0026-895X/95/030425-08$3.00/0

MOLECULAR PHARMACOLOGY, 48:425–432 (1995).

Structure-Activity Relationship of Novel Pentapeptide Neuropeptide Y Receptor Antagonists Is Consistent with a Noncontinuous Epitope for Ligand-Receptor Binding

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Received March 28, 1995; Accepted June 20, 1995

SUMMARY

We report the first systematic study on short peptide structure affinity and activity for the neuropeptide Y (NPY) receptor. A series of linear pentapeptides has been synthesized that display affinities in the low micromolar range toward rat brain NPY receptors. Furthermore, some of these compounds competitively antagonize the Y_1-type NPY receptor-mediated increase in cytosolic Ca^{2+} in human erythroleukemic (HEL) cells. The inactive NPY carboxyl-terminal pentapeptide (Thr-Arg-Gln-Arg-Tyr-NH_2; $IC_{50} > 100$ μM) was modified by replacing threonine with an aromatic amino acid and glutamine with leucine. This resulted in a series of pentapepides with dramatically improved affinity (IC_{50} = 0.5–4 μM) for the rat brain receptor. The structure-affinity data suggest that these peptides may represent a noncontinuous epitope containing the amino-terminal tyrosine and the carboxyl-terminal residues Arg-35 and Tyr-36 of NPY.

NPY is a linear 36-amino acid peptide amide (1, 2) found in both the peripheral and central nervous systems of mammals. In the periphery, NPY is costored with norepinephrine in sympathetic terminals and is released on nervous stimulation, causing a marked vasoconstriction both directly and by potentiation of other vasoactive substances (3, 4). In the central nervous system, NPY stimulates food intake (5) and modulates release of leutinizing hormone-releasing hormone (6). In addition, compelling evidence suggests that NPY plays an important role in modulating anxiety (7). The activity of NPY is mediated by at least two subtypes of NPY-specific G protein-coupled receptors, designated Y_1 and Y_2 (8–11). The existence of a third receptor subtype, Y_3, has been proposed (12, 13).

Wahlestedt *et al.* (8, 9) characterized pharmacologically the two receptor subtypes, Y_1 and Y_2, on the basis of their affinity for NPY and the amino-terminal truncated fragment, NPY(13–36). Optimal binding to Y_1 receptors required full-length NPY, whereas Y_2 receptors recognized NPY(13–36) with high affinity. More recently, the Y_1-selective agonist [Leu-31,Pro-34]NPY was described (14). On the basis of the relative binding affinity of these analogs, particular tissues or cell lines have subsequently been characterized as containing NPY receptors of the Y_1 or Y_2 type.

NPY is a member of the PP family of peptide hormones (15, 16). A high-resolution X-ray analysis of one member of this family, aPP, was reported by Blundell *et al.* (17, 18), who proposed a tertiary structure, referred to as the PP-fold, for the PP family. The PP-fold consists of a polyproline-like helix (residues 1–8) that is closely packed against an amphiphilic α-helix (residues 13–32). The two antiparallel helices are linked by a β turn (residues 9–12), whereas the carboxyl-terminal region (residues 33–36) adopts no regular structure and extends away from the molecule. Residues considered to be essential for maintaining the integrity of the PP-fold are also found in analogous positions in NPY, suggesting that the neuropeptide adopts a similar secondary structure motif (15, 19). Using the X-ray coordinates for aPP, Allen *et al.* reported a computer-generated three-dimensional model of NPY in which the PP-fold is maintained (20). More recently, solution structures of NPY (21) and bovine pancreatic polypeptide (22) were determined by NMR, and both were shown to contain the PP-fold motif present in the crystal structure of aPP.

The abbreviations for the amino acids are in accord with the recommendations of the International Union of Pure and Applied Chemistry/International Union of Biochemistry Joint Commission on Biochemical Nomenclature [*Eur. J. Biochem.* 138:9–37 (1984)]. The symbols represent the L-isomer except where indicated otherwise.

ABBREVIATIONS: NPY, neuropeptide Y; BOC, *tert*-butyloxycarbonyl; BOP (benzotriazol-1-yloxy)tri(dimethylamino)-phosphonium hexafluorophosphate; aPP, avian pancreatic polypeptide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HEL, human erythroleukemic; BSA, bovine serum albumin; IS-MS, ion-spray mass spectrometry; HPLC, high performance liquid chromatography.

Much research has been focused on understanding which regions of NPY are essential for optimal affinity, biological activity, and receptor-subtype selectivity. Most of the previous NPY structure-activity studies have focused on relatively large peptide fragments (>15 amino acids), which do not display significant antagonistic activity. Recently, however, NPY antagonists based on the nonpeptide benextramine have been described (23). Our strategy toward designing nonpeptide antagonists of NPY focused on examining a series of short peptides to determine minimal structural requirements for receptor binding. We describe a series of pentapeptides with low micromolar affinity for rat brain Y_2 receptors and antagonistic activity in a Y_1 receptor-mediated cell assay.

