Neuropeptide Y: Complete amino acid sequence of the brain peptide

(pancreatic polypeptide family/COOH-terminal α-amide/dansyl Edman subtractive technique)

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ABSTRACT The amino acid sequence of neuropeptide Y, a 36-residue peptide recently isolated from porcine brain, has been determined by using high performance liquid chromatography for separation of its tryptic and chymotryptic fragments and subsequent sequence analysis of the isolated fragments by an improved dansyl Edman subtractive technique. The amino acid sequence of neuropeptide Y has been found to be: Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Glu-Glu-Asp-Ala-Pro-Ala-Glu-Ala-Arg-Tyr-Tyr-Ser-Ala-Lys-Lys-His-Tyr-Ile-Leu-Ile-Leu-Thr-Arg-Glu-Arg-Tyr-NH₂. Neuropeptide Y has a high degree of sequence homology with peptide YY (70%), the newly isolated porcine intestinal peptide, and pancreatic polypeptide (50%). It is therefore proposed that neuropeptide Y, peptide YY, and pancreatic polypeptide are members of a newly recognized peptide family.

The selective isolation of peptide amides from tissue extracts resulted in the discovery of a series of new biologically active peptides (1, 2). The presence of peptide amides is detected by using a chemical technique based on fragmentation of peptides in tissue extracts and subsequent chromatographic identification of the resulting COOH-terminal amide fragments (3). By using this chemical assay technique, two previously unknown peptide amides, peptide H1 and peptide YY (PYY) were isolated from porcine intestinal extracts (1). Subsequent studies revealed that these peptides have structural similarities to known hormones and exhibit a number of bioactivities. Peptide H1 has been found to have structural similarities to vasoactive intestinal peptide, secretin, glucagon, and gastric inhibitory peptide (2) and to exhibit biological activities similar to vasoactive intestinal peptide and secretin (4–7). PYY is structurally similar to pancreatic polypeptide (PP) and inhibits secretin-stimulated exocrine pancreatic secretion (8) and jejunal and colonic motility (9). It also exhibits potent vasoconstrictor activities (9). By using similar techniques, another peptide amide, designated neuropeptide Y (NPY), has been isolated from porcine brain (10, *). NPY is abundantly present in the brain and is structurally similar to PYY and PP; like PP and PYY, it is capable of inhibiting secretin-stimulated pancreatic secretion (10).

During the course of characterization of these peptide amides, the strategies for structural analysis of biologically active peptides have been reexamined. This paper describes a strategy for amino acid sequence determination of peptides using a combination of HPLC for separation of peptide fragments and subsequent microsequence analysis by an improved dansyl Edman technique. The dansyl Edman procedure (11) was modified by addition of a subtractive procedure based on redansylation of the hydrolysate used for the NH₂-terminal determination in the dansyl procedure and subsequent identification of resulting dansyl amino acids. In addition, Asx and Glx residues were identified as their phenylthiohydantoin (>PhNCS) derivatives by HPLC.

MATERIALS AND METHODS

NPY was prepared from porcine brain as described (10). Amino acid analysis, NH₂-terminal determination, and HPLC separation techniques, as well as other chemicals used, have been described (8).

Edman Degradation Procedures. The dansyl Edman procedure described by Gray and Hartley (11) was modified to suit sequence determination on 2–10 nmol of peptide. Peptide (2–10 nmols) in a 5 × 50 mm test tube was dissolved in 20 μl of 50% (vol/vol) aqueous pyridine. One microliter of phenylisothiocyanate was delivered at the side wall of the test tube through a microsyringe. The tube was immediately placed under nitrogen gas for 20–30 sec and sealed carefully with Parafilm; then, the phenylisothiocyanate was allowed to mix with the pyridine solution with the aid of a Vortex mixer. The solution was incubated at 45°C for 60 min in a heating block and then evaporated to dryness at 60°C for 10 min under reduced pressure (<0.1 mbar (<10 Pa)); the residue was dissolved in 50 μl of ethanol and again evaporated to dryness at 60°C for 10 min under reduced pressure. To the dried residue, 50 μl of trifluoroacetic acid was added and the solution was incubated at 45°C for 20 min under nitrogen gas and then evaporated to dryness at room temperature for 10 min under reduced pressure. The residue was dissolved in water (40 μl) and extracted with butyl acetate (100 μl, three times). A microcap or microsyringe was used to remove most of the organic phase.

