased hydrophobicity compared to neutral conditions.

Circular dichroism

Circular dichroism (CD) spectra were measured from 250 to 180 nm on a JASCO J720A spectrapolarimeter at 22°C in a nitrogen atmosphere. The instrument was calibrated using an aqueous solution of 0.06% (w/v) ammonium-+-camphor-10-sulphonate ($\Theta = 8.058$ [deg.cm^2.dMol^{-1}] at 290.9 nm). The peptides were dissolved in different mixtures of trifluoroethanol and phosphate buffer (10 mM, pH 7.0) in concentrations of 0.8 to 2×10^{-4} M. Peptide concentrations were determined by quantitative amino acid analyses. All measurements were carried out in a sample cell with a path length of 0.2 mm. Response time was set to 8 s at a scan speed of 20 nm min^{-1}, a sensitivity range of 5 mdeg and a step resolution of 0.1 nm. Each spectrum was recorded in triplicate. The CD spectra of the solvents were subtracted from those of the peptide solutions to eliminate interference from the cell, solvent and optical equipment. The CD data are given as mean residue ellipticities $[\Theta]_m$. Helicity of the peptides was calculated using the spectra obtained in a 1 : 1 mixture of trifluoroethanol and phosphate buffer as described by Hennessey & Johnson (1981).

Receptor binding studies

Receptor binding studies were performed as described by Chang *et al.* (1985) with minor modifications. In brief, adult male white New Zealand rabbits were killed by i.v. administration of pentobarbitone, the kidneys removed rapidly and the cortex dissected. The tissue was homogenized in ice cold Tris-HCl (50 mM, pH 7.4 at 4°C) with a polytron and then centrifuged (5 min, 1,000 *g*, 4°C). The supernatant was centrifuged 3 times (25 min, 40,000 *g*). The final pellets were washed in 50 vols buffer and incubated for 40 min at room temperature. The membranes were then centrifuged and washed twice (as above). The resulting membranes were resuspended in 125 vols of the Tris-HCl buffer containing $MgCl_2$ (5 mM), soyabean trypsin inhibitor (0.1 mg ml^{-1}), bovine serum albumin (0.1%) and bacitracin (0.25 mg ml^{-1}).

For the binding studies the membrane solution (250 μl containing ca. 100 μg protein) was added to 10 μl buffer (for total binding) or 0.3 μM (final concentration) unlabelled NPY (for non-specific binding) or the displacer, each contained 10 μl [^{125}I]-NPY (Amersham Buchler, Braunschweig). After incubation (90 min, 21°C), the reaction was terminated by filtration under reduced pressure with Whatman GF-C glass filters (previously soaked for at least 2 h in 1% polyethylenamine to reduce binding to the filters) followed by 3 washes with ice-cold buffer (each 3 ml). The specific binding was estimated and the half maximal inhibition of the [^{125}I]-NPY is given as the IC_{50} value.

Blood pressure

Since NPY analogues cause hypotensive responses via a mast cell mediated action (Shen *et al.*, 1991), the hypotensive actions of the analogues used in this study were investigated. Male rats (Chbb:THOM, 270–300 g) were anaesthetized with urethane (1.25 g kg^{-1}, i.p.). A carotid artery was cannulated and connected to a Statham pressure transducer coupled to a Watanabe Multicorder. Increasing doses (0.1, 0.3 and 1.0 mg kg^{-1}) of NPY analogues were administered as aqueous solutions (in a volume of 0.1 ml 100 g^{-1} body weight) via a catheter im-

planted into a jugular vein. Resting mean arterial blood pressure levels were about 70 mmHg. The hypotensive effects ED_{10} values were estimated graphically and expressed as μmol kg^{-1} which induced a blood pressure decrease of 10 mmHg.

Histamine release

Mixed cells (containing ca. 5% mast cells) were obtained by peritoneal lavage of Hooded-Lister rats (male, 150–250 g body weight) as previously described (Ennis, 1992). These cells were washed twice in a modified Tyrode solution (composition mM: NaCl 154, glucose 5.6, HEPES 10, KCl 2.7, $MgCl_2$ 0.5, $CaCl_2$ 0.1, bovine serum albumin 0.1%; pH 7.3) and recovered by centrifugation (100 *g*, 4°C, 2 min).