Materials and Methods

Reagents and solvents. All solvents were analytical grade (Aldrich Chemical Co., Milwaukee, WI; Fisher Scientific, Springfield, NJ) and were used without further purification. The Aldrich Chemical Co. supplied 1-methylimidazole and trifluoroacetic acid. The BOP reagent was purchased from Richelieu Biotechnologies (St. Hyacinthe, Quebec, Canada). BOC-protected amino acids were obtained from Bachem Chemical Co. (Torrance, CA). *p*-Hydroxyphenylpropionic acid (des-NH_2-Tyr) was purchased from Fluka Chemical Corp. (Ronkonkoma, NY). Phe-Met-Arg-Phe-NH_2 was obtained from Peninsula Laboratories (Belmont, CA). 4-Methyl-benzhydrylamine resin was obtained from Advanced Chemtech (Louisville, KY). Porcine NPY was purchased from Dr. D. Klapper (University of North Carolina at Chapel Hill) and further purified with reverse-phase HPLC as previously described (24). [^{3}H]Propionyl NPY (specific activity, 70 Ci/mmol) was obtained from Amersham (Arlington Heights, IL).

Peptide synthesis. Peptides were prepared according to a modified version of the Merrifield (25) solid-phase method on a 4-methylbenzhydrylamine resin using the BOC protection strategy. BOC-protected amino acids were attached to the resin with a BOP coupling procedure (26) in dimenthylformamide in the presence of 1-methylimidazole. This procedure was performed automatically with an Applied Biosystems Model 430A peptide synthesizer (Foster City, CA). After the desired peptide was assembled, the peptide resin was cleaved with liquid anhydrous hydrogen fluoride in a variation of the method described by Sakakibara *et al.* (27). The crude peptides were precipitated with ethyl acetate, extracted with aqueous 1% acetic acid, lyophyllized, and purified by preparative reverse-phase HPLC. Some of the peptides were custom-synthesized by Multiple Peptide Systems (San Diego, CA).

Peptide characterization. Purified peptides (≥ 97% by HPLC) were subjected to HPLC analysis, ion-spray mass spectrometry, or fast atom bombardment mass spectrometry and amino acid analysis as previously described (28). None of the peptides described in Table 1 showed significant impurities based on any of these characterization techniques.

Molecular modeling. A protocol for generating a structural model of NPY has been described in the literature (20, 29). We used an analogous procedure with MacroModel Version 2.5 software (30). The heavy atom aPP crystallographic coordinates at 1.4 Å resolution were extracted from the Brookhaven Protein Data Bank. Each nonhomologous residue was sequentially replaced with the corresponding residue for NPY and subjected to a localized energy minimization using the torsional search option. The conformation obtained for the resulting NPY model was used, without further modification, solely as a qualitative guide and visual aid to assist in our small peptide design.

[^{3}H]NPY rat brain binding assay. Rat brain membranes were prepared following a modified procedure of Chang *et al.* (31). Male Sprague-Dawley rats (250–300 g) were killed by decapitation, and the brains (minus cerebellum) were rapidly removed, placed on ice, and homogenized (1:10, w/v) in 0.32 M sucrose with a Brinkman Polytron. The homogenate was centrifuged at 4300 × *g* (Sorvall, SA-600 rotor) for 10 min at 4°, and the supernatants were decanted and placed on ice. The pellets were rehomogenized and centrifuged again. The combined supernantants were then centrifuged at 30,000 × *g* for 30 min, and the pellets were again suspended by homogenization (Polytron) in 12 volumes of 5 mM Tris buffer, pH 7.4, and

TABLE 1

NPY receptor binding data for NPY fragments and small peptides

Comparative potencies of NPY, NPY fragments, and novel pentapeptides in displacing [^{3}H]NPY from rat brain membranes. Membranes were incubated with 0.2 nM [^{3}H]NPY ($\leq K_d$) for 60 min at 37° in the presence of increasing concentrations of the test peptides. Results are presented as the concentration of peptide that produces a 50% displacement (IC_{50}) of specifically bound [^{3}H]NPY.

