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Conformational and Biological Studies of Neuropeptide Y Analogs Containing Structural Alterations

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SUMMARY

We evaluated the α-helix content, the biological activities and the affinities of a series of neuropeptide Y (NPY) analogs containing structural alterations, mainly in the central portion of the molecule for which a putative α-helix arrangement has been proposed. First, we investigated the conformational and pharmacological characteristics of derivatives containing the N-terminal tetrapeptide linked to C-terminal peptide-amide segments of various lengths. In some of these, the missing portion was replaced with ε-aminocaproic acid, a flexible arm-linker. Data revealed that (1–4)-Aca-(18–36)NPY is a discontinuous analog almost as potent as the native peptide in a pharmacological preparation enriched in Y_2 receptors (rat vas deferens), whereas it is about 5 times less potent in a Y_1 bioassay (rabbit saphenous vein). This analog showed a similar profile in [^{125}I]PYY binding assays performed in rat frontoparietal cortex (Y_1) and hippocampus (Y_2) membrane preparations. In a series of truncated derivatives obtained with the successive removal of the 5–13 to 5–17 segments of the NPY molecule, no apparent correlation was observed between the affinity or potency in bioassays and the α-helix content, as measured by circular dichroism spectroscopy. Other truncated analogs, obtained by linking the C-terminal 31–36 fragment to various N-terminal tetrapeptides were also investigated. None showed any affinity in brain membrane preparations (frontoparietal cortex and hippocampus) or activity in the rat vas deferens bioassay. However, a weak short-lasting contraction was measured with some of these analogs in the rabbit saphenous vein, thus suggesting that the 1–4 and 31–36 segments of the molecule contains pharmacophores recognized by the Y_1 receptor subtype. The contribution of the arginine residues also was evaluated in relation with the α-helix. Their successive substitution with lysine, an excellent helix-promoter, showed that the replacement of Arg-19 or Arg-25, two residues found in the putative α-helix, gave active analogs. Furthermore, the substitution of Arg-19 with lysine increased the activity in the rat vas deferens as well as the affinity in the brain membrane binding assays. On the other hand, the substitution of Arg-33 produced a weak agonist, whereas the replacement of Arg-35 generated an inactive analog in the Y_2-pharmacological preparation and a very weak competitor in the CNS binding assays. Interestingly, this latter analog was still active in the rabbit saphenous vein, thus identifying the position 35 as an additional potential target for the development of Y_1 versus Y_2 specific molecules. Overall, these pharmacological and spectral data showed that the α-helix content is not a predominant factor for the maintenance of affinity and activity of the NPY analogs. Instead, our results suggest that the key parameter is the folding of the NPY molecule, which ensures an adequate orientation of the N- and C-terminal residues.

NPY is a 36-amino-acid peptide (Table 1) isolated from porcine brain by Tatemoto *et al.* (1, 2) in 1982. Characterization studies revealed that NPY shares a high degree of sequence homology with pancreatic polypeptide and peptide YY, and it is considered as a member of this regulatory peptide family (1, 3). NPY is distributed widely in various areas of the central nervous system (4, 5) where it plays, for instance, a key role in the control of appetite (6, 7) and blood pressure (8). NPY is also found in abundance in the peripheral nervous system, and more particularly in the reproductive organs (9), the perivascular nerve fibers (10), the heart (11, 12), and the gastrointestinal tract (13, 14). NPY exhibits a potent vasoconstrictor activity (15) and participates in the tonus control of smooth

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The abbreviations for the amino acids are in accord with the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature (Eur. J. Biochem, 1984, 138, 9–37). L-isomers of amino acids were used.

ABBREVIATIONS: NPY, neuropeptide Y; pNPY, porcine neuropeptide tyrosine; Boc, tert-butoxycarbonyl; BOP, benzotriazol-1-yl-oxy-tris (dimethylamino) phosphonium hexafluorophosphate; TFA, trifluoroacetic acid; DIEA, diisopropylethylamine; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; SAR, structure-activity relationship; CNS, central nervous system; Pro, proline; Lys, lysine; HPLC, high performance liquid chromatography; BSA, bovine serum albumin; CD, circular dichroism.

TABLE 1

Amino acid sequence of various NPY analogs and fragments

Amino acids are represented by their one-letter symbols. pNPY, porcine neuropeptide-tyrosine. Subscript D indicates D-amino acids.

Peptide	Amino acid							
	1	5	10	15	20	25	30	35
1 pNPY	YPSK	PDNPG	EDAPA	EDLAR	YYSAL	RHYIN	LITRQ	RY[a]
2 [Lys[19]]pNPY	YPSK	PDNPG	EDAPA	EDLAK	YYSAL	RHYIN	LITRQ	RY[a]
3 [Lys[25]]pNPY	YPSK	PDNPG	EDAPA	EDLAR	YYSAL	KHYIN	LITRQ	RY[a]
4 [Lys[33]]pNPY	YPSK	PDNPG	EDAPA	EDLAR	YYSAL	RHYIN	LITKQ	RY[a]
5 [Lys[35]]pNPY	YPSK	PDNPG	EDAPA	EDLAR	YYSAL	RHYIN	LITRQ	KY[a]
6 (1–4)-Aca-(14–36)pNPY	YPSK	Aca	A	EDLAR	YYSAL	RHYIN	LITRQ	RY[a]
7 (1–4)-Aca-(15–36)pNPY	YPSK	Aca		EDLAR	YYSAL	RHYIN	LITRQ	RY[a]
8 (1–4)-Aca-(16–36)pNPY	YPSK	Aca		DLAR	YYSAL	RHYIN	LITRQ	RY[a]
9 (1–4)-Aca-(17–36)pNPY	YPSK	Aca		LAR	YYSAL	RHYIN	LITRQ	RY[a]
10 (1–4)-Aca-(18–36)pNPY	YPSK	Aca		AR	YYSAL	RHYIN	LITRQ	RY[a]
11 pNPY(18–36)				AR	YYSAL	RHYIN	LITRQ	RY[a]
12 (1–4)-(31–36)pNPY11	YPSK						ITRQ	RY[a]
13 (1–4)-Aca-(31–36)pNPY	YPSK			Aca			ITRQ	RY[a]
14 (4–1)-(31–36)pNPY	KSPY						ITRQ	RY[a]
15 (4–1)-Aca-(31–36)pNPY	KSPY			Aca			ITRQ	RY[a]
16 $(4–1)_D$-(31–36)pNPY	kspy						ITRQ	RY[a]
17 $(4–1)_D$-Aca-(31–36)pNPY	kspy			Aca			ITRQ	RY[a]

[a] Presence of an amidated terminus.

muscle by playing a cotransmitter role with norepinephrine in noradrenergic neurons (16).