Cell aliquots were prewarmed for 5 min at 37°C and then NPY or the analogues were added. Histamine release was allowed to proceed for 10 min. The reaction was terminated by placing the tubes in an ice cold water bath, followed by the addition of ice cold Tyrode buffer. Cells were immediately separated from the supernatants by centrifugation (100 *g*, 4°C, 2 min). The remaining cell pellets were resuspended in buffer and boiled for 10 min to release the residual histamine.

Histamine was measured in both the supernatants and cell pellets by an automated fluorometric assay based on that described by Shore and coworkers (Shore *et al.*, 1959; Ennis, 1991). Histamine release was expressed as a percentage of the total cellular content of the amine, corrected for the spontaneous release occurring in the absence of any stimulus. Each concentration of peptide was tested in duplicate in the experiments and all experiments were performed 4–7 times.

Data presentation and statistical analysis

Unless otherwise stated all values are given as mean ± standard error of the mean (s.e.mean). Statistical analyses were performed using Student's *t* test. For multiple comparisons, firstly an analysis of variance was performed followed by the protected *t* test (Howell, 1989). Regression analyses were performed using Fig P software.

Results

Peptide synthesis

The peptides were synthesized by Fmoc/tBu strategy, characterized by electrospray mass spectroscopy, amino acid analysis and h.p.l.c. (Table 1). The purity was >90% for all peptides from both h.p.l.c. and mass spectroscopy and the masses corresponded to the expected data. Chromatography was performed at pH 2 (0.1% trifluoroacetic acid in water) in order to protonate the peptides fully and increase peak resolution.

Hydrophobicity

The calculated overall hydrophobicity and the r.p.-h.p.l.c. retention times of the synthezised NPY peptides are listed in Table 1. All calculations and experimental investigations indicated that peptide [$Glu^{4,25,33,35}$]-NPY(1-4)-Ahx-(25-36) is one of the most hydrophobic peptides. Calculations based on the method of Fauchère *et al.* (1988) showed that native NPY is very hydrophilic, whereas those based on the methods of Wolfenden *et al.* (1981) and the retention time suggest a more hydrophobic behaviour. However, peptides of this length exhibit a pronounced secondary structure, which can influence the retention time (Gauss *et al.*, 1993). NPY exposes a strong hydrophobic binding domain via the α-helical organisation of the amphiphatic sequence when it interacts with the h.p.l.c. stationary phase (Krause *et al.*, 1995). Although peptides Ac-NPY (25–36) and Ala-Pro-Leu-Glu-Ahx-NPY-(25–36) have the same net charge, the former is less hydrophobic probably due to the lack of the hydrophobic Ahx spacer; a similar effect occurred for NPY (19–36) compared to NPY (1-4)-Ahx-(25–36) using the method of Wolfenden *et al.* (1981). No differences in calculated hydrophobicity were found between the peptides NPY (1–4)-Ahx-(25–36), NPY (36–25)-Ahx-(1–4) and all D-NPY(1–4)-Ahx-(25–36) since they differ only in sequence or configuration. However, different h.p.l.c. retention times were found, suggesting that NPY (36–25)-Ahx-(4–1) is less hydrophobic.

Circular dichroism

Circular dichroism measurements demonstrated that all peptides had an α-helical conformation in trifluoroethanol : phosphate buffer (pH 7, 1 : 1 and 9 : 1) (Figure 1). In all cases the typical Cotton effects (positive band at 193 nm and 2 negative bands at 207 and 222 nm) were present. In phosphate buffer (pH 7.0) alone, only native NPY revealed a helical structure, all segments and discontinuous peptides had a random coil formation. This is because the size of the native hormone is 2–3 times longer than the segments and its conformation is therefore much more pronounced and stable. However the high helical content of the segments indicates a strong tendency to form a helical secondary structure. As mixtures of trifluoroethanol are frequently used to imitate membranes it can be presumed that both the segments and the native hormone will exhibit helical conformations when approaching the cell surface.

The spectra of the peptides in trifluoroethanol : phosphate buffer (pH 7, 1 : 1) were used to calculate the α-helical content according to the method of Hennessey & Johnson (1981) (Table 2). The highest percentage α-helicity was found with the parent peptide (30%). Exchanging Lys^4 and/or Arg^{25} resulted in analogues with slightly lower helicity (Table 2). NPY(36–25)-Ahx-(4–1), which consists of the same amino acids as NPY(1–4)-Ahx-(25–36) but synthesized with the opposite orientation had the least pronounced conformation (<10% helicity) (Figure 1). All D NPY(1–4)-Ahx-(25–36) exhibited two positive Cotton effects at 207 and 222 nm and a negative band at 192 nm and was, as expected, the mirror image of the spectrum produced by the all L analogue (Figure 1). In this case the method of Hennessy & Johnson (1981) could not be applied.