Compound	Structure	IC_{50}
		μM
1	NPY(1–36)	0.00045 ± 0.000055
2	NPY(1–20)	>100
3	NPY(2–36)	0.0037 ± 0.0013
4	NPY(13–36)	0.015 ± 0.002
5	NPY(20–36)	0.077 ± 0.012
6	NPY(25–36)	1.26 ± 0.4
7	NPY(26–36)	2.5 ± 0.6
8	NPY(27–36)	30 ± 7
9	NPY(32–36)	>100
10	[D-Tyr^1]NPY(2–36)	0.0015 ± 0.001
11	[His^1]NPY(2–36)	0.0015 ± 0.003
12	Tyr-Arg-Met-Arg-Tyr-NH_2	3.5 ± 0.3
13	Tyr-Arg-Leu-Arg-Tyr-NH_2	3.8 ± 0.6
14	Tyr-Arg-Nle-Arg-Tyr-NH_2	3.6 ± 0.3
15	Tyr-Arg-Gln-Arg-Tyr-NH_2	71 ± 4.5
16	Tyr-Arg-Val-Arg-Tyr-NH_2	35.1 ± 2.3
17	Tyr-Arg-Pro-Arg-Tyr-NH_2	34.5 ± 1.8
18	Tyr-Arg-His-Arg-Tyr-NH_2	29.7 ± 4.3
19	D-Tyr-Arg-Leu-Arg-Tyr-NH_2	1.2 ± 0.18
20	D-Tyr-Arg-Gly-Arg-Tyr-NH_2	33.1 ± 3.1
21	D-Tyr-Arg-Ser-Arg-Tyr-NH_2	21.1 ± 2.7
22	D-Tyr-Arg-Thr-Arg-Tyr-NH_2	32.3 ± 4.1
23	D-Tyr-Arg-Ile-Arg-Tyr-NH_2	13.1 ± 0.5
24	D-Tyr-Arg-Ala-Arg-Tyr-NH_2	35.3 ± 2.9
25	D-Tyr-Arg-Phe-Arg-Tyr-NH_2	5.8 ± 1.6
26	D-Tyr-Arg-Met-Arg-Tyr-NH_2	1.9 ± 0.7
27	D-Tyr-Arg-Asp-Arg-Tyr-NH_2	>100
28	D-Tyr-Arg-Glu-Arg-Tyr-NH_2	>100
29	Trp-Arg-Leu-Arg-Tyr-NH_2	2.5 ± 0.27
30	His-Arg-Leu-Arg-Tyr-NH_2	0.45 ± 0.05
31	D-His-Arg-Leu-Arg-Tyr-NH_2	2.9 ± 0.18
32	Thr-Arg-Leu-Arg-Tyr-NH_2	17.7 ± 1.9
33	Tyr-Arg-Met-Arg-Phe-NH_2	5.3 ± 2.2
34	Tyr-Arg-Leu-Arg-His-NH_2	98 ± 6
35	D-Tyr-Arg-Leu-Arg-Trp-NH_2	29.2 ± 3.8
36	D-Tyr-Arg-Leu-Arg-His-NH_2	94 ± 4.2
37	D-Tyr-Arg-Leu-Arg-Pro-NH_2	>100
38	Tyr-Arg-Leu-Lys-Tyr-NH_2	31.8 ± 1.6
39	Tyr-Lys-Leu-Arg-Tyr-NH_2	3.2 ± 0.6
40	Tyr-Lys-Leu-Lys-Tyr-NH_2	93 ± 3.3
41	Tyr-Gly-Met-Arg-Tyr-NH_2	>100
42	Tyr-Phe-Met-Arg-Tyr-NH_2	6.8 ± 0.5
43	His-His-Leu-Arg-Tyr-NH_2	2.9 ± 0.15
44	D-Tyr-His-Leu-Arg-Tyr-NH_2	3.5 ± 0.5
45	D-Tyr-Tyr-Leu-Arg-Tyr-NH_2	0.53 ± 0.1
46	D-Tyr-Arg-Leu-D-Arg-Tyr-NH_2	2.5 ± 0.4
47	D-Tyr-D-Arg-Leu-Arg-Tyr-NH_2	5.9 ± 1.1
48	His-D-Arg-Leu-Arg-Tyr-NH_2	0.6 ± 0.17
49	His-Arg-Leu-Arg-D-Tyr-NH_2	9.2 ± 0.8
50	Ac-Tyr-Arg-Leu-Arg-Tyr-NH_2	4.8 ± 0.2
51	Ac-D-Tyr-Arg-Leu-Arg-Tyr-NH_2	11.4 ± 0.4
52	des-NH_2-Tyr-Arg-Leu-Arg-Tyr-NH_2	1.9 ± 0.5
53	Phe-Met-Arg-Phe-NH_2	40.0 ± 3

allowed to stand on ice for 30 min. The suspension was then centrifuged at 30,000 × *g* for 15 min, the supernatants were discarded, and the pellets were resuspended and centrifuged again. The pellets were homogenized in 12 volumes of 50 mM Tris containing 0.1 M NaCl and allowed to stand for 1 hr on ice. The suspension was then centrifuged at 30,000 × *g* for 15 min, and the pellets were homogenized in 12 volumes of 50 mM Tris. The latter procedure was repeated once, and the final pellet was resuspended (1.5 vol/mg original tissue) in 50 mM Tris and stored in frozen aliquots at −70° until required for binding assay. Proteins were determined by the BCA method (32) with BSA as a standard.

Binding assays were performed in 200 μl of brain membranes (0.075 mg protein) incubated for 1 hr at 37° in 500 μl of 50 mM Tris buffer, pH 7.4, containing 0.05% BSA, 5 mM $CaCl_2$, 2 mM $MgCl_2$, 0.22% bacitracin, 0.4% leupeptin, and porcine [^{3}H]propionyl NPY at concentrations ranging from 0.1 to 1.5 nM. To verify the presence of more than one NPY binding site, we extended the concentration range of the ligand from 0.005 to 1.0 nM by using ^{125}I-NPY (Amersham, specific activity, 2000 Ci/mmol). Specific binding was defined as that displaced by excess (100 nM) unlabeled NPY and represented 65% of the total binding at a ligand concentration of 0.2 nM. All conditions were tested in triplicate. Displacement curves were obtained by incubation of various dilutions of the test peptides (dissolved in 50 mM Tris buffer containing 0.5% BSA) in the presence of 0.2 nM [^{3}H]NPY under the same experimental conditions as described. Incubations were terminated by the addition of 500 μl of ice-cold 50 mM Tris buffer (containing 0.02% BSA, 2.5 mM $CaCl_2$, and 2 mM $MgCl_2$) followed by centrifugation at 14,000 × *g* for 10 min in a Sorvall refrigerated centrifuge (SH-MT rotor). The supernatants were carefully aspirated, and the pellets were dissolved in 200 μl of 1 N NaOH containing 0.02% Triton X-100. Aliquots of the pellet suspension (in 5 ml of Optifluor) were analyzed for radioactivity content by liquid scintillation (Beckman Model LS5801). Binding kinetic parameters, K_d, B_{max}, and IC_{50} were calculated using the Lundon ReceptorFit Saturation Two-Site software.