Structural studies allowed the elaboration of a model containing a N-terminal poly-Pro type II helix and a C-terminal α-helix (17, 18) connected by a type II β-turn. Circular dichroism spectra (19, 20) and molecular modeling (21) proposed an α-helix for residues 14–32, antiparallel to the N-terminal poly-Pro structure and stabilized by intramolecular interactions. According to several authors, this amphipathic α-helix would play an important role in stabilizing the spatial arrangement of the N- and C-terminal segments of the peptide when bound to its receptor (22, 23). It appears that in certain tissues; the N-terminal portion of the molecule would play an important role for the affinity, whereas the C-terminal segment would contain the message for triggering the biological response (24). Moreover, SAR investigations, which described the presence of receptor subtypes identified as Y_1 and Y_2, suggested that the Y_1 subtype is more dependent on the N-terminal segment than the Y_2 receptor site (25).

To further evaluate the role of the helical core of NPY, we synthesized analogs containing structural modifications in the putative α-helix segment. A first series of analogs was obtained by the successive replacement of the arginine residues in positions 19, 25, 33, and 35 by lysine, an amino acid considered an excellent helix-promoter. Also, for a better understanding of the interaction between the N- and C-terminal segments of the molecule, and according to Beck-Sickinger's strategy (26), we synthesized analogs containing the N-terminal tetrapeptide of NPY linked *via* ϵ-aminocaproic acid to C-terminal segments of various lengths. The binding of the analogs to NPY receptors was evaluated in the rat frontoparietal cortex (Y_1-enriched) and hippocampus (Y_2-enriched) preparations (27), and the biological activity was measured with the rabbit saphenous vein (a Y_1-receptor preparation) (28) and the rat vas deferens (a Y_2-receptor preparation) (25) bioassays.

Material and Methods

Synthesis Procedures

Reagents and solvents. Boc-protected amino acid derivatives and BOP reagent were purchased from Richelieu Biotechnologies (St-Hyacinthe, Québec, Canada). ACS-grade dimethylformamide and methylene chloride were obtained from Anachemia Canada Inc. (Ville St-Pierre, Québec, Canada), and Biograde trifluoroacetic acid was purchased from Halocarbon (Hackensack, NJ). Diisopropylethylamine was obtained from Pfaltz and Bauer (Waterbury, CT) and was distilled from ninhydrin before use. Finally, methylbenzhydrylamine resin (copolystyrene-1% divinylbenzene, 0.41 mequiv/g) was also from Richelieu Biotechnologies.

Peptide synthesis and cleavage. NPY and its analogs were synthesized according to the solid-phase peptide synthesis method after a protocol that we described (30, 31). A methylbenzhydrylamine resin was used as the solid support, and BOP reagent (29) was used for the coupling step. The syntheses were carried out with a homemade manual multireactor synthesizer. Side-chain protection of α-Boc-amino acids was as follows: Arg(Tos), Asp(OcHx), Glu(Bzl), His(Tos), Lys(ClZ), Ser(Bzl), Thr(Bzl) and Tyr(Dcb). Peptides were cleaved from the polymeric solid support with liquid hydrofluoric acid (HF; 9 ml/g) in presence of *m*-cresol (1 ml/g) at 0° for 90 min. After precipitation and washings with anhydrous diethylether, the crude peptides were extracted with pure trifluoroacetic acid followed by evaporation.

Peptide purification. All crude peptides (ca. 750 mg) were purified by preparative reverse-phase HPLC on a Waters Prep 590 System equipped with a Waters Associates Model 441 absorbance detector. Peptides were dissolved in H_2O containing 0.06% TFA before injection on a Vydac C_{18} (15–20 μm, 300 Å) column (60 × 5 cm). The material was eluted with linear gradients of (A) H_2O containing 0.06% TFA and (B) CH_3CN (40%) in H_2O containing TFA (0.06%). The gradients used were 0% (B) to 100% (B) in 2.5 hr for the truncated derivatives, and 0% (B) to 40% (B) in 20 min followed by 40% (B) to 100% (B) in 2 hr for the Lys-containing NPY analogs. The flow rate was constant at 64 ml/min, and detection was at 230 nm. Fractions were analyzed by analytical reverse-phase HPLC on a 600 Multisolvent Delivery System with a Lambda-Max Model 481 LC spectrophotometer (Millipore/Waters, Ville St-Laurent, Québec, Canada). Analyses were carried out with a Vydac C_{18} (10 μm) column (30 × 0.39 cm) and an eluant of (A) H_2O with 0.06% TFA and (B) CH_3CN in a linear gradient mode. The gradient used was 20% (B) to 50% (B) in 15 min. The flow rate was maintained at 1.5 ml/min, and detection was at 230 nm. The fractions corresponding to the purified peptide were pooled and lyophilized.

Peptide characterization. NPY and its analogs were characterized by amino acid analysis, analytical HPLC, and capillary electrophoresis. Peptides were hydrolyzed with 6N HCl containing 0.1% phenol during 24 hr at 110°. The samples were analyzed after drying and derivatiza-

tion with PITC, according to the method described by Waters Chromatography. The analyses were carried out with a HPLC system comprising two Waters 510 pumps, a Waters 715 Ultra Wisp sample processor, a Waters TCM temperature controller coupled to a column heater module, and a Waters PICO.TAG amino acid analysis column. During the analysis, the column was kept at 30°C, and the elution of the PTC amino acid derivatives was achieved with successive gradients of CH_3CN in a sodium acetate buffer, according to the operation table described by Waters. The system was controlled, and the data were processed with the Waters Baseline 810 chromatography workstation software.

Analytical HPLC was performed with the system described above using a Pharmacia Super Pak C_{18} (5 μm) column (4 × 250 mm) (Baie D'Urfé, Québec, Canada). The peptides were eluted with a linear gradient of (A) H_2O containing 0.06% TFA and (B) CH_3CN. The gradient used was 10% (B) to 60% (B) in 50 min at a flow rate of 1.0 ml/min, and the detection was at 230 nm. Capillary electrophoresis was carried out with a Applied Biosystem 270A system using the following conditions: 20 mM sodium citrate buffer, pH 2.5; capillary, 45 cm × 50 μm; voltage, 20 kV; T°, 30°; injection, 3 sec in vacuum mode and detection at 200 nm.

As confirmed by means of the characterization techniques, highly purified (≥98%) preparations were obtained and yields, although they were not optimized, were satisfactory (10–40%).

