

Synthesis of receptor antagonists of neuropeptide Y

(hormone/analog/peptide YY)

KAZUHIKO TATEMOTO*[†], MICHAEL J. MANN*, AND MEIKYO SHIMIZU[‡]

*Peptide Research Laboratory, Department of Psychiatry and Behavioral Sciences, Stanford University School of Medicine, Stanford, CA 94305; and
[‡]Peninsula Laboratories, Inc., Belmont, CA 94002

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ABSTRACT We report the synthesis of receptor antagonists of neuropeptide Y (NPY) by a strategy based on synthesis of mixtures of analogs and the subsequent isolation and identification of receptor antagonists from these mixtures. After screening a series of mixtures of NPY analogs by using an NPY antagonist assay, two potent receptor antagonists, designated PYX-1 and PYX-2, were isolated from an antagonist-containing mixture. Structural analysis revealed these analogs to be Ac-[3-(2,6-dichlorobenzyl)Tyr²⁷,D-Thr³²]NPY-(27–36) amide and Ac-[3-(2,6-dichlorobenzyl)Tyr^{27,36},D-Thr³²]NPY-(27–36) amide, respectively. The receptor antagonists inhibited release of intracellular calcium elicited by NPY in human erythroleukemia cells and displaced ³H-labeled NPY from NPY receptors in rat brain membrane. The approach of screening and identifying useful analogs from synthetic mixtures may significantly reduce the time and resources previously required for development of receptor antagonists.

Despite the importance of developing receptor antagonists of hormones and neurotransmitters, the synthesis of such antagonists has generally proved to be a difficult, time-consuming, and often elusive task. In fact, this process still relies on a hit-or-miss repetition of synthesis and screening of individual analogs.

To reduce the time and resources previously required for development of receptor antagonists, we investigated a synthetic strategy based on synthesis of mixtures of analogs and the subsequent isolation and identification of receptor antagonists from these mixtures. Using such a strategy, one can screen for and identify antagonist-containing mixtures and then isolate individual antagonists from the mixtures in a manner similar to the isolation of natural peptides from tissue extracts. After determination of their structures, compounds identical to the isolated analogs can then be resynthesized for further study. By combining synthetic and isolation methodologies, the analog mixture screening strategy allows large numbers of analogs to be synthesized simultaneously and then screened in a simple and efficient manner. The use of such a strategy therefore increases the probability of identifying receptor antagonists in a short period of time.

Based on this strategy, we carried out a study to develop receptor antagonists of neuropeptide Y (NPY). NPY is a 36-amino acid peptide that belongs to a family of peptides including peptide YY (PYY) and pancreatic polypeptide (1, 2). NPY, the most abundant peptide in the central nervous system, is involved in a broad spectrum of brain functions, including food intake, blood pressure regulation, hormone secretion, sexual behavior, and circadian rhythmicity. NPY is also abundantly distributed throughout the peripheral nervous system; it plays important roles in sympathetic vascular control and modulates the release of catecholamines (3–5). PYY, a 36-amino acid peptide hormone (6, 7), is

located in the gastrointestinal tract, particularly in the distal intestine, and is involved in various functions including vascular control, gut motility, and pancreatic secretion (8). Since NPY and PYY are structurally similar (2) and bind to each other's receptors (9, 10), NPY receptor antagonists are likely to act as PYY receptor antagonists as well.

The development of NPY receptor antagonists may be useful for studying the mechanisms of action of NPY and may be important in the treatment of clinical problems such as obesity, hypertension, coronary artery disease, sleep disorders, sexual dysfunction, and gastrointestinal disorders. In the present study, two receptor antagonists of NPY were isolated and characterized from a mixture of NPY analogs by the analog mixture screening strategy.

A preliminary study has been published (11).