Intracellular calcium measurements. Cytosolic calcium was measured as previously described by Daniels *et al.* (24). Briefly, HEL cells were centrifuged and resuspended in a buffer containing 120 mM NaCl, 20 mM HEPES, 5 mM KCl, 1 mM $Mg(OAc)_2$, 1 mM $CaCl_2$, and 1 mg/ml glucose, pH 7.4, and were loaded with Fura-2 acetoxymethylester (1 μM) by incubation for 1 hr at room temperature. Then, cells were washed by centrifugation and resuspended in fresh buffer at 10^6 cells/ml. Fluorescence was measured at room temperature with an SLM AMINCO DMX-1000 spectrofluorometer, with dual excitation at 340 and 380 nm and emission at 510 nm using 0.5 ml of cell suspension and buffer up to a total volume of 2.5 ml and constant stirring. Compounds were added to the media 30 sec before stimulation with NPY, and results were recorded as the ratio of the fluorescence at 340 nm to that at 380 nm. Inhibitory activity was expressed as IC_{50} (the concentration that produces 50% inhibition of the response to a half-maximal dose of NPY).

Results

Displacement of [^{3}H]NPY from rat brain membranes. Fig. 1A shows a typical saturation isotherm for the binding of [^{3}H]NPY to rat brain membranes. The nonlinear regression analysis of the data from 10 different experiments, using the Lundon ReceptorFit Saturation Two-Site software is shown in Fig. 1B. The data best fits a single binding site model with an apparent homogeneous population of receptors with a K_d of 0.29 ± 0.04 nM and a B_{max} of 281 ± 13 fmol/mg protein (mean ± standard error of 10 experiments). Identical kinetic parameters were found when we used ^{125}I-NPY as a ligand and extended the range of concentrations an additional 20-fold down to 0.005 nM. Analysis of the data under the latter conditions also indicated the presence of a single binding site. The IC_{50} values for NPY and compounds that were obtained from the displacement curves of specifically bound [^{3}H]NPY were calculated by nonlinear regression analysis (Hill plot) of the data. The Hill slope for the NPY displacement curve was also consistent with a single binding site (n_H = 0.99 ± 0.01; 10 experiments). The results of the displacement of [^{3}H]NPY by fragments of NPY and a series of small peptides are shown in Table 1 and Fig. 3.

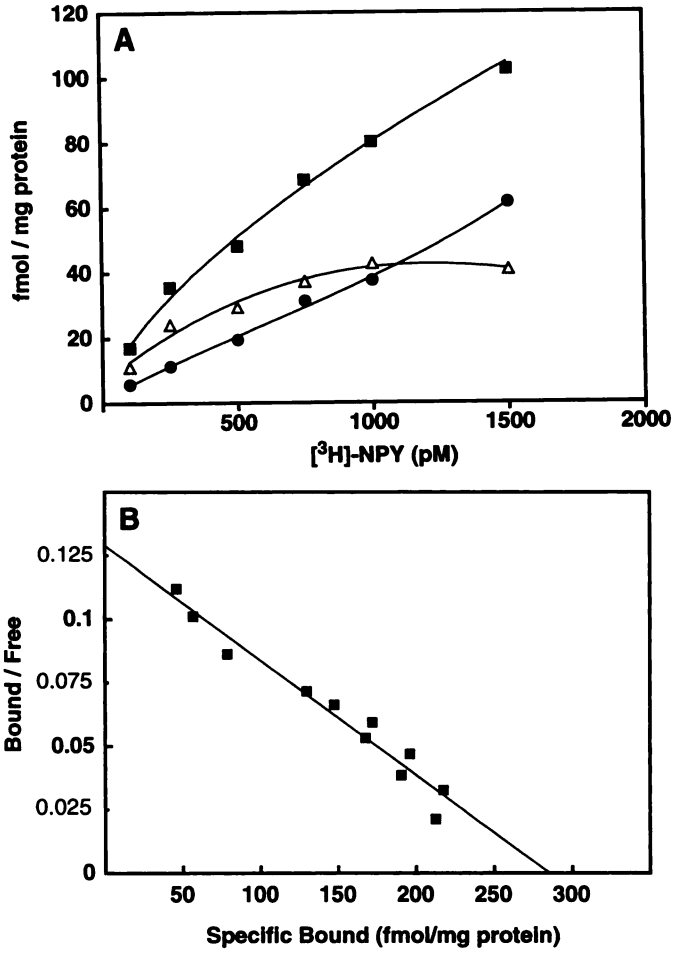

Fig. 1. Saturation isotherm of [^{3}H]propionyl NPY binding to rat brain membranes. A, Binding of [^{3}H]NPY to brain membranes incubated with increasing concentrations of the radiolabeled peptide. Shown are botal binding (■), nonspecific binding (●), and specific binding (△, the difference between total and nonspecific binding). B, Scatchard analysis of the saturation experiments. Results represent a typical saturation experiment repeated with different membrane preparations (more than 10 experiments); K_d = 0.29 ± 0.04 nM, B_{max} = 281 ± 13 fmol/mg protein).