Circular dichroism spectroscopy. CD spectra were recorded on a Jobin Yvon Dichrograph CD6 (Spex Industries Inc., Edison, NJ). The instrument was calibrated with (+)-10-camphorsulfonic acid and isoandrosterone. The peptide samples were contained between cylindral quartz windows with a pathlength of 0.1 cm. Spectra were recorded with a 2-nm bandwidth, a 0.5-nm step, and an integration time of 0.5 sec. Each spectrum was the mean of four scans, and it was corrected for solvent contribution. A "smooth" routine was applied by using a digital low-pass filter. The peptides were studied in increasing concentrations (0–80%; v/v) of HFIP in water, at a final concentration of 0.06 mg/ml, as verified by HPLC in comparison with a standard. The molecular ellipticities ($[\Theta]_\lambda$) are reported in degree-cm^2-$dmol^{-1}$ using the mean residue weight of each peptide for the calculations.

Bioassays and Binding Experiments

Rabbit saphenous vein-Y_1. Albino New-Zealand rabbits of either sex weighing 1.5 to 2.0 kg (Charles River, St-Constant, Québec, Canada) were sacrificed by stunning and exsanguination. Saphenous veins were quickly removed, placed in cold buffer [Krebs-Ringer buffer solution (mmol/l): NaCl (118), KCl (5.4) $CaCl_2$ (2.5), KH_2PO_4 (1.2), $MgSO_4$ (1.2), $NaHCO_3$ (23.8), and D-glucose (11.1)], and cut into helicoidal strips. Segments of 1.5 to 2.0 cm length were set up for isometric recording (Grass Instruments, Quincy, MA) in 5-ml organ baths under a resting tension of 0.5 g. The effects of NPY and the analogs were examined by testing cumulatively applied peptides against the resting tension (for details, see Ref. 28).

Rat vas deferens-Y_2. Adult male Sprague-Dawley rats (200–250 g) from Charles River were sacrificed by decapitation. The abdominal cavity was incised, and the vasa deferentia were dissected out and placed immediately in oxygenated (95% O_2/5% CO_2) Krebs-Ringer buffer solution at 37°. The vasa deferentia were freed carefully from connective tissue and mounted on platinum electrodes in a double-jacketed tissue bath containing oxygenated Krebs-Ringer buffer and maintained at 37°. The tissues were equilibrated for 1 hr at a tension of 0.5 to 1 g. The tissues were stimulated with square electrical pulses (frequency, 0.15 Hz; duration, 0.5 msec; amplitude 60–90 V), and the force of the twitch response was recorded on a Grass polygraph with a force transducer. The effects of NPY and its analogs were measured by adding cumulative doses of the peptides in the concentration range from 1 to 1000 nM. A dose-response curve was measured for each analog.

Membrane binding assays. Homogenate membrane binding assays were performed as previously described (32). Briefly, male Sprague-Dawley rats (200 g) were sacrificed by decapitation, and their brains were rapidly removed. Frontoparietal cortex (Y_1) and hippocampus (Y_2) were dissected rapidly on ice, homogenized in Krebs-Ringer buffer using a Brinkman polytron (at setting 6 for 15–20 sec) (Fisher Scientific, Montréal, Québec, Canada) and centrifuged at 49,000 × g for 15 min. Supernatants were discarded, and pellets were washed, resuspended, and recentrifuged. Final pellets were rinsed and resuspended in Krebs-Ringer buffer to a final protein concentration of 1.0 to 1.5 and 0.4 to 0.6 mg/ml for the frontoparietal cortex and hippocampus, respectively.

Binding assays were initiated by adding 100 μl of membrane preparations in a final volume of 500 μl of Krebs-Ringer buffer containing 0.1% BSA, 0.05% bacitracin, 50,000 cpm [^{125}I]PYY (corresponding to 20–25 pM PYY), and various concentrations (up to 20–21) of competitors (10^{-12}-10^{-6} M). After a 2-hr incubation at room temperature, the reaction was terminated by rapid filtration through filters (previously soaked in 1.0% polyethyleneimine) using a cell harvester filtering apparatus (Brandel Instruments, Gaithersburg, MD).

Filters were rinsed 3 times with 3 ml of cold Krebs-Ringer buffer, and the radioactivity remaining on filters was quantified using a gamma counter. Specifically bound [^{125}I]PYY was determined as the difference in binding observed in the presence and absence of 1 μM unlabeled NPY and represented between 80 and 90% of total binding.

Binding data from each competition curve were analyzed using the Lundon Software program (Lundon Software Inc., Chagrin Falls, OH). All values were expressed as percentage of specific binding and represented the mean ± standard error of four to seven individual determinations, each in triplicate.

Analysis of *in vitro* bioassay results. On all tissue preparations, concentration-response curves were constructed by plotting the molar concentration of drug versus response expressed as grams of tension. From these plots, EC_{50} values were calculated by linear regression analysis and all points between 20 and 80% of maximal responses. EC_{50} values were calculated from each individual curves, and the mean ± standard error was calculated from these data.

Results

Circular dichroism studies. CD spectra of NPY and various analogs showed that these molecules did not adopt, as is seen with most linear peptides, a well defined secondary structure when dissolved in water. However, with the addition of increasing concentrations of structure-promoting co-solvents, the far-UV spectra (250–190 nm) indicated the formation of a helical stretch in these molecules (19, 20, 26). Thus, a CD evaluation of NPY and a series of analogs containing structural alterations designed to evaluate the contribution to the affinity and activity of the amphiphilic α-helix found in the molecule was carried out using HFIP as co-solvent. NPY exhibited Cotton effects typical for a right-handed α-helix. We first investigated the effect of decreasing the length of the putative helical core using discontinuous analogs formed by linking, *via* an ϵ-aminocaproic acid spacer, the N-terminal tetrapeptide Tyr-Pro-Ser-Lys to C-terminal segments of various lengths. Table 2 gives the CD data of pNPY and its analogs measured in a 40% HFIP/H_2O mixture. An approximate amount of 28% of helical content was obtained with pNPY. No significant differences of helix stability were observed between the discontinuous analogs (1–4)-Aca-(14–36)pNPY, (1–4)-Aca-(15–36)pNPY, and (1–4)-Aca-(16–36)pNPY. However, as seen with (1–4)-Aca-(17–36)pNPY and (1–4)-Aca-(18–36)pNPY, it appears that deletions beyond Glu-15 are detrimental to the helix stability.