Receptor binding studies

Rabbit kidney membranes are used as a model for the Y_2 receptor. Native NPY bound with the greatest affinity to these receptors (Table 2). As previously shown, the centrally truncated analogue NPY(1–4)-Ahx-(25–36) binds with a similar affinity to NPY 19–36 and is 50 times more active than Ac-NPY(25–36) (Table 2) (Beck-Sickinger *et al.*, 1990b). Binding was not altered by the exchange of Pro^2 by His or Ile^{31} by Tyr. It was moderately reduced by Lys^4 to Ala (3 fold difference) or Arg^{25} to Ala (7 fold difference) (Table 2). A hundred fold reduction in affinity was however found when Asp was substituted for Lys^4 and Arg^{25}. Previous studies have shown that both residues are not sensitive to a single substitution by Gly, L-Ala or the D amino acid (Beck-Sickinger *et al.*, 1990a; 1994b). In contrast to the Y_1 receptor, binding at the Y_2 receptor is very sensitive to the exchange of Gln^{34} by Pro both in the truncated form (Table 2) and with native NPY (Fuhlendorff *et al.*, 1990). No receptor binding was detected with the all D amino acid analogue or the retro peptide (NPY(36–25)-Ahx-(4–1).

Blood pressure

Typically, the NPY analogues induced an initial blood pressure increase, lasting for about 1 min. In the case of hypotensive activity, this pressor phase was directly followed by a more sustained depressor phase lasting up to 5 min or more. The hypotensive effects elicited by the analogues are shown in Table 2, expressed as the ED_{10} values [μmol kg^{-1}, i.v.]. Native

Table 1 Chemical details of synthesized neuropeptide Y (NPY), C-terminal segments of NPY and centrally truncated NPY analogues

Number	*Abbreviation*	MS_{theo} (Da)	MS_{exp} (Da)	R_T (min)	*W*	*F*
1	hNPY	4271.78	4272	23.20	-5.84	0.20
2	Ac-NPY (25-36)	1673.96	1673	17.36	-8.30	0.39
3	NPY (19-36)	2385.78	2385	20.51	-7.37	0.36
4	NPY (1-4)-Ahx-(25-36)	2220.63	2221	16.48	-6.94	0.36
5	NPY (36-25)-Ahx-(4-1)	2220.63	2221	16.30	-6.94	0.36
6	All D-NPY(1-4)-Ahx-(25-36)	2220.63	2220	16.65	-6.94	0.36
7	[His^{2}]-NPY(1-4)-Ahx-(25-36)	2220.65	2261	16.17	-7.55	0.33
8	[Pro^{34}]-NPY(1-4)-Ahx-(25-36)	2189.61	2189	16.49	-6.39	0.42
9	[Tyr^{31}]-NPY(1-4)-Ahx-(25-36)	2270.65	2271	14.63	-7.43	0.31
10	[Ala^{4}]-NPY(1-4)-Ahx-(25-36)	2163.53	2163	17.12	-6.27	0.44
11	[Ala^{25}]-NPY(1-4)-Ahx-(25-36)	2135.53	2135	17.51	-5.66	0.44
12	[$Gly^{25,26}$]-NPY(1-4)-Ahx-(25-36)	2041.40	2042	17.45	-4.99	0.41
13	Ala-Pro-Leu-Glu-Ahx-NPY(25-36)	2155.55	2156	18.02	-6.08	0.45
14	[$Asp^{4,25}$]-NPY(1-4)-Ahx(25-36)	2166.44	2166	17.42	-6.50	0.39
15	[$Glu^{4,25}$]-NPY(1-4)-Ahx(25-36)	2194.50	2194	17.75	-6.41	0.40
16	[$Asp^{4,25,33,35}$]-NPY(1-4)-Ahx(25-36)	2084.24	2084	18.06	-5.44	0.42
17	[$Glu^{4,25,33,35}$]-NPY(1-4)-Ahx(25-36)	2140.35	2139	19.23	-5.27	0.45

MS_{theo} represents the theoretical and MS_{exp} the experimental mass spectrum, R_T the retention time (min) on h.p.l.c. (10% to 70% acetonitrile in water, pH 2 within 30 min). For the calculation of hydrophobicity per residue parameters of Wolfenden *et al.* (1981) (W = water affinity) or Fauchère *et al.* (1988) (F = hydrophobicity) were used. The abbreviations Ahx and Ac refer to aminohexanoic acid and an acetylated residue respectively.