The butyl acetate extracts were discarded except those that contained the thiazolinone derivative of Asx or Glx. The extracts were combined and subjected to the conversion and identification steps. From the water phase, an aliquot of 0.05–0.1 nmol of the peptide was withdrawn, transferred to a 4 × 50 mm conical-bottom test tube, and subjected to the dansyl procedures. The remaining water phase was evaporated to dryness and the residue was subjected to the next Edman degradation cycle.

Dansyl Procedures. The aliquot of the water phase in the micro test tube was evaporated to dryness, the residue was dissolved in 2 μl of 0.05 M sodium bicarbonate buffer (pH 9.5), and an equal volume of dansyl chloride in acetonitrile (2 mg/ml) was added. The reaction mixture was incubated at 37°C for 30 min and evaporated to dryness. The residue was dissolved in 10 μl of 6 M HCl, sealed under reduced pressure, and incubated at 105°C for 16 hr. The hydrolysate was evaporated to dryness.

Abbreviations: NPY, neuropeptide Y; PYY, peptide YY; PP, pancreatic polypeptide; >PhNCS, phenylthiohydantoin. 

* Tatemoto, K., Eighth Meeting of the International Society for Neurochemistry, Nottingham, United Kingdom, Sept. 6–11, 1981, p. 392 (abstr.).
dryness under reduced pressure and the residue was dissolved in 2 μl of 50% ethanol; 1 μl was applied to a polyamide thin-layer plate (5 × 5 cm or 2.5 × 2.5 cm, A1700 aluminum plate, Schleicher & Schuell). The plate was developed with the following solvent systems: (i) 1.5% formic acid; (ii) acetic acid/benzene, 1:9 (vol/vol); and (iii) ethyl acetate/methanol/acetic acid, 20:1:1 (12). For the separation of the basic amino acid derivatives, 0.3% ammonia solution was used as the fourth solvent system in place of the triphosphate buffer system described by Hartley (12) for a better separation. The use of a polyamide sheet on aluminum foil improved the detection limit of dansyl amino acids severalfold compared with conventional polyamide sheets on polyester (13). The peptide loss due to withdrawal of peptide material for the NH2-terminal determination was minimal in the present procedure because only 50–100 pmol of peptide was used per cycle for the NH2-terminal determination.

The remaining aliquot (1 μl) of the hydrolysate used for the identification of NH2-terminal dansyl amino acid also contained the undansylated amino acid components resulting from acid hydrolysis of the peptide. These amino acids could be identified in the form of dansyl amino acids after redansylation of the hydrolysate. The remaining hydrolysate was evaporated to dryness and the residue was dissolved in 2 μl of 0.05 M sodium bicarbonate-buffer (pH 9.5), an equal volume of dansyl chloride in acetonitrile (2 mg/ml) was added, and the mixture was incubated at 57°C for 30 min. After evaporation to dryness, it was dissolved in 2 μl of 50% pyridine and applied to the polyamide thin-layer plate. The dansyl amino acids were identified and compared with those obtained from the next cycle of the Edman degradation. When adjacent steps were compared, the amino acid found to be missing from the latter step represented the NH2-terminal amino acid removed from the peptide by the Edman reaction and should be identical to the amino acid identified by the NH2-terminal determination in the former step. This subtractive technique not only confirms results of the NH2-terminal determination but also monitors the Edman reaction cycle for factors such as peptide loss in each cycle, unusual degradations, the presence of fluorescent side products, the presence or absence of a blocked NH2-terminus, and determination of the last residue to be degraded. When the subtractive technique is used, the amino acid composition of the peptide is monitored after each degradation step and therefore it may not be necessary to use an amino acid analyzer to determine the amino acid composition of a peptide subject to the Edman degradation.

Identification of Asp/Asn and Glu/Gln Residues. The butyl acetate extracts that contained the thiazoline derivatives of Asx or Glx were evaporated to dryness, and the residue was dissolved in 1 M HCl (100 μl) and incubated at 80°C for 5 min under nitrogen gas. The resulting >PhNCS amino acid was extracted from the water phase by ethyl acetate (100 μl, twice). The extracts were combined and subsequently evaporated to dryness. Identification of the >PhNCS amino acid was carried out by using the same HPLC system as described for the separation of tryptic fragments. Chen (14) has reported a procedure for identification of Asp/Asn and Glu/Gln residues by using the >PhNCS derivatives derived from the Edman degradation products in the butyl acetate extracts which are usually discarded in dansyl Edman procedures. In the present procedure, the >PhNCS amino acids were identified by using HPLC (instead of the TLC used by Chen) because of its greater sensitivity and reliability. The presence of excess side products and side products from the Edman reaction did not interfere with the identification of these >PhNCS amino acids because most of them were eluted after the HPLC identification of the >PhNCS amino acids.