The contribution of the arginine residues of the NPY molecule was also evaluated in relation to the α-helix. Thus, the

TABLE 2

Molecular ellipticity of pNPY and its analogs at 208 and 222 nm, as determined by circular dichroism spectroscopy, and content of α-helix structure

Mean residue molecular ellipticity of the peptide at 208 and 222 nm, determined in 40% HFIP/H_2O (see Materials and Methods) ± standard error of four experiments. The percentage of α-helix content was calculated from $[\Theta]_{222}$ (deg − cm²/dmol) using the formula % helix content = 100 × ($[\Theta]_{222}$ − 3000)/−39000.[a]

No.	pNPY and its analogs	Molecular ellipticity	% α-Helix content
		$-[\Theta]_{208/222}$ ± SE	Mean ± SE
		deg − cm²/dmol	
1	pNPY	7609 ± 849 /7699 ± 258	27.5 ± 0.7
2	[Lys[19]]pNPY	6775 ± 582 /6261 ± 301	23.7 ± 0.8[b]
3	[Lys[25]]pNPY	8407 ± 321 /8352 ± 498	29.1 ± 1.3
4	[Lys[33]]pNPY	7735 ± 1094/6702 ± 496	24.9 ± 1.3
5	[Lys[35]]pNPY	9143 ± 833 /8937 ± 625	30.6 ± 1.6
6	(1–4)-Aca-(14–36)pNPY	5896 ± 497 /5540 ± 163	21.9 ± 0.4
7	(1–4)-Aca-(15–36)pNPY	5411 ± 119 /5746 ± 316	22.4 ± 0.8
8	(1–4)-Aca-(16–36)pNPY	5686 ± 415 /6319 ± 409	23.9 ± 1.0
9	(1–4)-Aca-(17–36)pNPY	4461 ± 209 /3765 ± 250	17.3 ± 0.7[c]
10	(1–4)-Aca-(18–36)pNPY	3855 ± 176 /3495 ± 71	16.7 ± 0.2[c]

[a] From Ref. 45.
[b] $p < 0.02$ with pNPY as reference.
[c] $p < 0.001$ with (1–4)-Aca-(14–36)pNPY as reference.

arginines were substituted successively with lysine, a good helix-promoter carrying a positive charge on its side-chain. Circular dichroism spectra of NPY and its Arg/Lys-substituted derivatives, in 40% HFIP/H_2O (Table 2), showed that the substitution of arginine with lysine did not modify considerably the α-helix arrangement of the molecule. However, among the analogs, it seems that the replacement of Arg-19 would give some disturbance in the secondary structure.

***In vitro* bioassays.** The biological activities of the analogs were evaluated in the rabbit saphenous vein, a pharmacological preparation that we identified as enriched in Y_1 receptors (28), and the rat vas deferens bioassay, a Y_2-receptor model (25). In the saphenous vein paradigm, pNPY and its analogs were shown to produce a concentration-dependent contraction (Fig. 1). The relative potency (Table 3) of the Arg/Lys-substituted NPY analogs was pNPY ≥ [Lys[19]]NPY ≥ [Lys[25]]NPY ⋙ [Lys[35]]NPY > [Lys[33]]NPY. Thus, it appears that substitutions of Arg-19 or Arg-25 with lysine is well tolerated by the Y_1 receptor subtype, whereas the replacement of Arg-33 and Arg-35 causes a decrease of the potency in the Y_1 assay.

On the other hand, the discontinuous analogs had, on the saphenous vein, a contractile effect distinct from that of pNPY. As seen in Fig. 1B, a maximum of approximately 0.7 g of tension was reached with NPY, whereas the analogs (1–4)-Aca-(18–36)NPY and (1–4)-Aca-(15–36)NPY developed greater tensions in this tissue. It is yet unclear why the analogs possessed this behavior. However, this particular activity may be related to variations in metabolism or to enhanced expressions of second messenger, giving rise to a potentiation of the contractile response.

Neuropeptide Y inhibited in a concentration-dependent manner the electrically induced muscle twitches of the rat vas deferens (Fig. 2). In this Y_2-pharmacological preparation (25), the substitution of the arginine residues generated analogs with the following relative potency of inhibition (Table 3): [Lys[19]]NPY ≥ pNPY > [Lys[25]]NPY ≫ [Lys[33]]NPY. The derivative [Lys[35]]NPY was found to be inactive in this preparation, whereas the substitution of Arg-25 or Arg-19 produced potent analogs of NPY. Although [Lys[25]]pNPY was about 3-fold less

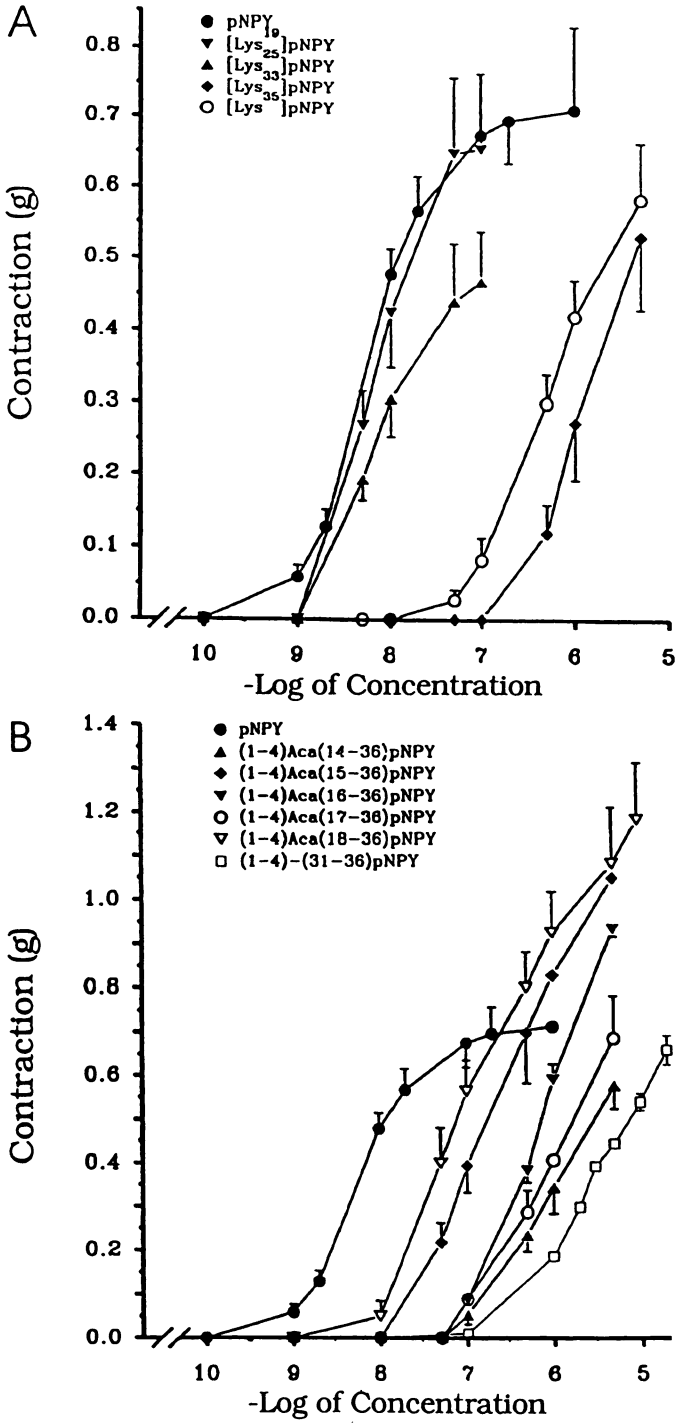

Fig. 1. Concentration-response curves of the contractile response of the rabbit saphenous vein for NPY and related analogs. Values are means of at least six experiments.

potent than NPY, [Lys[19]]pNPY exhibited a potency slightly higher than that of the parent molecule (IC_{50}, 7 versus 15 nM).