MATERIALS AND METHODS

t-Butoxycarbonyl (*t*-Boc) amino acids and *t*-Boc amino acid resins were obtained from Peninsula Laboratories. *N*-9-fluorenylmethoxycarbonyl (Fmoc) amino acids were purchased from Bachem/Bioscience and 4-aminomethyl-3,5-dimethylphenoxy resin was from MilliGen/Bioscience (Novato, CA). Trifluoroacetic acid (TFA) and dansyl chloride were obtained from Pierce, polyamide thin-layer sheets (A1700) were from Schleicher & Schuell, and trypsin was from Boehringer Mannheim. Reagents for sequence analysis were obtained from Applied Biosystems. Dimethylformamide (analytical grade) was redistilled under reduced pressure. Male Sprague-Dawley rats (300 g) were purchased from Simonson Laboratories (Gilroy, CA) and [propionyl-³H]-NPY was from Amersham. Human erythroleukemia (HEL) cells were a generous gift from T. Papayannopoulou (University of Washington) and H. J. Motulsky (University of California, San Diego). Acetone, ethyl acetate, acetic acid, and acetonitrile were of HPLC grade, and other reagents were of analytical grade.

Synthesis of NPY Analogs and NPY Analog Mixtures. NPY analogs and NPY analog mixtures were synthesized manually with *p*-methylbenzhydrylamine resin and *t*-Boc amino acids with conventional side-chain protecting groups, and the mixtures were deprotected and cleaved from the resin by hydrofluoric acid (12). A series of NPY analogs and NPY analog mixtures were also synthesized manually by using an *N*^α-Fmoc protection strategy on a 4-aminomethyl-3,5-dimethylphenoxy resin and were deprotected and cleaved from the resin by treatment with TFA/thioanisole/ethanedithiol/anisole (90:5:3:2) (13).

Synthesis of PYX-1 and PYX-2. Manual solid-phase synthesis of PYX-1 and PYX-2 was carried out with an *N*^α-

Abbreviations: NPY, neuropeptide Y; PYY, peptide YY; TFA, trifluoroacetic acid; *t*-Boc, *t*-butoxycarbonyl; Fmoc, *N*-9-fluorenylmethoxycarbonyl.

[†]To whom reprint requests should be sent at present address: The Institute of Endocrinology, Gunma University, Maebashi, 371 Japan.

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Fmoc protection strategy on a 4-aminomethyl-3,5-dimethylphenoxy resin (13). The side chains of D-threonine and arginine were protected with *t*-butyl and 4-methoxy-2,3,6-trimethylphenylsulfonyl groups, respectively. Ac-3-(2,6-dichlorobenzyl)tyrosine and 3-(2,6-dichlorobenzyl)tyrosine were prepared from Ac-*O*-(2,6-dichlorobenzyl)tyrosine and *O*-(2,6-dichlorobenzyl)tyrosine, respectively, by treatment with 2.5% trifluoromethanesulfonic acid in TFA for 3 hr at 0°C (14) and subsequent purification by HPLC. Fmoc-3-(2,6-dichlorobenzyl)tyrosine was prepared from 3-(2,6-dichlorobenzyl)tyrosine using Fmoc chloride (15). The coupling reaction was done using a 5-fold excess of Fmoc amino acid and diisopropylcarbodiimide in dimethylformamide in the presence of 1-hydroxybenzotriazole. The *N*^α-Fmoc group of the growing peptide was deblocked with 20% piperidine in dimethylformamide for 10 min. The peptides were deprotected and cleaved from the resin by treatment with TFA/thioanisole/ethanedithiol/anisole (90:5:3:2) for 8 hr at room temperature. After filtration, the cleavage reagents were removed under vacuum and the peptide was precipitated by addition of anhydrous ether. The precipitate was redissolved in 0.1 M acetic acid and lyophilized. The crude peptide preparation was purified by using a reverse-phase HPLC column (MCI gel ODS-1HU; 10 × 300 mm; Mitsubishi Kasei, Japan) with a flow rate of 2 ml/min and a linear gradient of solvent A (0.1% TFA in water) and solvent B (70% acetonitrile containing 0.1% TFA). The synthetic peptide preparations obtained were subjected to amino acid analysis and mass spectrometric determinations.