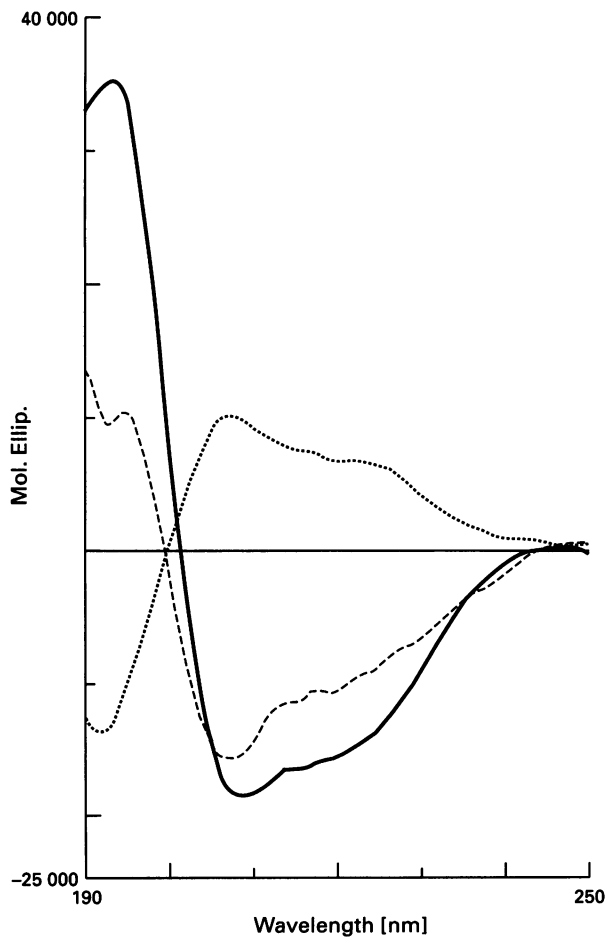

Figure 1 Circular dichroism spectra of peptides 4 (NPY (1-4)-Ahx-(25-36)) (continuous line), 5 (NPY (36-25)-Ahx-(4-1)) (dashed line) and 6 (All D NPY (1-4)-Ahx-(25-36)) (dotted line). Negative Cotton effects at 207 and positive Cotton effect at 193 nm are typical for α-helical conformation, whereas peptide 6 shows the opposite orientation due to the all D configuration of the amino acids.

NPY (only tested up to 10 $\mu g\ kg^{-1}$) was without hypotensive activity. None or only marginal hypotensive activity was observed with the peptides: [Ala^{25}]-NPY(1-4)-Ahx-(25-36), [$Gly^{25,26}$]-NPY(1-4)-Ahx-(25-36), [$Asp^{4,25}$]-NPY(1-4)-Ahx-(25-36) and [$Glu^{4,25}$]-NPY(1-4)-Ahx-(25-36). With the latter peptide, a hypotensive response was observed only in 1 of the 4 rats examined. For this animal, the ED_{10} value was 0.13 $\mu mol\ kg^{-1}$.

Histamine release

With two exceptions, ([$Glu^{4,25,33,35}$]-NPY(1-4)-Ahx-(25-36) and [$Asp^{4,25,33,35}$]-NPY(1-4)-Ahx-(25-36)) which lacked any histamine releasing ability, all analogues tested evoked a dose-dependent histamine release but there were marked differences between the peptides (Figure 2a-e, Table 2). Of the analogues which caused histamine release, native NPY was the least active with 9.2 μM being required to elicit release of 25% of the total cellular histamine content (EC_{25}). In most cases, an increased net positive charge was reflected in increased histamine release and a smaller EC_{25}. Thus the C-terminal fragments were significantly more active than the native NPY, due to the removal of the anionic region (Figure 2, Table 2). Examination of the EC_{25} values for all analogues with a net charge of +5 reveals up to a 12 fold difference in activity (Table 2). Stereochemistry did not exert major effects since the all L and the all D peptides of the centrally truncated type (1-4)-Ahx-(25-36) exhibited similar EC_{25} values and maximal responses (Figure 2, Table 2). The conformation was crucial as shown in the dose-response curves from the truncated peptide and the retro truncated peptide (Figure 2). Single amino acid substitutions in positions 2, 31 and 34 did not markedly alter activity (Figure 2, Table 2). Substitutions at positions 4, 25, 33 and 35 with Glu or Asp removed all histamine releasing ability from the molecule.