All the truncated NPY analogs of the series obtained by linking, *via* aminocaproic acid, the N-terminal tetrapeptide of NPY to C-terminal segments of lengths varying from 14–36 to 18–36 exhibited significant potencies in the rat vas deferens bioassay. As shown in Table 3, their relative potency of inhibition was as follows: pNPY ≥ (1–4)-Aca-(18–36)pNPY ≥ (1–4)-Aca-(16–36)pNPY = (1–4)-Aca-(15–36)pNPY > (1–4)-Aca-

TABLE 3

Contractile effects in the rabbit saphenous vein (Y_1) and inhibitory activity in the rat vas deferens (Y_2) of NPY and its analogs

EC_{50}*, the concentration of peptide producing 50% of the maximum effect induced by NPY, in the rabbit saphenous vein. The value is obtained from the concentration-response curves and is expressed as means ± standard error of at least six determinations. The asterisk indicates that some of the analogs develop a force of contraction superior to that of NPY. IC_{50}, the concentration of peptide producing a 50% inhibition of the maximum effect induced by NPY in the rat vas deferens. The value is obtained from concentration-response curves and is expressed as mean ± standard error of at least six determinations. RP, relative potency as compared with pNPY; NE, no effect at a concentration of 1 μM and ND, not determined, although a weak short-lasting contraction was measured with these analogs.

No.	pNPY and its analogs	Rabbit saphenous vein (Y_1 enriched)		Rat vas deferens (Y_2 enriched)	
		EC_{50}* ± SE	RP	IC_{50} ± SE	RP
		nM	%	nM	%
1	pNPY	4.6 ± 0.6	100	15 ± 4	100
2	[Lys^{19}]pNPY	6.6 ± 1.5	70	7 ± 2	214
3	[Lys^{25}]pNPY	8.2 ± 1.5	56	54 ± 15	26
4	[Lys^{33}]pNPY	770 ± 10	0.6	818 ± 279	2
5	[Lys^{35}]pNPY	325 ± 5	1	NE	—
6	(1–4)-Aca-(14–36)pNPY	607 ± 153	0.8	135 ± 91	11
7	(1–4)-Aca-(15–36)pNPY	70 ± 17	7	31 ± 5	48
8	(1–4)-Aca-(16–36)pNPY	297 ± 44	2	31 ± 14	48
9	(1–4)-Aca-(17–36)pNPY	228 ± 69	2	135 ± 21	11
10	(1–4)-Aca-(18–36)pNPY	29 ± 7*	16	24 ± 4	63
11	pNPY(18–36)	454 ± 117	1	322 ± 13	5
12	(1–4)-Aca-(31–36)pNPY	NE	—	NE	—
13	(1–4)-(31–36)pNPY	1833 ± 33	0.3	NE	—
14	(4–1)-Aca-(31–36)pNPY	ND	—	NE	—
15	(4–1)-(31–36)pNPY	NE	—	NE	—
16	$(4–1)_D$-Aca-(31–36)pNPY	NE	—	NE	—
17	$(4–1)_D$-(31–36)pNPY	ND	—	NE	—

* EC_{50} was measured for (1–4)-Aca-(18–36)pNPY. A value of 120 ± 40 nM was obtained.

(14–36)pNPY ≥ (1–4)-Aca-(17–36)pNPY. From these relative potencies, it clearly appeared that no direct correlation was noticeable between the biological activity found in the vas deferens, a Y_2-receptor pharmacological preparation, and the successive removal of the 14–17 residues.

Finally, several short truncated analogs, obtained by linking the C-terminal hexapeptide 31–36 to various N-terminal tetrapeptides, were prepared, and their biological activity was determined. None of these exhibited any effect in the rat vas deferens bioassay (Table 3). However, in the rabbit saphenous vein, a weak effect was measured with some of the short discontinuous analogs, such as (4–1)-Aca-(31–36)pNPY and $(4–1)_D$-(31–36)pNPY.

CNS binding assays. Using CNS membrane binding assays, estimations of the affinities of the analogs have been made in comparison with the native peptide (Figs. 3 and 4). The results (Table 4) showed that the truncated analogs are better competitors in the hippocampus (Y_2-enriched) than in the frontoparietal cortex (Y_1-enriched) preparations. Their affinities are, in magnitude, usually between one and two orders lower than that of NPY. However, (1–4)-Aca-(18–36)pNPY exhibited an effect distinct from those of the other analogs of the series. In the cortex, the affinity of (1–4)-Aca-(18–36)pNPY was 5- to 7-fold higher than that of the other truncated analogs of this series (IC_{50}, 5 ± 1 nM versus values ranging from 20 ± 3 to 36 ± 7 nM for the other truncated analogs), whereas in the hippocampus, its affinity was 5- to 14-fold higher (IC_{50}, 0.6 ± 0.2 nM versus values ranging from 2.6 ± 0.4 to 6.5 ± 2.3 nM for the other truncated analogs). NPY exhibited in the cortex and the hippocampus IC_{50} values of 0.39 ± 0.15 and 0.28 ± 0.10 nM respectively. Thus, (1–4)-Aca-(18–36)pNPY is about half as