NPY Agonist and Antagonist Assay. NPY agonist activity was detected by measuring peptide-stimulated increases in intracellular calcium concentration in HEL cells according to Motulsky and Michel (16). HEL cells were grown in RPMI 1640 medium (Sigma) supplemented with 2 mM glutamine, 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (0.1 mg/ml) at 37°C with 95% air/5% CO₂. For intracellular calcium measurements, cells were suspended in growth medium with 5 μg of Indo 1-AM per ml (Sigma) and incubated in the dark for 30 min at 37°C. The Indo 1-AM-loaded cells were then centrifuged and resuspended in buffer (120 mM NaCl/20 mM Hepes/5 mM KH₂PO₄/1 mM magnesium acetate/1 mM CaCl₂/1 mg of glucose per ml, pH 7.40) at 10⁶ cells per ml. Intracellular calcium concentration was measured using a fluorometer (SLM) that measured the ratio of emissions at 405 and 485 nm with excitation at 350 nm according to Grynkiewicz *et al.* (17). Increases in intracellular calcium were measured as the difference between baseline and peak calcium concentration. NPY antagonist activity was measured by the addition of the potential antagonist to the Indo 1-AM-loaded cell suspension followed by the immediate addition of NPY. The inhibitory effect of the antagonist is expressed as a percentage of the increase in intracellular calcium concentration with NPY alone.

Receptor Binding Studies. NPY receptor binding was measured by a competitive binding assay with [*propionyl*-³H]-NPY and rat brain membrane preparations according to Chang *et al.* (9).

Structural Analysis. Peptide sequence analysis was performed with a gas-phase protein sequencer (Applied Biosystems) and amino acid analysis was performed with a Beckman 6300 amino acid analyzer after hydrolysis of samples in 5.7 M HCl containing 1.0% phenol at 110°C for 24 hr. Peptide hydrolysates were also reacted with dansyl chloride, and the resulting dansyl amino acids were analyzed by thin-layer chromatography (18). The C-terminal amide assay was carried out by a chemical method (19) that measures the amounts of tyrosine amide released by treatment of samples with trypsin. Fast atom bombardment mass spectrometry was performed with a VG analytical ZAB 2-SE high-field mass spectrometer operating at $V_{acc} = 8$ kV.

RESULTS

Synthesis of NPY Analog Mixtures. Since the C-terminal region of NPY is known to be important for receptor binding (20), a series of NPY analogs, each containing one D-amino acid substitution in the C-terminal region of the NPY molecule, was synthesized. The NPY antagonist assay revealed that none of these analogs exhibited NPY antagonist activity. Some of these analogs, however, were found to be devoid of any NPY agonist activity as well. Since it is anticipated that the presence of agonist activity would seriously interfere with screening for antagonists, the analogs having no NPY agonist activity were selected as a basis for designing analog mixtures containing potential antagonists. Thus, peptide analog mixtures were generated based on the structures of the NPY analogs with one D-amino acid substitution at positions 32–35. A series of mixtures of analogs with several amino acid substitutions was synthesized by dividing the solid-phase resin into several portions at desired positions during synthesis, coupling a different amino acid to each portion, and then recombining the portions for further coupling steps. Mixtures of peptide analogs with various chain lengths were synthesized by withdrawing portions of the resin at desired positions, and then recombining the portions for cleavage from the resin.

Isolation of NPY Receptor Antagonists. The analog mixtures were screened for NPY antagonism by measuring inhibition of the NPY-stimulated release of intracellular calcium in HEL cells. Although some of the analog mixtures were found to exhibit NPY antagonist activity, attempts to isolate NPY antagonists from these mixtures were unsuccessful.