The initial indication that small peptides could bind to rat brain NPY receptors was based on our observation that the commercially available cardioactive tetrapeptide Phe-Met-Arg-Phe-NH_2 interacted with NPY receptors, albeit weakly (Table 1, compound 53, IC_{50} = 40 μM). Compound 53 may represent an active conformation of the carboxyl-terminal fragment of NPY (Thr-Arg-Gln-Arg-Tyr-NH_2; compound 9), suggesting that the carboxyl-terminal region alone is sufficient for binding to brain receptors. Alternatively, Phe-Met-Arg-Phe-NH_2 may correspond to a composite of the amino- and carboxyl-terminal residues of NPY, as is suggested by visual inspection of a model of NPY (20, 29) generated from

the published X-ray structure of aPP (Fig. 2). This latter observation led to the preparation of Tyr-Arg-Gln-Arg-Tyr-NH_2 (compound 15), which corresponds to the NPY amino-terminal Tyr and the carboxyl-terminal sequence NPY (33–36). Compound 15 displayed binding (IC_{50} = 71 μM) comparable to that of 53. However, when glutamine was replaced by methionine (compound 12), a 20-fold increase in affinity was obtained. Substitution of Met-3 by leucine or norleucine (Nle), compounds 13 and 14, afforded peptides with similar affinity to that of compound 12.

Subsequently, we used Tyr-Arg-Leu-Arg-Tyr-NH_2 (compound 13) as the reference peptide to examine the effect of substitutions at position 1 or 5. Replacement of the amino-terminal tyrosine by D-tyrosine or histidine resulted in a 3–8-fold increase in affinity (compounds 19 and 30). Other aromatic replacements at the amino terminus (compound 29) were better tolerated than aliphatic residues (compound 32). Deamination (compound 52) or *N*-acetylation (compound 50) did not decrease the affinity, but *N*-acetylation (compound 51) of the D-tyrosine-substituted analogue (compound 19) reduced the affinity by 10-fold. Substitution of the carboxyl-terminal tyrosine by either aromatic (except phenylalanine, compound 33) or aliphatic amino acids uniformly afforded analogues with significantly reduced affinity (compounds 34-37).

Using the more potent analogue D-Tyr-Arg-Leu-Arg-Tyr-NH_2 (compound 19) as a reference, we reexamined the effect of substitutions at position 3. This series (compounds 20-28) revealed that with the exception of the phenylalanine and methionine analogues (compounds 25 and 26), all other replacements significantly reduced affinity.

The relative importance of the two arginine residues in this series was determined by varying the position, nature, and number of positively charged amino acids. Replacement of Arg-4 in 13 with lysine (compound 38) reduced binding 8-fold; however, no change in affinity was found for the correspond-

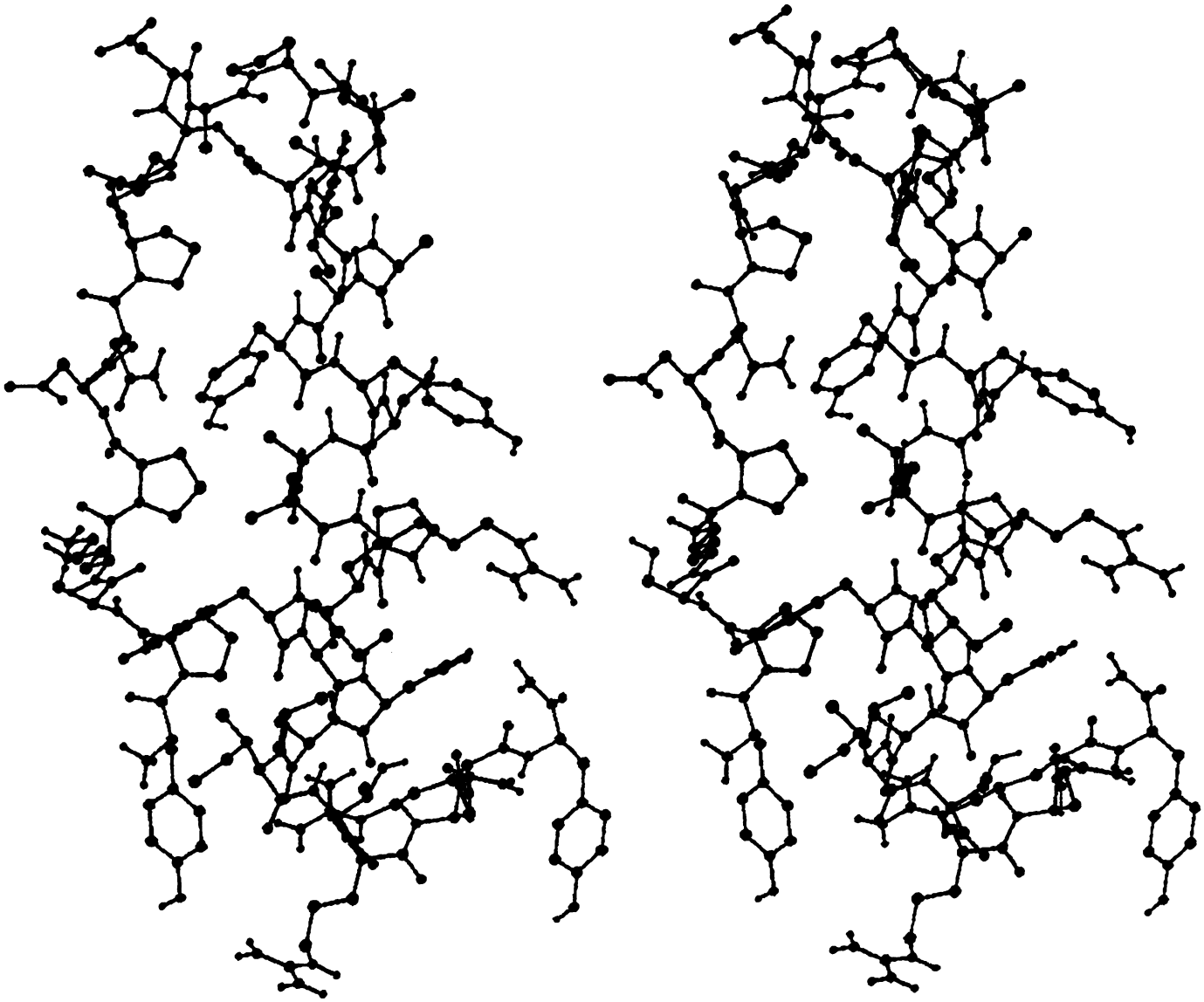

Fig. 2. Stereo view of a computer-derived model of porcine NPY based on the crystal structure of aPP (17, 18). Tyr-1, Arg-35, and Tyr-36 are blue. NPY sequence: Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Leu-Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-NH_2.