**RESULTS**

**Amino Acid Composition of NPY.** The results of amino acid analysis of the NPY preparation indicated that NPY consists of 36 amino acid residues (Table 1). Cysteine, phenylalanine, methionine, and valine were not detectable, but a high content of tyrosine (5 residues per mol) was noted. The amino acid compositions of NPY and PYY are remarkably similar.

**Terminal Analysis.** NPY contained NH2-terminal tyrosine, determined by the method of Gray (15) using dansyl chloride. No other detectable NH2-terminal amino acid was present in the preparation. Treatment of NPY with trypsin or thermolysin yielded the COOH-terminal tyrosine amide which was identified by the TLC method (3). The results of NH2- and COOH-terminal determination indicated that NPY has the same termini as those of PYY and the same COOH-terminus as PP.

**HPLC of the Tryptic and Chymotryptic Fragments.** Tryptic and chymotryptic digestions were carried out as described in Fig. 1. Use of trifluoroacetic acid in the HPLC solvent system is convenient because it is easily removed by lyophilization and results in fairly good resolution and symmetric elution peaks (16, 17). By using the reversed-phase HPLC column, it was possible to prepare tryptic and chymotryptic fragments from 10–20 nmol of the NPY preparation in good yields (50–80%). Treatment of NPY with trypsin yielded five fragments which were separated by HPLC (Fig. 1). Amino acid analysis of these fragments indicated that two of them, Gln-Arg and Tyr-NH2, were identical to the corresponding fragments of PYY (8). Treatment of NPY with chymotrypsin yielded a total of seven fragments which were isolated by HPLC. The numbers of amino acid residues of these fragments were all identical to the corresponding chymotryptic fragments of PYY (8) but three of them had different NH2-terminal amino acids—that is, serine (C-2) and isoleucine (C-3 and C-7) in the place of alanine and leucine in the corresponding PYY fragments (Table 2).

**Sequence Analysis of the Tryptic Fragments.** The amino acid sequences of the isolated tryptic fragments were determined by using the dansyl Edman subtractive technique. The isolated tryptic fragments (2–12 nmol) obtained from 17 nmol of NPY were more than sufficient to complete the sequences of all tryptic fragments. All of the 19 residues of the T-1 fragment were determined by using the modified sequence technique with 11 nmol of the peptide. Asp/Asn and Glu/Gln residues of the frag-

**Table 1. Amino acid compositions (residues per mol) and NH2-termini of NPY and its isolated tryptic fragments**

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**NH2-terminus**  
TyroTyroHisGlyTyrTyr

**Recovering**  
9.5 10.4 17.2

Values in parentheses represent theoretical number of residues.
ments were identified by using the HPLC method. No difficulty was encountered in identification of the residues even after 15 Edman degradation steps.

The amino acid sequence of fragment T-1 was determined to be Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Ala-Pro-Ala-Glu-Leu-Ala-Arg.

The T-2 was found to be Tyr-Tyr-Ser-Ala-Leu-Arg, and T-3 was His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg. The sequence of fragment T-4 was confirmed to be Gln-Arg by the Edman reactions.

Fragment T-5 contained only tyrosine and ammonia after acid hydrolysis and it was identified to be Tyr-NH2 by the amide determination method (3). The fragment T-5 therefore should be the COOH-terminal fragment.

Complete Amino Acid Sequence of NPY. The NH2-terminal sequence of NPY was determined by subjecting 10 nmol of the untreated NPY preparation to the Edman degradations, which yielded the following sequence: Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Ala-Pro-Ala. This indicates that the T-1 fragment is the NH2-terminal tryptic fragment. Because chymotryptic fragments C-3 and C-7 contained Ile2, Asx, Leu, Thr, Arg3, Glx, and Tyr and fragment C-2 consisted of Ser, Ala, Leu, Arg, His, and Tyr, the COOH-terminal sequence should be His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-NH2. The complete amino acid sequence of NPY was therefore found to be: Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Ala-Pro-Ala-Glu-Leu-Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-NH2 (Fig. 2). The NH2-terminal half of the molecule contains all of its five carboxyl groups, forming a acidic region; the other half contains all of its four arginyl residues, forming a highly basic region. All of its four prolyl residues are located in the NH2-terminal region.

DISCUSSION

A comparison of the primary structures of NPY, PYY, and PP indicates that a high degree of sequence homology exists between NPY and PYY and also that a lesser degree of homology exists between NPY and PP (Fig. 3). The entire molecules of

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Values in parentheses represent theoretical number of residues.