Finally, we decided to screen some of the crude peptide preparations obtained during the syntheses of NPY analogs with one D-amino acid substitution at positions 32–35, since these preparations contained large amounts of NPY analogs as side products. After the screening, an analog mixture was found to contain potent NPY antagonists. This mixture was obtained during an unsuccessful attempt to synthesize [D-Thr³²]NPY. The preparation contained a large amount of analogs because of incomplete couplings that occurred during synthesis with *p*-methylbenzhydrylamine resin and *t*-Boc amino acid derivatives. Cleavage from the resin was accomplished by treatment with hydrogen fluoride. This crude preparation was subjected to HPLC (Fig. 1) and the antagonist-containing fractions were identified by measuring NPY antagonist activity. Two potent NPY receptor antagonists, designated PYX-1 and PYX-2, were isolated from this mixture after three consecutive HPLC steps. Approximately 120 μg of PYX-1 and 30 μg of PYX-2 were obtained as the final products from 200 mg of the crude analog mixture.

Structural Analysis of NPY Receptor Antagonists. Edman degradation of PYX-1 and PYX-2 using a gas-phase protein sequencer yielded no PTH amino acid, indicating that both peptides have a blocked N-terminal residue. Since peptide chains with uncoupled N termini were acetylated during synthesis of the original preparation, the N termini of these peptides were likely to be blocked by acetyl groups. Amino acid analysis indicated that PYX-1 consists of Asp ($n = 1$), Thr ($n = 1$), Glx ($n = 1$), Ile ($n = 2$), Leu ($n = 1$), Tyr ($n = 1$), Arg ($n = 2$), and that PYX-2 consists of Asp ($n = 1$), Thr ($n = 1$), Glx ($n = 1$), Ile ($n = 2$), Leu ($n = 1$), Arg ($n = 2$). The primary structures of both peptides thus correspond to C-terminal fragments of [D-Thr³²]NPY. Thin-layer chromatography of the dansylated peptide hydrolyzates revealed the presence of one modified tyrosine residue in PYX-1 and two identical residues in PYX-2. PYX-2 reacted with the Pauly reagent, indicating that the modified tyrosine residue contains a phenol group. The tyrosine residues were protected by *O*-(2,6-dichlorobenzyl) groups during synthesis of the original preparation. Since these protected tyrosine residues can