ing Lys-2 analogue (compound 39). Substitution of both arginine residues with lysine (compound 40) markedly weakened binding. Replacement of Arg-2 with histidine in either the His-1 or D-Tyr-1 series (compounds 43 and 44) lowered affinity 3–6-fold. Analogues in which Arg-2 was replaced with a hydrophobic bulky neutral residue such as tyrosine (compound 45) maintained binding, but the glycine analogue (compound 41) showed poor affinity.

The effect of substitutions by the corresponding D-amino acids was also examined (compounds 19, 31, and 46–49). No clear trends emerged from this series, although the tolerance for D-amino acids diminished somewhat from the amino to the carboxyl terminus. Several laboratories have shown that D-amino acid substitution in large (33) and centrally truncated fragments of NPY weakens but often does not eliminate bioactivity, particularly at the carboxyl-terminal tyrosine (34).

The displacement of [^{3}H]NPY from rat brain membranes by NPY, NPY(13–36), the most potent pentapeptides, and compound 9 (the inactive carboxyl-terminal fragment of NPY) is shown in Fig. 3.

Intracellular Ca^{2+} responses to NPY and pentapeptides in HEL cells. We previously demonstrated that HEL cells, in response to NPY, mobilize intracellular Ca^{2+} through activation of a Y_1-type of receptor coupled to phospholipase C (24). Some of the pentapeptides that displayed the highest affinity at the brain receptor were assayed in this functional test to assess whether they were NPY agonists or antagonists.

Fig. 4A (*C1*) shows a typical increase in cytosolic Ca^{2+} in HEL cells in response to NPY. Compound 30, at 1 μM, did not induce cytosolic calcium increase on its own; however, it inhibited by 50% the response to a subsequent addition of NPY (Fig. 4A, *C2*). The dose-response curves for the NPY-induced Ca^{2+} mobilization in the presence or absence of compound 30 indicates that this pentapeptide is a competitive antagonist of NPY in HEL cells (Fig. 4B). Table 2 shows that His-Arg-Leu-Arg-Tyr-NH_2 (compound 30) was the most potent compound evaluated in this assay. Compounds that displayed low affinity for the brain receptor (16, 17) were inactive.

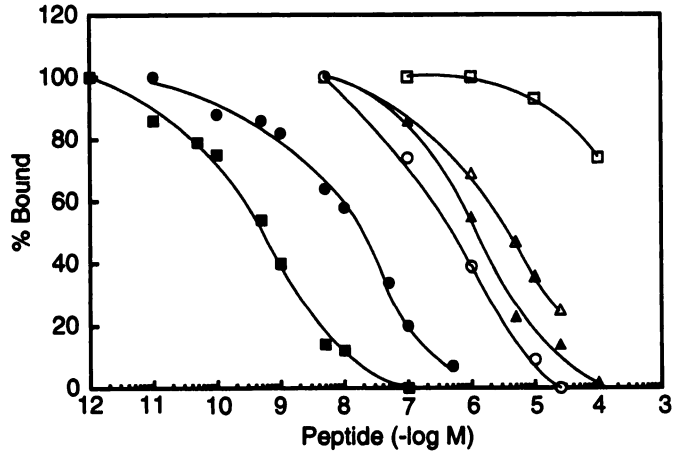

Fig. 3. Inhibition isotherms for the specific binding of [^{3}H]NPY to rat brain membranes as a function of competitor concentration. Amount of [^{3}H]NPY bound at each concentration of competitor is expressed as a percentage of [^{3}H]NPY specifically bound in the absence of competitor. ■, NPY (compound 1); ●, NPY(13–36) (compound 4); ○, His-Arg-Leu-Arg-Tyr-NH_2 (compound 30); ▲, D-Tyr-Arg-Leu-Arg-Tyr-NH_2 (compound 19); △, Tyr-Arg-Leu-Arg-Tyr-NH_2 (compound 13); and □, Thr-Arg-Gln-Arg-Tyr-NH_2 (compound 9).