Correlation between histamine release and other properties of NPY analogues

Using h.p.l.c. retention time as a measure of the hydrophobicity of the peptides, there was a good correlation between the log EC_{25} values (where available) and the hydrophobicity ($r = 0.65$, $P = 0.008$). This was apparent with the other measures of hydrophobicity (water affinity method of Wolfenden

Table 2 Comparison of helicity, net charge, action on the Y_2 receptor, changes in blood pressure and histamine releasing effects of neuropeptide Y (NPY), C-terminal segments of NPY and centrally truncated NPY analogues

Number	*Peptide*	*Helicity* (%)	*Net charge*	ED_{10} (μmol kg^{-1})	IC_{50} (nM)	EC_{25} (μM)
1	hNPY	30	+1	>0.0023	0.5	9.20±2.20
2	Ac-NPY (25–36)	20	+3	ND	160	1.44±0.44*
3	NPY (19–36)	26	+5	0.12±0.12	4	0.47±0.1*
4	NPY (1–4)-Ahx-(25–36)	26	+5	0.06±0.03	3	0.04±0.01*
5	NPY (36–25)-Ahx-(4–1)	10	+5	0.28±0.14	1×10^{-5}	0.23±0.03*
6	All D-NPY(1–4)-Ahx-(25–36)	**	+5	0.03±0.01	8.4×10^{-6}	0.08±0.04*
7	[His^2]-NPY(1–4)-Ahx-(25–26)	24	+5	0.04±0.01	4	0.04±0.01*
8	[Pro^{34}]-NPY(1–4)-Ahx-(25–36)	28	+5	0.40±0.18	3200	0.11±0.02*
9	[Tyr^{31}]-NPY(1–4)-Ahx-(25–36)	29	+5	0.22±0.09	4	0.09±0.01*
10	[Ala^4]-NPY(1–4)-Ahx-(25–36)	21	+4	0.28±0.04	9	0.22±0.05*
11	[Ala^{25}]-NPY(1–4)-Ahx-(25–36)	18	+4	>0.47	22	0.32±0.09*
12	[Gly^{25,26}]-NPY(1–4)-Ahx-(25–36)	17	+4	>0.47	31	0.25±0.07*
13	Ala-Pro-Leu-Glu-Ahx-NPY(25–36)	29	+3	ND	20	0.43±0.08*
14	[Asp^{4,25}]-NPY(1–4)-Ahx(25–36)	20	+1	>0.46	310	4.87±0.72
15	[Glu^{4,25}]-NPY(1–4)-Ahx(25–36)	22	+1	NA	640	5.74±1.30
16	[Asp^{4,25,33,35}]-NPY (1–4)-Ahx(25–36)	14	−3	ND	ND	NA
17	[Glu^{4,25,33,35}]-NPY(1–4)-Ahx(25–36)	10	−3	ND	ND	NA

ND represents not determined, NA not attained and ** opposite handed helix. ED_{10} is the dose (μmol kg^{-1} body weight) which elicits a blood pressure decrease of 10 mmHg; these values are expressed as means±s.e.mean for 2–4 independent experiments. NA For peptide 15 only 1 of the 4 rats tested exhibited a hypotensive response. IC_{50} is the concentration which elicits a half maximal inhibition of the binding of [^{125}I]-NPY on membrane preparations of rat kidney (Y_2 receptor). EC_{25} (μM) is the concentration required to elicit release of 25% of the total histamine content. These values are expressed as means±s.e.mean for 4–7 independent experiments performed in duplicate. *$P<0.04$ verses native NPY. The abbreviations Ahx and Ac refer to aminohexanoic acid and an acetylated residue respectively.