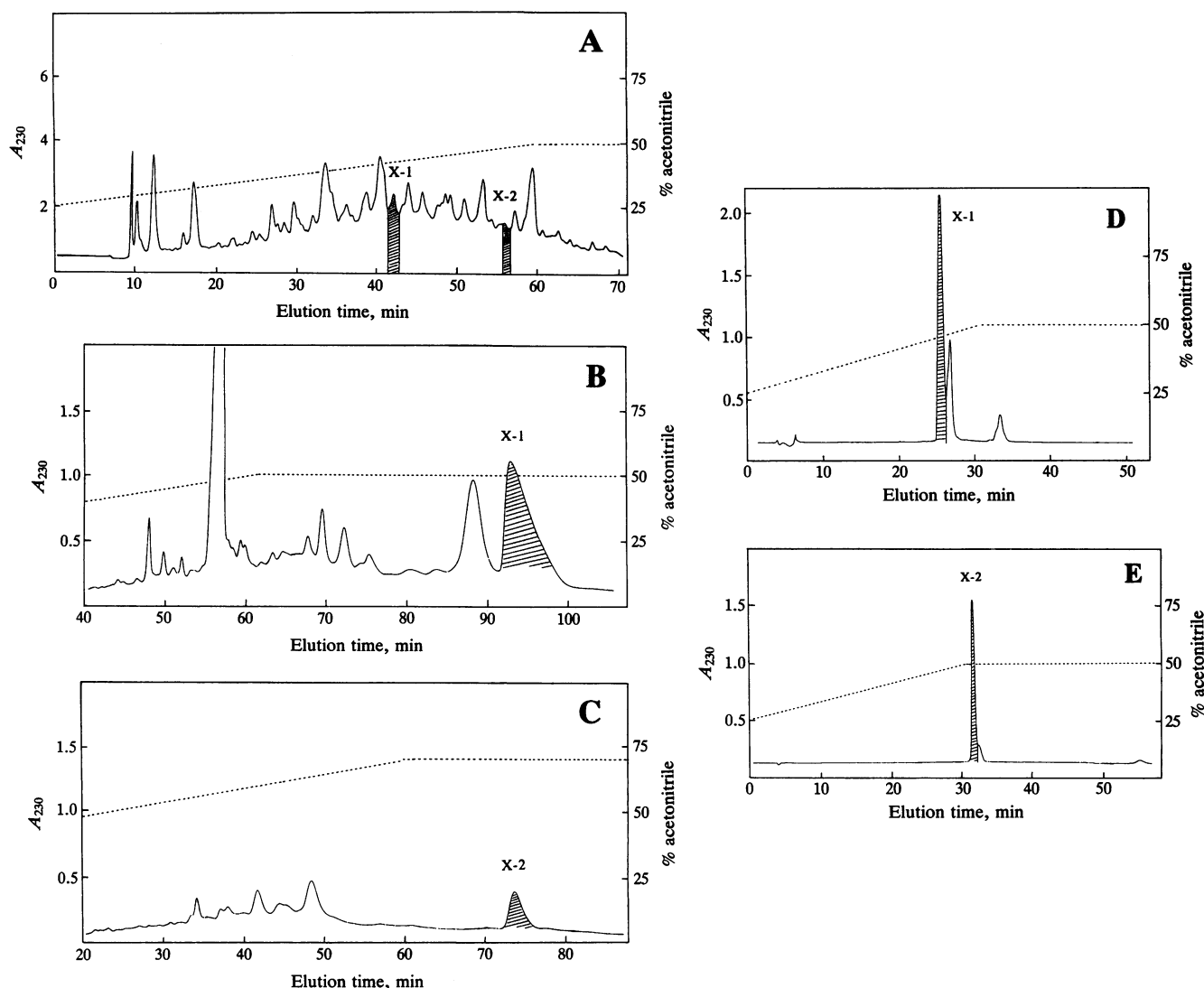


FIG. 1. Reverse-phase HPLC purification of the NPY receptor antagonists from a [D-Thr³²]NPY analog mixture. (A) The synthetic analog mixture (20 mg) was subjected to HPLC by using a MCI gel ODS column (10 × 250 mm; Mitsubishi Kasei) and a linear gradient of acetonitrile in 0.1% TFA at a flow rate of 2.0 ml/min. The antagonist-containing fractions (X-1 and X-2; shaded areas) were identified by measuring the inhibition of NPY-stimulated increases in intracellular calcium concentration in HEL cells. This HPLC was repeated 10 times and the antagonist-containing fractions were combined and evaporated to dryness. (B) Second HPLC purification of X-1 fraction from A. (C) Second HPLC purification of X-2 fraction from A. The second HPLC steps were carried out using the same HPLC column as in the first HPLC with a linear gradient of acetonitrile in 5 mM phosphate buffer (pH 6.5) at a flow rate of 2 ml/min. (D) Third HPLC purification of X-1 fraction from B. (E) Third HPLC purification of X-2 fraction from C. The third HPLC steps were carried out using a Chemcosorb 5-ODS-H column (4.6 × 250 mm; Chemco, Osaka, Japan) and a linear gradient of acetonitrile in 0.1% TFA at a flow rate of 1.0 ml/min.

be rearranged to 3-(2,6-dichlorobenzyl)tyrosine during deprotection with hydrogen fluoride (21), the modified tyrosine is likely to be 3-(2,6-dichlorobenzyl)tyrosine. Degradation of PYX-1 with trypsin yielded a tyrosine amide, but the degradation of PYX-2 did not, indicating that PYX-1 contains a tyrosine amide while PYX-2 contains the modified tyrosine amide at its C terminus. The results of structural analysis therefore predicted that PYX-1 is an N-acetylated C-terminal decapeptide amide of [D-Thr³²]NPY with a 3-(2,6-dichlorobenzyl)tyrosine residue at the N terminus, and that PYX-2 is the decapeptide amide with the same modified tyrosine residues at both the N and C termini. PYX-1 and PYX-2 would thus correspond to Ac-[3-(2,6-dichlorobenzyl)Tyr²⁷,D-Thr³²]NPY-(27–36) amide, and Ac-[3-(2,6-dichlorobenzyl)Tyr^{27,36},D-Thr³²]NPY-(27–36) amide, respectively (Fig. 2).