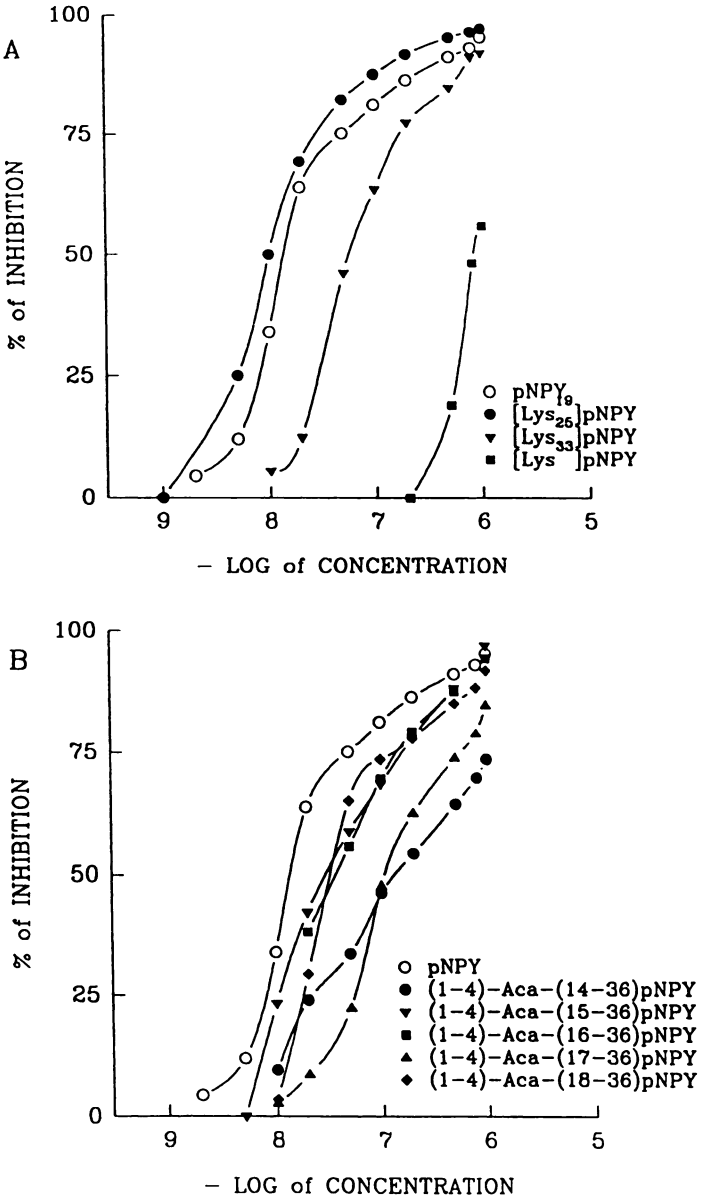

Fig. 2. Concentration-response curves of inhibition of the electrically stimulated twitch response of the rat vas deferens preparation for pNPY and related analogs. Values are means of at least six experiments.

potent as pNPY in the hippocampus, whereas it is 10 times less potent than the parent molecule in cortical homogenates.

The NPY analogs obtained by substituting the arginine residues with lysine possessed affinity rankings comparable to activities measured in the rat vas deferens and the rabbit saphenous vein bioassays. Although [Lys^{33}]- and [Lys^{35}]pNPY were very weak competitors, [Lys^{25}]pNPY exhibited relative affinities of 4 and 3% in the Y_1 and Y_2 assays, respectively. In contrast, the analog [Lys^{19}]pNPY appeared to be slightly more potent than pNPY. Interestingly, the replacement of Arg-35 with lysine had more detrimental effects in the Y_2 preparation than in the Y_1 assay.

Discussion

It has been hypothesized that the amphipathic α-helix would play an important role in stabilizing the spatial arrangement

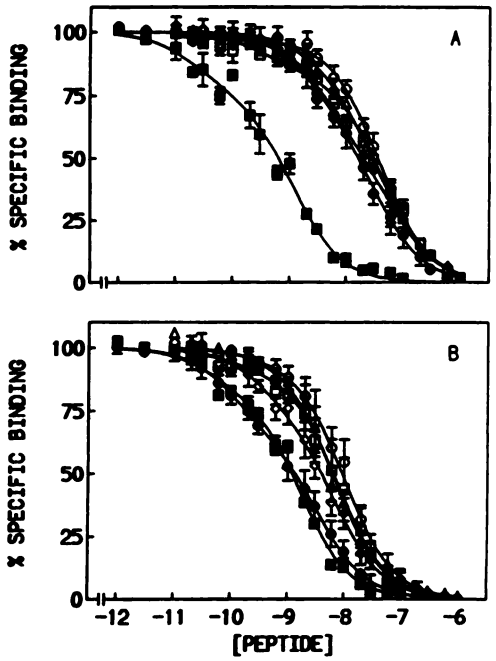

Fig. 3. Competition binding profiles of pNPY (■) and various truncated analogs such as (1–4)-Aca-(18–36)pNPY (●), (1–4)-Aca-(17–36)pNPY (○), (1–4)-Aca-(16–36)pNPY (△), (1–4)-Aca-(15–36)pNPY (◇), and (1–4)-Aca-(14–36)pNPY (□) against specific [^{125}I]PYY labeling in *A*, rat frontoparietal cortex Y_1-enriched membrane homogenates and *B*, rat hippocampus Y_2-enriched membrane homogenates. Each point represents the mean ± standard error of data obtained from four to seven experiments, each performed in triplicate and expressed as the percentage of specific binding.

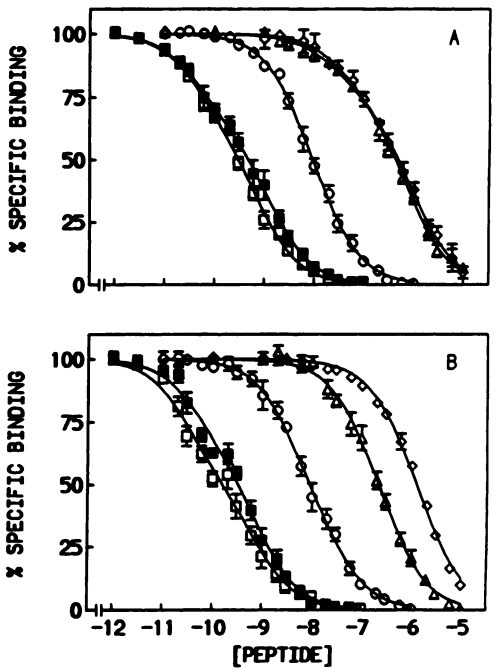

Fig. 4. Competition binding profiles of pNPY (■) and various substituted analogs such as [Lys^{19}]pNPY (□), [Lys^{25}]pNPY (○), [Lys^{33}]pNPY (△), and [Lys^{35}]pNPY (◇) against specific [^{125}I]PYY labeling in *A*, rat frontoparietal cortex Y_1-enriched membrane homogenates and *B*, rat hippocampus Y_2-enriched membrane homogenates. Each point represents the mean ± standard error of data obtained from four to seven experiments, each performed in triplicate and expressed as the percentage of specific binding.

of the N- and C-terminal segments of NPY and in providing conformational prerequisites of residues 33–36 for receptor binding (22, 23, 33). Therefore, the role of the helical core of NPY was evaluated by synthesizing analogs containing structural modifications, mainly in the putative α-helix segment, and by measuring their biological activities, as well as their affinities in Y_1 and Y_2 receptor paradigms. In parallel, conformational features of the analogs were analyzed by means of CD spectroscopy.