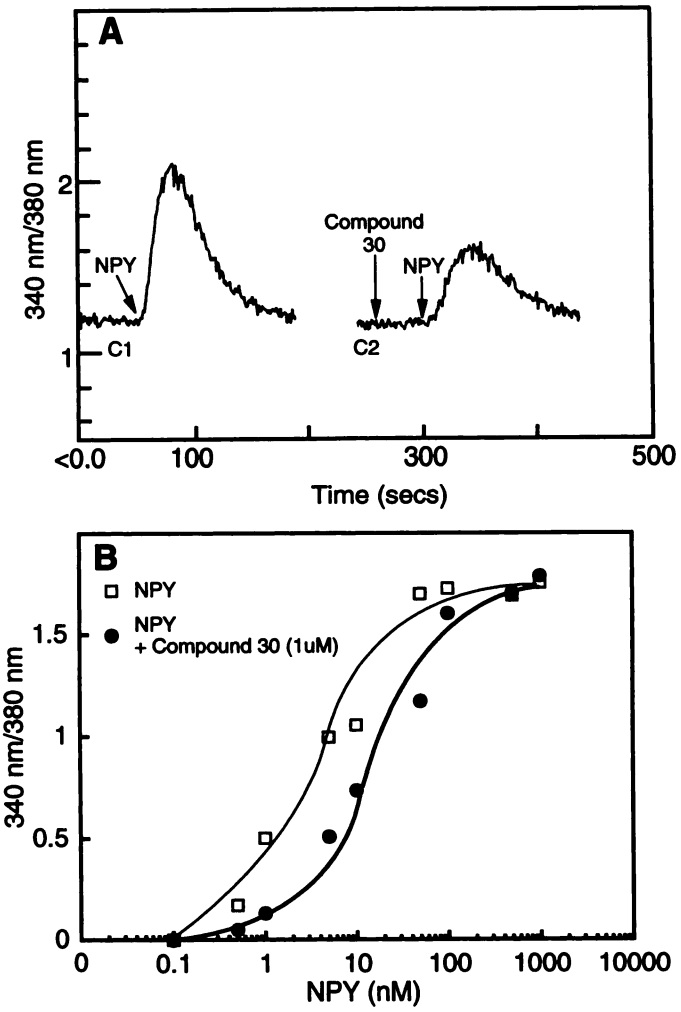

Fig. 4. Characterization of the inhibitory effect of His-Arg-Leu-Arg-Tyr-NH_2 (compound 30) on the NPY-induced increase of cytosolic Ca^{2+} in HEL cells. A, Cytosolic Ca^{2+} increase in response to a half-maximal concentration (5 nM) of NPY (*C1*) and cytosolic Ca^{2+} response of 5 nM NPY 30 sec after the addition of 1 μM His-Arg-Leu-Arg-Tyr-NH_2 to the medium (*C2*). B, Dose-response curves for the NPY-induced increase in cytosolic Ca^{2+} in the presence (●) and absence (□) of 1 μM His-Arg-Leu-Arg-Tyr-NH_2. Inhibitor was added to the medium 30 sec before stimulation with NPY. Results are presented as the 340/380 nm fluorescence ratio and represent a typical experiment repeated with different cell preparations and with similar results.

Discussion

The precise molecular determinants that govern NPY binding to its receptors are still unclear, although some general principles have emerged from extensive structure-activity and -affinity studies on NPY and large NPY fragments (9, 15). For example, both the amino- and carboxyl-terminal amino acids of NPY are essential for strong receptor binding and activity. These observations are consistent with the published NPY model in which these regions are held in close proximity. In native NPY, this proximity is enforced by the PP-fold, which aligns the proposed binding pharmacophore, amino acids located in the carboxyl- and amino-termini, into

TABLE 2

Inhibition of NPY-induced increase in cytosolic Ca^{2+} in HEL cells

Results are expressed as IC_{50} of the increase in the 340/380-nm fluorescence ratio in Fura-2-loaded cells induced by a half-maximal concentration (5 nM) of NPY.

Compound	Structure	IC_{50}
		μM
30	His-Arg-Leu-Arg-Tyr-NH_2	0.65 ± 0.12
19	D-Tyr-Arg-Leu-Arg-Tyr-NH_2	1.5 ± 0.2
13	Tyr-Arg-Leu-Arg-Tyr-NH_2	>10
12	Tyr-Arg-Met-Arg-Tyr-NH_2	5.4 ± 0.5
16	Tyr-Arg-Val-Arg-Tyr-NH_2	>10
17	Tyr-Arg-Pro-Arg-Tyr-NH_2	>10

the correct spatial orientation for presentation to the receptor (15, 19). Beck *et al.* (35) and Krstenansky *et al.* (36) demonstrated that a formal PP-fold is not required for high bioactivity. Both laboratories have shown that large central segments of NPY can be deleted, provided that the remaining amino- and carboxyl-terminal regions are linked by appropriate flexible nonpeptide spacers, disulfide constraints, or both.

Systematic substitution by the amino acids glycine and alanine in both NPY and analogues linking amino- and carboxyl-terminal fragments (37–41) consistently reveals the importance of residues 33–36. Earlier studies showed a significant loss of affinity and bioactivity when either the amino- (40, 42) or carboxyl- (33, 43) terminal tyrosine was modified or deleted. The absolute requirement for carboxyl-terminal amidation has also been demonstrated (9, 44, 45).

Based on our observations of structure and affinity to the rat brain receptor for the series of pentapeptides described in the present report, we conclude the following.

First, a wide range of aromatic but not aliphatic amino acid residues can be accommodated at the amino-terminus. Neither the amino-terminal amino group (blocked or deleted), specific aryl substituents (e.g., a tyrosine hydroxyl), nor specific amino acid stereochemistry is required. These structure-affinity relations deduced from the various pentapetides of this study are also observed with the full-length NPY analogues 10 and 11, where replacement of Tyr-1 with histidine or D-tyrosine had minimal affect on affinity. Overall, the structure-affinity pattern found for the pentapeptide series is in good agreement with previously reported structural requirements at the amino terminus of NPY (33, 40, 43) and with centrally truncated NPY analogues (46).