Fast atom bombardment mass spectrometry indicated quasi-molecular ions at m/z 1538 and 1698 for PYX-1 and PYX-2, respectively, which correspond to the molecular weights of

PYX-1 (1539.6) and PYX-2 (1698.6) as calculated according to the proposed structures.

Solid-phase syntheses of PYX-1 and PYX-2 were carried out based on the proposed structures using Fmoc amino acids. The protected amino acids included 3-(2,6-dichlorobenzyl)tyrosine derivatives that were synthesized from *O*-(2,6-dichlorobenzyl)tyrosine prepared according to Kiso *et al.* (14). The synthetic peptide preparations thus obtained

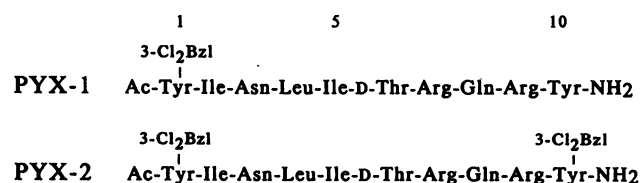


FIG. 2. Structures of NPY receptor antagonists PYX-1 and PYX-2. 3-(Cl₂Bzl), 3-(2,6-dichlorobenzyl); NH₂, amide; Ac, acetyl.

coeluted with PYX-1 and PYX-2 in HPLC and exhibited identical NPY antagonist activity, thereby confirming the proposed structures of the receptor antagonists.

Characterization of NPY Receptor Antagonists. PYX-1 or PYX-2 at 10^{-3} – 10^{-7} M exhibited no NPY agonist activity in releasing intracellular calcium in HEL cells, indicating that both antagonists are devoid of NPY agonist activity. In contrast, these analogs strongly inhibited the action of NPY in HEL cells. PYX-1 at 10^{-6} M and PYX-2 at 10^{-7} M inhibited the release of intracellular calcium elicited by 10^{-7} M NPY or PYY by $\approx 50\%$ (Fig. 3 A and B), indicating that PYX-2 is a more potent antagonist than PYX-1. The results suggest that both PYX-1 and PYX-2 act not only as NPY receptor antagonists but also as PYY receptor antagonists.

To verify the specific binding of these analogs to NPY receptors, the NPY antagonists were subjected to a competitive binding assay with [*propionyl*- 3 H]-NPY using rat brain membrane preparations (9). PYX-2 was found to have an IC_{50} similar to that of NPY or PYY in displacing 3 H-labeled NPY from NPY receptors, while the IC_{50} of PYX-1 was found to be ≈ 10 times higher than that of NPY or PYY (Fig. 3C). These results confirm the specific binding of both PYX-1 and PYX-2 to NPY receptors.

Biological Activity of NPY Analogs Related to PYX-1 and PYX-2. The decapeptides NPY-(27–36) and [D-Thr 32]NPY-(27–36) exhibited no NPY antagonism. Analogs of PYX-1 and PYX-2 in which the 3-(2,6-dichlorobenzyl)tyrosine residues were replaced by *O*-(2,6-dichlorobenzyl)tyrosine failed to exhibit any antagonist activity.