Thus, structural characterizations confirmed that NPY exhibits Cotton effects typical for a right-handed α-helix and that the amount of helical content in the molecule is approximately 28%. This value is similar to those previously reported in different solvent conditions (19, 20, 34). Our investigation of the discontinuous analogs in which a peptide segment was replaced with ϵ-aminocaproic acid showed a decrease of α-helix stability, even if the segment believed to contain the helical arrangement was kept intact, as seen with (1–4)-Aca-(14–36)pNPY. This suggested that the missing portion exhibited a helix-stabilizing effect. A similar conclusion was reached by Bouvier and Taylor (20), who proposed such a stabilizing effect for the portion 1–9 of the molecule after observing that N^{α}-Ac-NPY (10–36) possessed only a 13% α-helix content, compared to 32% for NPY. No significant differences in helix stability were observed between the discontinuous analogs (1–4)-Aca-(14–36)pNPY, (1–4)-Aca-(15–36)pNPY, and (1–4)-Aca-(16–36)pNPY. However, as seen with (1–4)-Aca-(17–36)pNPY and (1–4)-Aca-(18–36)pNPY, it appears that deletions beyond Glu-15 are detrimental for the helix stability. A stabilizing role, therefore, can be hypothesized for Asp-16. The stabilization phenomenon might be produced by an ionic interaction involving the negative charge of Asp-16 and a basic residue of the peptide chain, such as Arg-19, which is in close proximity in the α-helix. However, as recently described by Bjørnholm *et al.* (35), the presence of a cluster of negatively charged residues in the N-terminal end of the α-helix and the existence of a positive charge (Arg-33 and Arg-35) in the C-terminal segment of the helical core give rise to a dipole moment antiparallel to that created by the helical structure. Thus, the complete removal of the negative cluster would destroy the dipole moment, which appears to play an important role in the structural stability of the PP-fold arrangement (35, 36).

The contribution of the arginine residues of the NPY molecule was also evaluated in relation to the α-helix. Their successive replacement with lysine, a good helix-promoter carrying a positive charge on its side chain, showed that the substitution of arginine with lysine did not modify considerably the α-helix arrangement of the molecule. However, among the analogs, it was observed that the replacement of Arg-19 induced a disturbance in the secondary structure. This decrease in α-helix content measured with [Lys^{19}]pNPY, as compared to pNPY, is compatible with the postulated participation of the Arg-19 side-chain in an ionic bridge. Indeed, the lower basicity of the lysine side-chain would give rise to a weaker stabilizing intramolecular bond.

The biological activities and the affinities of the analogs were evaluated in biological preparations enriched in Y_1 receptors (28, 32). The relative potencies of the Arg/Lys-substituted NPY analogs in the rabbit saphenous vein bioassay and the frontoparietal cortex binding preparation suggested that substitutions of Arg-19 or Arg-25 with lysine is tolerated by the Y_1 receptors,

TABLE 4

Relative potencies of NPY and its analogs in the [^{125}I]PYY CNS binding assay

IC_{50}, concentrations of competitor needed to inhibit 50% of specific [^{125}I]PYY binding in A, the frontoparietal cortex, a Y_1-enriched membrane preparation, and in B, the hippocampus, a Y_2-enriched membrane preparation. Data are expressed as mean ± standard error of four to seven determinations, each performed in triplicate. RA, relative affinity as compared with pNPY; N_H, Hill coefficient.

No.	pNPY and its analogs	A: Frontoparietal cortex (Y_1-enriched)			B: Hippocampus (Y_2-enriched)		
		IC_{50} ± SE	RA	N_H	IC_{50} ± SE	RA	N_H
		nM	%		nM	%	
1	pNPY	0.39 ± 0.15	100	0.70 ± 0.05	0.28 ± 0.10	100	0.75 ± 0.06
2	[Lys^{19}]pNPY	0.26 ± 0.14	150	0.75 ± 0.04	0.15 ± 0.08	187	0.69 ± 0.04
3	[Lys^{25}]pNPY	8.9 ± 3.2	4	0.94 ± 0.05	8.7 ± 2.4	3	0.85 ± 0.05
4	[Lys^{33}]pNPY	354 ± 58	0.1	0.75 ± 0.05	205 ± 83	0.1	0.93 ± 0.06
5	[Lys^{35}]pNPY	410 ± 84	0.1	0.74 ± 0.06	1172 ± 340	0.02	0.94 ± 0.05
6	(1–4)-Aca-(14–36)pNPY	24 ± 4	2	0.64 ± 0.03	4.5 ± 1.3	6	0.84 ± 0.03
7	(1–4)-Aca-(15–36)pNPY	20 ± 4	2	0.73 ± 0.04	2.6 ± 0.4	11	0.76 ± 0.02
8	(1–4)-Aca-(16–36)pNPY	26 ± 3	2	0.88 ± 0.05	5.6 ± 1.9	5	0.92 ± 0.04
9	(1–4)-Aca-(17–36)pNPY	36 ± 7	1	0.94 ± 0.03	6.5 ± 2.3	4	0.96 ± 0.05
10	(1–4)-Aca-(18–36)pNPY	5 ± 1	8	0.63 ± 0.04	0.6 ± 0.2	47	0.71 ± 0.03
11	pNPY(18–36)	16 ± 3	3	0.68 ± 0.03	0.5 ± 0.2	56	0.63 ± 0.02

whereas the replacement of Arg-33 or Arg-35 produces a decrease in the potency. As described before, the lysine incorporation into the NPY peptide chain did not modify largely the α-helical arrangement of the molecule. Therefore, no correlation can be established between the Y_1-mediated biological activity and the stability of the helical stretch of these analogs. Moreover, the analog of this series having the lowest α-helix content ([Lys^{19}]pNPY) showed a potency not significantly different to that of NPY. These results contrast to those reported by Jung *et al.* (33), who observed that the helicity of a series of small NPY analogs correlated well with biological activity and receptor binding affinity. Furthermore, it is worthwhile to mention that in the rabbit saphenous vein Y_1 bioassay, the analogs possessing a lysine residue at position 33 or 35 still exhibit a contracting effect. Previous studies (26, 37) had suggested that Arg-33 and Arg-35 cannot be substituted for lysine without considerable decrease of activity. Since [Lys^{33}]- and [Lys^{35}]NPY have similar α-helical content to that of NPY, it can be concluded that the Arg-33 and Arg-35 guanidinium moieties are relatively important, but not critical, features for the full expression of Y_1-mediated biological activities.