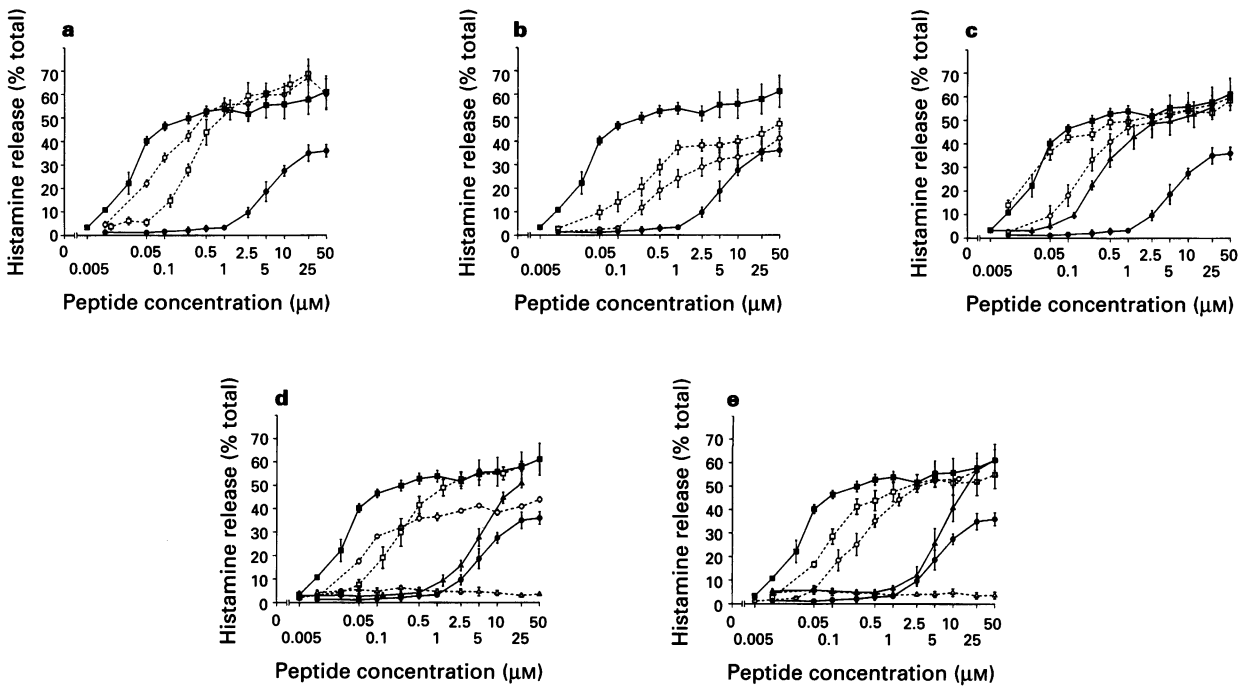

Figure 2 Dose-response relationships for histamine release from rat peritoneal mast cells induced by NPY and its analogues. For comparative purposes all figures contain native NPY (●) and NPY (1–4)-Ahx-(25–36) (■). All data are presented as mean±s.e.mean for $n=4-7$ independent experiments performed in duplicate. (a) All D NPY (1–4)-Ahx-(25–36) (○); NPY (36–25)-Ahx-(4–1) (□). (b) Ac-NPY (25–36) (○); NPY (19–36) (□). (c) [Ala^4]-NPY (1–4)-Ahx-(25–36) (○); [His^2]-NPY (1–4)-Ahx-(25–36) (□); Ala-Pro-Leu-Glu-NPY (1–4)-Ahx-(25–36) (▲). (d) [Tyr^{31}]-NPY (1–4)-Ahx-(25–36) (○); [Gly^{25,26}]-NPY (1–4)-Ahx-(25–36) (□); [Asp^{4,25}]-NPY (1–4)-Ahx-(25–36) (▲); [Asp^{4,25,33,35}]-NPY (1–4)-Ahx-(25–36) (△). (e) [Ala^{25}]-NPY (1–4)-Ahx-(25–36) (○); [Pro^{34}]-NPY (1–4)-Ahx-(25–36) (□); [Glu^{4,25}]-NPY (1–4)-Ahx-(25–36) (▲); [Glu^{4,25,33,35}]-NPY (1–4)-Ahx-(25–36) (△).

et al., 1981: $r=0.21$, $P=0.45$; method of Fauchère *et al.*, 1988: $r=0.183$, $P=0.51$). There was no correlation between helicity (%) or binding at the Y_2 receptor and the log EC_{25} values ($r=-0.026$, $P=0.93$ and $r=-0.113$ $P=0.688$ respectively). There was a strong negative correlation between net charge and log EC_{25} ($r=-0.93$, $P=5.74\times10^{-7}$). The log of the dose required to elicit a 10 mmHg drop in blood pressure (ED_{10}) correlated with the log EC_{25} values ($r=0.62$, $P=0.04$).