DISCUSSION

Structural analysis of the NPY receptor antagonists suggests that modification of the side chain of the tyrosine residue at position 27, together with a D-substitution of threonine at position 32, converts the C-terminal decapeptide amide of NPY to a receptor antagonist. Analogs structurally closely related to PYX-1 and PYX-2, such as NPY-(27–36), [D-Thr 32]NPY-(27–36), Ac-[*O*-(2,6-dichlorobenzyl)Tyr 27 , D-Thr 32]NPY-(27–36), and Ac-[*O*-(2,6-dichlorobenzyl)Tyr 27,36 , D-Thr 32]NPY-(27–36), exhibited no antagonist activity. Therefore, the 3-(2,6-dichlorobenzyl) group of PYX-1 or PYX-2 at position 27 is important for producing NPY antagonism. The additional 3-(2,6-dichlorobenzyl) group of PYX-2 at position 36 seems to potentiate NPY antagonist activity, since PYX-2 is a more potent NPY antagonist than PYX-1. The presence of hydrophobic 3-(2,6-dichlorobenzyl) groups might allow tighter binding of the decapeptide amides to NPY receptors. These results also suggest that both phenol groups at positions 27 and 36 in the NPY molecule play important roles in receptor binding.

NPY receptors have been classified into two subtypes, Y1 and Y2 receptors (22). Only Y2 receptors bind to C-terminal fragments of NPY (19). Both PYX-1 and PYX-2 bind to the NPY receptors in rat brain membrane, which are predominantly of the Y2 type (23). In view of these observations, PYX-1 and PYX-2, modified C-terminal fragments of NPY, may interact with Y2 receptors. A preliminary study, however, indicated that PYX-2 inhibited the binding of NPY to Y1, as well as Y2, receptors in neuroblastoma cells (C. Wahlestedt, personal communication). The NPY antagonists may therefore interact with both Y1 and Y2 receptors.

Although two nonpeptidergic NPY antagonists have previously been reported, they are not specific antagonists to the NPY receptors. One of them, He 90481, is a potent H2 histamine receptor agonist that also binds to α_2 -adrenoceptors (24). The other, benextramine, is a nonspecific and irreversible antagonist of α_2 -adrenoceptors (25). Recently, the C-terminal octadecapeptide NPY-(18–36) has been shown to be a competitive NPY antagonist in rat cardiac ventricular membrane (26). NPY-(18–36), however, has been