Definite activities and affinities were measured with the various discontinuous analogs. This confirmed that a large portion of the NPY molecule plays only a structural role. However, this contribution would not be in relation to the α-helix secondary structure, but rather to the folding of the peptide chain. As shown by the CD evaluation, and in contrast to previous reports concerning NPY and PYY (33, 38), the most potent analog of this series (analog number 10) is the one with the lowest α-helix content. Moreover, it can be concluded that the length of the C-terminal segment comprising the helical structure is not a determinant factor, because (1–4)-Aca-(18–36)NPY is much more potent than (1–4)-Aca-(14–36)NPY. Interestingly, it seems that leucine-17 would be a boundary residue. Indeed, as seen with (1–4)-Aca-(17–36)NPY and (1–4)-Aca-(18–36)NPY, the removal of Leu-17 caused an 8-fold increase of contracting effect in the saphenous vein bioassay. A relative affinity of 8%, the highest of this series, was also measured for (1–4)-Aca-(18–36)NPY in the frontoparietal Y_1-enriched membrane preparation. Thus, the replacement of the 5–17 segment of NPY with ε-aminocaproic acid generated an analog in which the pharmacophores of the molecule are oriented in positions adequate for receptor recognition.

The biological activity and the affinity were also measured in Y_2-enriched preparations. Interestingly, we observed that the substitution of the arginine residues gave analogs with relative potencies distinct to those measured in the Y_1 paradigms. For instance, the derivative [Lys^{35}]NPY was found to be inactive. We already pinpointed, using Y_1-related assays, the putative functions of the guanidinium moieties of Arg-35. However, in the Y_2-enriched preparations, it is clear that position 35 is more critical and is crucial for the maintenance of biological activity and binding affinity. This observation cannot be related to a modification of the secondary structure because the helical content of [Lys^{35}]NPY is not significantly different from that of pNPY. On the other hand, the substitution of Arg-19 with Lys, as seen in frog NPY (melanostatin) (42), produced an analog exhibiting a potency equal to or slightly higher than that of the parent molecule. Because the truncated analog (1–4)-Aca-(25–36)NPY was shown by Beck-Sickinger *et al.* (26, 37) to be active in a Y_2-pharmacological preparation, it can be assumed that Arg-19 is not an essential residue for the effects observed in Y_2 assays. Moreover, as shown with our CD analyses, a significant α-helix destabilization effect occurred with the replacement of arginine-19 with lysine, suggesting that the biological activity and affinity are not directly related to the secondary structure of the molecule.

All the truncated NPY analogs of the series obtained by linking, *via* ε-aminocaproic acid, the N-terminal tetrapeptide of NPY to C-terminal segments of lengths varying from 14–36 to 18–36 exhibited substantial activity and affinity in Y_2-enriched assays. Noteworthy, and as previously reported (39–41), is that the truncation of mid-segments of the NPY molecule did not impair seriously the interaction of the NPY analogs with the Y_2 receptor subtype, and they gave analogs that usually appeared to be better agonists or competitors in Y_2 than in Y_1 assays. However, it clearly appeared that no correlation can be established between the binding properties or biological activity, the successive removal of the 14–17 residues and the stability of the helical core. Indeed, the derivative showing the lowest α-helix content, (1–4)-Aca-(18–36)pNPY, was almost as potent as NPY in inhibiting the twitch response of the rat vas deferens or in competing for the Y_2 receptors found in the

hippocampus. Furthermore, lower biological activities and affinities were observed with the longer discontinuous analogs. This might result from a limited ability of these derivatives to interact with the receptor after an unfavorable spatial orientation of the C-terminal amino acid residues when the initiation of the α-helix is located before Ala-18.

Although the length of the C-terminal portion of the NPY molecule for eliciting the pharmacological Y_2-effect is not identified precisely, it can be concluded that activity is obtained with segments beginning between Arg-25 and Ile-31. Indeed, Beck-Sickinger *et al.* (26) showed that (1–4)-Aca-(25–36)NPY exhibited a potent prejunctional activity, and we observed that (1–4)-Aca-(31–36)NPY had no effect in the rat vas deferens bioassay, or in the CNS binding assays. In fact, none of the short truncated analogs of our study, obtained by linking the C-terminal hexapeptide 31–36 to various N-terminal tetrapeptides, exhibited any effect or affinity in the rat vas deferens bioassay or the rat CNS membrane preparations. However, it is worth mentionning that in the rabbit saphenous vein, a weak biological response was measured with some of the short discontinuous analogs (numbers 13, 14, and 17), which suggested that certain amino acid residues located in the N-terminal tetrapeptide and the C-terminal hexapeptide are recognized by the NPY receptors present in this preparation. It is yet unclear, however, whether this weak short-lasting effect is mediated by Y_1 receptors or *via* another PP family-related receptor subtype.

Taken together, the results of the present study revealed that the α-helix content is not a predominant factor for the maintenance of adequate affinity or biological activities of NPY analogs. Moreover, the data with [Lys^{35}]pNPY showed that the affinity of the hippocampal receptor site and the twitch response of the electrically stimulated rat vas deferens (Y_2 preparations) were influenced more by the substitution of arginine-35 with lysine than by the binding affinity to cortex receptors and the contractile effect of the saphenous vein (Y_1 preparations). This residue, therefore, might represent another useful target for the development of Y_1/Y_2 selective agonists or antagonists. On the other hand, the discontinuous analogs exhibited better affinities in the hippocampus than in the cortex membrane preparations, and higher potencies in the vas deferens assay than in the saphenous vein preparation. This implies that alterations in the mid-portion of the NPY molecule are better tolerated by the Y_2 than the Y_1 receptor subtypes. Interestingly, the analog (1–4)-Aca-(18–36)pNPY is a highly potent agonist, especially in the Y_2 bioassay. This suggests that among the analogs of the series, this derivative closely mimics the conformation adopted by NPY. The biological effect of this truncated analog is related to the presence of both N- and C-terminal segments. Indeed, our previous investigations showed that C-terminal fragments, such as $pNPY_{16-36}$ and $pNPY_{18-36}$ were weak agonists in the rat vas deferens assay (43, 44). Moreover, Chang *et al.* (45) and Michel *et al.* (46) observed that NPY_{18-36} was 3- to 5-fold less potent than NPY in their Y_2-pharmacological preparations. Michel *et al.* (46) also demonstrated that this fragment can to some extent discriminate between Y_1 and Y_2 receptor subtypes. Hence, our data demonstrated that the linking of the N-terminal tetrapeptide of NPY to its C-terminal 18–36 fragment improved considerably the biological activity of NPY_{18-36}. Moreover, the results with (1–4)-Aca-(18–36)pNPY demonstrated that a certain ability for discriminating between Y_1 and Y_2 receptors was preserved with this truncated analog.

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