**FIG. 1.** HPLC separation of tryptic NPY fragments (T-1 to T-5). NPY (70 ng) was dissolved in 50 μl of 1% NH4HCO3, and 2 μl of the trypsin solution (2 mg/ml) was added. The solution was incubated at room temperature for 4 hr, boiled for 6 min, and lyophilized. The lyophilized digest was dissolved in 50 μl of water and injected into a (Waters) HPLC instrument consisting of an injector (U6K), two pumps (M-6000 A), a UV detector (450 variable), and a solvent programmer (model 660). The tryptic fragments were separated on a Waters reversed-phase HPLC column (μBondapak C18, 3.9 x 300 mm) with a linear gradient elution system of 0.12% CF3COOH/H2O (solvent A) and CF3COOH/CH3CN (solvent B, 0-50% for 40 min) at a flow rate of 1 ml/min. The eluate that contained peptides was collected and concentrated to approximately one-fifth of the volume under reduced pressure. It was then diluted by addition of water and lyophilized.
NPY and PYY involve a total of 25 identities in their 36 residues, and many of the remaining 11 nonidentical residues seem to be paired with structurally similar amino acid residues. These include four Ser/Ala (or Ala/Ser), two Asp/Glu, one Ile/Leu, and one Ile/Val replacement. The other replacements are Asn/Ala at position 7 and Pro-Ala/Ser-Pro replacements at position 13–14. A shift of Pro from position 13 to 14 is observed between the structures of NPY and PYY and also between those of avian PP and bovine PP. There is an unusually high number of Ser/Ala (including Pro-Ala/Ser-Pro) interchanges between the sequences of NPY and PYY. These interchanges might play a role in functional differences between the two peptides. The differences in 10 of the 11 nonidentical residues can be explained genetically by single nucleotide replacements, but the difference at position 7 requires two nucleotide changes. This means that a total of 12 base changes in the 108 nucleotide positions is the minimum needed to change NPY to PYY or vice versa, suggesting that the two peptides may have evolved from a common ancestor.

Several gut/brain peptides have been isolated and shown to have identical amino acid sequences in both these tissues. These include substance P (18), neurotensin (19), cholecystokinin octapeptide (20), somatostatin-28 (21–23), dynorphin (24, 25), the hydra head activator (26), and the vasoactive intestinal peptide (27). Although NPY and PYY are remarkably similar with respect to their structures, they do not have identical sequences. PYY is present in endocrine cells of gut, being especially abundant in the distal intestine, but is absent from brain (9). On the other hand, NPY is abundantly present in brain but, to date, has not been detected in the intestine (10). However, because of their intense structural similarities, we cannot exclude the possibility that together they form one pair of gut/brain peptides which are not strictly identical in their sequences.

NPY also has distinct sequence homologies to porcine, bovine, and avian PP (Fig. 3), NPY is identical to porcine PP in 18 of its 36 residues and to bovine PP in 17. The sequence homologies are marked in the COOH-terminal region where 9 of the 13 residues at position 24–36 are identical. On the other hand, NPY and avian PP are identical in 20 residues with the homology being most notable in the middle region at positions 8–17, where 8 of the 10 residues are identical. It has been reported that the brain contains a peptide that reacts with antisera raised against bovine and avian PP (28–30). NPY is abundantly present in brain and possesses distinct structural homologies to both bovine and avian PP, suggesting that the anti-PP antibodies may crossreact with NPY. One recent study indicated that NPY was able to crossreact with antisera raised against avian and bovine PP (31). It remains to be determined whether NPY solely is responsible for the PP immunoreactivities in brain.

NPY and PYY have sequence homologies to some extent to members of other peptide families (unpublished data), but the structural similarities of the two peptides to PP are not only in their distinct sequence homologies but also in the identical number of residues and their unique COOH-terminal tyrosine amide structures. Moreover, they have biological activities that resemble each other. It therefore is proposed that NPY, PYY, and PP are members of a previously unrecognized peptide family.

Intravenous injection of NPY, like PYY and PP inhibits secretin-stimulated pancreatic exocrine secretion (10), but rela-
tively high doses are needed to produce a significant reduction of the flow of pancreatic juice. NPY has potent vasoconstrictor properties, being more potent than avian and bovine PP but less potent than PYY (31). It remains to be determined whether these biological effects of NPY are of physiological significance. Because NPY is abundant in the brain, it is of importance to identify the biological effects of NPY in the central nervous system as well as those of NPY in the gut.

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