The role of the amino terminus with regard to binding and activation of NPY receptors is not well understood. Forest *et al.* (40) proposed that Tyr-1 of NPY helps maintain the integrity of the PP-fold by associating with hydrophobic residues in the amphiphilic helix. In doing so, the amino terminus can assist in inducing an appropriate conformation at the carboxyl terminus for presentation to the receptor. Alternatively, Tyr-1 may bind directly to the receptor. Such different modes of binding have been discussed by McLean *et al.* (47) and may be a factor in distinguishing receptor subtypes. Regardless of the precise role of this residue, the data presented in this report strongly suggest that the amino-terminal tyrosine in compounds such as Tyr-Arg-Leu-Arg-Tyr-NH_2 functions as a surrogate for Tyr-1 of NPY.

Second, at position 3, the highest affinity is found for pentapeptides with leucine or residues of similar size and hydrophobicity, such as phenylalanine, methionine, and Nle. In contrast, analogues containing residues with special conformational properties, such as glycine and proline, or those with branched amino acids, such as isoleucine and valine, showed weaker affinity. Charged amino acids at position 3 displayed no detectable binding. It is intriguing that the Gln-3 analogue (compound 15) does not show higher affinity. It has been convincingly demonstrated for NPY that Gln-34 is important in distinguishing between receptors in the PP family as well as between NPY receptor subtypes (15, 19). Furthermore, Gln-34 is essential for activity in larger, centrally truncated NPY fragments, as evaluated in a rabbit kidney preparation (37). Residue 34 may play an important role in stabilizing specific amino acid side-chain/backbone conformations or secondary structure in NPY and medium-size NPY fragments, neither of which have relevance to the pentapeptides described in the present study.

Third, in the pentapeptide series, a carboxyl-terminal fragment containing arginine at position 4 and tyrosine at position 5 is essential for tight binding to the brain receptor. There is no strict requirement for arginine or for any positively charged residue at position 2. These results differ significantly from those reported for full-length or long carboxyl-terminal fragments of NPY, where both Arg-33 and Arg-35 are needed for high affinity and activity (37, 39, 41, 46).

Our results can be compared to those of Perlman *et al.* (48), who examined the affinity of a different set of carboxyl-terminal NPY pentapeptide analogues toward aPP receptors. In that study, the carboxyl-terminal sequence -Arg-X-NH_2 (X is aromatic amino acid) was shown to be essential for binding to aPP receptors. In addition, it was noted that the arginine residue corresponding to position 2 of their series of pentapeptides was nonessential. These results are in general agreement with our NPY binding data. However, in contrast to our results, the carboxyl-terminal NPY pentapeptide fragment NPY(33–37), Thr-Arg-Gln-Arg-Tyr-NH_2 (compound 9), was reported to bind competitively to aPP receptors (IC_{50} = 3 μM). We found that compound 9 had no significant affinity for NPY receptors. Moreover, even when threonine was replaced with tyrosine (Tyr-Arg-Gln-Arg-Tyr-NH_2, compound 15), receptor binding was still among the poorest in the series of position 3-substituted analogues. Thus, important differences are observed in the structural requirements for pentapeptide binding, particularly at positions 1 and 3, to aPP and NPY receptors. The affinity of compound 9 toward aPP but not NPY receptors is noteworthy and may provide insight into the function of the amino-terminal residue in NPY (Tyr-1) versus that of aPP (Gly-1).

In summary, from this first systematic report on pentapeptide structure-affinity and -activity relations for the NPY receptor, it can be concluded that Tyr-1, Arg-4, and Tyr-5-NH_2 represent the discontinuous epitope Tyr-1, Arg-35, and Tyr-36-NH_2 of full-length NPY (highlighted in blue in Fig. 2). Incorporation of these residues into appropriately substituted pentapeptides is sufficient to allow interaction, with low micromolar affinity, to NPY rat brain receptors. Furthermore, these same pentapeptides competitively antagonized the NPY-induced increase in cytosolic Ca^{2+} in the Y_1 receptor-mediated HEL cell assay. Modification of these highly flexible linear peptides by cyclization, by introduction of a backbone, or by side-chain constraints may lead to a more well-defined solution structure and enhanced affinity and/or

receptor subtype selectivity. Toward that end, we have recently reported (28) the design, structure, and biological activity of a series of decapeptides and nonapeptides derived from these pentapeptides that display greatly enhanced affinities for Y_2 and Y_1 receptors.

Despite notable advances in understanding the physiology, pharmacology, and biochemistry of NPY, the development of potent and specific antagonists of NPY receptors remains an elusive goal. Such antagonists are essential for evaluating the physiological and pathophysiological role of NPY and may have significant therapeutic potential. The peptides described in the present report suggest a simple model for a minimal essential pharmacophore that could provide a useful starting point for the design of potent and selective small peptide and nonpeptide antagonists. While this manuscript was in preparation, Rudolf *et al.* reported on a small-molecule NPY antagonist, the structure of which is consistent with our proposed model (49).

Acknowledgments

We thank Dr. Janet Allen for the generous gift of [His-1]NPY and [D-Tyr-1]NPY. We also thank Dr. C. Webster Andrews for assistance with molecular modeling, Dr. George Painter for constructive discussions, Mr. Antonio Landavazo for peptide synthesis, Dr. Ann Aulabaugh for NMR data, Mr. W. Chestnut and Ms. Resa Rorie for amino acid analysis, and Dr. Lester Taylor and associates for mass spectral data.

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