Discussion

These investigations have examined the relationships between histamine releasing activity, Y_2 receptor binding ability, conformation and *in vivo* blood pressure effects for native NPY, C-terminal segments of NPY and centrally truncated NPY analogues. Since NPY-related drugs may be developed for systemic use, the structural requirements for NPY-induced mast cell activation and the hypotensive component of the blood pressure response are of interest.

A series of different peptide groupings can be identified: those containing linear segments Ac-25–36 and 19–36 of the native NPY molecule; which are distinguished from the discontinuous analogues NPY(1–4)-Ahx-(25–36) which contain the N-terminal tetrapeptide of NPY linked by Ahx to the C-terminal dodecapeptide, whereby 1 to 4 amino acids of this centrally truncated analogue were substituted resulting in peptides with different overall charge. In addition, the all D NPY(1–4)-Ahx-(25–36) and the retro NPY(36–25)-Ahx-(4–1) contain the same amino acids as NPY(1–4)-Ahx-(25–36) but in the first analogue in a different configuration and in the opposite arrangement for the latter.

The histamine releasing ability of the peptides did not appear to be linked to their ability to displace radiolabelled NPY from specific Y_2 receptors. Although the all D amino acid analogue and the retro peptide (NPY(36–25)-Ahx-(1–4)) exhibited no affinity for the Y_2 receptor ($IC_{50} > 10{,}000$ nM), both were able to induce a marked histamine release (Figure 2, Table 2). Indeed the all D truncated analogue was one of the most active as judged by its EC_{25} and the maximum release obtained, which was identical to that obtained with the all L truncated analogue. An involvement of the Y_1 receptor is also unlikely since the truncated analogue has previously been shown to have only a low affinity for this receptor (4000 nM) (Beck-Sickinger *et al.*, 1994a). Thus this provides further evidence that histamine release from rat peritoneal mast cells by NPY and its analogues is mediated by a receptor-independent pathway or an as yet uncharacterized receptor subtype. Thus these structure-activity studies including truncated Ahx-analogues of NPY are in agreement with the conclusions of Shen *et al.* (1991) drawn on the basis of experiments with the C-terminal fragments NPY(22–36) and NPY(26–36) and the study of Emadi-Khiav *et al.* (1995).

For the centrally truncated peptides, those with the smallest EC_{25} values {NPY(1–4)-Ahx-(25–36) 0.04 μM; [His^2]-NPY(1–4)-Ahx-(25–36) 0.04 μM; all D NPY(1–4)-Ahx-(25–36) 0.08 μM} also had the lowest ED_{10} values (Table 2). However, two of the analogues with relatively low EC_{25} values {[Ala^{25}]-NPY(1–4)-Ahx-(25–36) and [$Gly^{25,26}$]-NPY(1–4)-Ahx-(25–36); 0.32 μM and 0.25 μM respectively} surprisingly showed only marginal or no blood pressure lowering effects. Thus although, histamine release is implicated in the blood pressure changes, it may not be the sole determinant. This may in part reflect differences between the *in vivo* and *in vitro* models used. The dose-dependant decrease in blood pressure elicited by higher NPY concentrations in *in vivo* rat models disappears after repeated NPY injections or pretreatment with histamine antagonists or the histamine liberator compound 48/80 (Grundemar *et al.*, 1990). Furthermore, histamine antagonists enhance the pressor effect of NPY in pithed rats, indicating that histamine may interact with NPY- and hence modulate the pressor response (Sun *et al.*, 1994). However, α-trinositol inhibits the pressor response to NPY to a similar extent in pithed rats both in the presence and absence of histamine antagonists (Sun *et al.*, 1994). Thus further studies are necessary to investigate the exact role of histamine release in the blood pressure responses to NPY and its analogues.