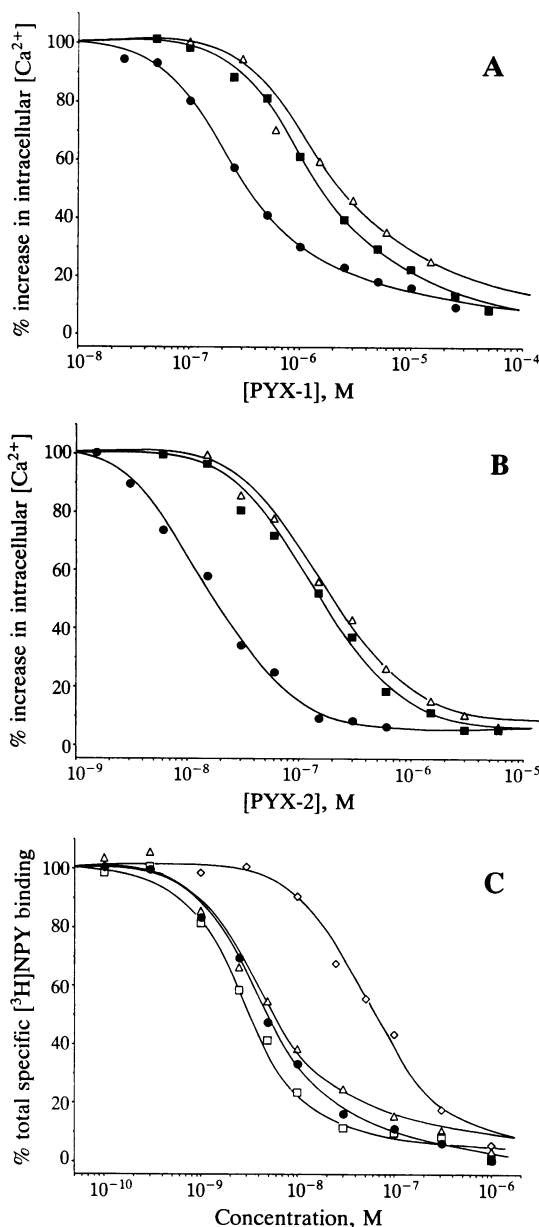


FIG. 3. (A) Inhibitory effects of PYX-1 on NPY- or PYY-stimulated increases in intracellular $[Ca^{2+}]$ in HEL cells. Intracellular calcium concentration was measured by using Indo 1-Am-loaded HEL cells and an SLM fluorimetry system. The increase in intracellular $[Ca^{2+}]$ was measured by the addition of 10 nM NPY (\bullet), 100 nM NPY (\blacksquare), and 100 nM PYY (Δ) in the presence of various concentrations of PYX-1. Inhibition is shown as a percentage of the increase in the absence of antagonist. (B) Inhibitory effects of PYX-2 on NPY- or PYY-stimulated increases in intracellular $[Ca^{2+}]$ in HEL cells. The increase in intracellular $[Ca^{2+}]$ was measured by addition of 10 nM NPY (\bullet), 100 nM NPY (\blacksquare), and 100 nM PYY (Δ) in the presence of various concentrations of PYX-2. Other conditions are as described in A. (C) Binding of PYX-1 and PYX-2 to NPY receptors in rat brain membrane. Rat brain membrane preparations were incubated with various concentrations of NPY (\bullet), PYY (\square), PYX-1 (\diamond), or PYX-2 (Δ) in the presence of 100 pM [*propionyl*- 3 H]-NPY. Competitive displacement of [*propionyl*- 3 H]-NPY is shown as a percentage of total specific binding.

known to exhibit NPY agonist activity in reducing cardiac output (27). Furthermore, a similar analog, NPY-(19–36) exhibits no NPY antagonistic effect in the Y1/Y2 receptor systems (20). It has therefore been suggested that NPY-(18–36) acts as a specific antagonist to a newly discovered class

of NPY receptors, Y3, which may be present in the cardiac ventricular system (26).

The NPY receptor subtype of HEL cells has not previously been characterized. The receptors interact not only with NPY but with PYY as well. The NPY antagonists were capable of blocking the actions of both NPY and PYY in releasing intracellular calcium in the cells. They were also able to block the binding of PYY to the NPY receptors in rat brain membrane. Since the structure of the C-terminal decapeptide of PYY is very similar to that of the corresponding fragment of NPY (2), both PYX-1 and PYX-2 may act as PYY receptor antagonists as well.

Although we were unsuccessful in our first attempt to isolate useful compounds from analog mixtures designed to contain NPY receptor antagonists, further development of the design and synthesis of such mixtures may allow identification of other potent NPY antagonists. Since the mixture screening strategy allows us to search for antagonists in a wide variety of analog sources, we decided to screen side products in synthetic NPY analog preparations. The NPY receptor antagonists PYX-1 and PYX-2 were isolated from a crude peptide preparation that was produced during an attempt to synthesize [D-Thr³²]NPY. HPLC analysis indicated that the preparation contained hundreds of NPY analogs as side products, two of which turned out to be potent NPY receptor antagonists. PYX-1 and PYX-2 represented <0.1% of the total weight of the preparation, demonstrating the viability of searching for useful analogs in such a synthetic mixture.

The unusual tyrosine structure of PYX-1 and PYX-2 seems to play an important role in producing antagonist activity, and the usefulness of such a structure would have been difficult to predict by using traditional approaches of antagonist design. This finding underscores one of the distinct advantages of using such an analog mixture screening strategy; namely, that useful analogs with unexpected structures can be identified. The approach of screening and isolating useful analogs in synthetic analog mixtures may be applied not only to the identification of receptor antagonists but also to the identification of agonists of peptides and other bioactive compounds.

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