The results indicate that NPY and the analogues tested here cause histamine release via a receptor-independent pathway. A number of features have been described as important for this type of mast cell activation. An overall positive charge is believed to be important (Repke & Bienert, 1987; Devillier *et al.*, 1989). Comparing the EC_{25} values for native NPY with an overall charge of +1 to the C-terminal peptides, Ac-NPY (25–36) and NPY (19–36) with a net charge of +3 and +5 respectively, the EC_{25} values decrease as the charge increases (Table 2). The maximum histamine release observed at 50 μM is almost identical for these 3 peptides (Figure 2b). A similar pattern was observed for the discontinuous analogues containing the spacer Ahx with the EC_{25} decreasing as the number of positive charges increased (Table 2). Peptides number 7–9 (see Table 2), which also have a net charge of +5, exhibit a similar helical content and the slight difference in histamine release may be caused by their different hydrophobicities. Histamine release is reduced in peptides 10–12 (see Table 2) (net charge +4), further reduced for Ala-Pro-Leu-Glu-Ahx-NPY-(25–36) (net charge +3), less for [$Asp^{4,25}$]-NPY(1–4)-Ahx-(25–36) and [$Glu^{4,25}$]-NPY(1–4)-Ahx-(25–36) (net charge +1) and abolished for [$Asp^{4,25,33,35}$]-NPY(1–4)-Ahx-(25–36) and [$Glu^{4,25,33,35}$]-NPY(1-4)-Ahx-(25-36) (net charge −3) suggesting a strong correlation between net charge and EC_{25} for most of the analogues. However a comparison of the centrally truncated peptides, NPY(1–4)-Ahx-(25–36), NPY(36–25)-Ahx-(4–1) and all D NPY(1–4)-Ahx-(25–36), all with a net charge of +5, suggests that other factors in addition to net positive charge influence the potency of the peptides. Histamine release induced by NPY(36–25)-Ahx(4–1) is three fold less than that of all D-NPY(1–4)-Ahx-(25–36) and 6 fold reduced compared to NPY (1–4)-Ahx-(25–36). The helicity of the retro peptide is considerably reduced (Table 2) probably because the Arg residues are placed at the N-terminal segment, which leads to a reduced dipole moment of the helix (Tonan *et al.*, 1990). However, whether the reduced helicity of NPY (36–25)-Ahx-(4–1) is responsible for the diminished histamine releasing activity is questionable, since analysis of EC_{25} values versus helicity revealed no correlation. Moreover, NPY and magainin-2 amide analogues as well as lysine-containing model peptides, exhibiting diminished or abolished helicity due to the insertion of D-amino acids or proline residues, are better histamine releasers than the α-helical parent peptides (Hook *et al.*, 1990; Cross *et al.*, 1995).

In addition to net charge, lipophilicity also contributes to the histamine releasing properties of a peptide. The centrally truncated peptides contain the lipophilic spacer molecule 6-aminohexanoic acid. There are three fold differences in the activity of the peptides Ac-NPY(25–36) and Ala-Pro-Leu-Glu-Ahx-NPY-(25–36) (both +3) and the peptides NPY(19–36) and NPY(1–4)-Ahx-(25–36) (both +5). The analogues containing the spacer are more active, with lower EC_{25} values and higher maximal release (Table 2, Figure 2).

Surprisingly, NPY(36–25)-Ahx-(4–1) behaves less lipophilically than the reverse sequence NPY(1–4)-Ahx-(25–36). This may be explained by the different dipole moment due to the different orientation of the peptides destabilising the helicity, as demonstrated by the circular dichroism findings. The change in h.p.l.c. retention times supports the change of conformation and suggests a decrease of lipophilicity due to the reduced stability of the conformation and the less pronounced helix.

In conclusion, the histamine release evoked by various NPY analogues depends strongly on the number of positive charges. Lipophilicity also contributes to the mast cell activating properties of the peptides, whereas the helicity of the sequences is not a requirement for peptide-induced histamine release. The histamine releasing activity of the NPY analogues with net positive charge is at least in part responsible for the observed depressor effects observed in anaesthetized rats. Wahlestedt & Reis (1993) suggested that with respect to the development of NPY-related drugs for systemic use it might be advantageous to avoid compounds with too many positive charges. This study has provided further evidence for the importance of positive charge and lipophilicity in the induction of mast cell activation and hence of unwanted *in vivo* effects.

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