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Primary structures of PYY, [Pro^{34}]PYY, and PYY-(3–36) confer different conformations and receptor selectivity

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[1]*The Beckman Research Institute of the City of Hope, Duarte, California, 91010-0269;* [2]*Durham Veterans Affairs and Duke University Medical Centers, Durham, North Carolina, 27705;* [3]*CURE Digestive Diseases Research Center, Greater Los Angeles Veterans Affairs Healthcare System, Los Angeles 90073; and* [4]*Digestive Diseases Division, UCLA School of Medicine, Los Angeles, California 90095*

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Keire, David A., Peter Mannon, Mitsuo Kobayashi, John H. Walsh, Travis E. Solomon, and Joseph R. Reeve, Jr. Primary structures of PYY, [Pro^{34}]PYY, and PYY-(3–36) confer different conformations and receptor selectivity. *Am J Physiol Gastrointest Liver Physiol* 279: G126–G131, 2000.—We synthesized PYY-(1–36) (nonselective between Y_1 and Y_2 receptor subtype agonists), [Pro^{34}]-PYY (selective for Y_1), and PYY-(3–36) (selective for Y_2) to determine whether solution conformation plays a role in receptor subtype selectivity. The three peptides exhibited the expected specificities in displacing labeled PYY-(1–36) from cells transfected with Y_1 receptors (dissociation constants = 0.42, 0.21, and 1,050 nM, respectively) and from cells transfected with Y_2 receptors (dissociation constants = 0.03, 710, and 0.11 nM, respectively) for PYY-(1–36), [Pro^{34}]PYY, and PYY-(3–36). Sedimentation equilibrium analyses revealed that the three PYY analogs were 80–90% monomer at the concentrations used for the subsequent circular dichroism (CD) and ^{1}H-nuclear magnetic resonance (NMR) studies. CD analysis measured helicities for PYY-(1–36), [Pro^{34}]PYY, and PYY-(3–36) of 42%, 31%, and 24%, suggesting distinct differences in secondary structure. The backbone ^{1}H-NMR resonances of the three peptides further substantiated marked conformational differences. These patterns support the hypothesis that Y_1 and Y_2 receptor subtype binding affinities depend on the secondary and tertiary solution state structures of PYY and its analogs.

peptide YY; Y_1 receptor; Y_2 receptor; circular dichroism; nuclear magnetic resonance; three-dimensional structure

THE PANCREATIC POLYPEPTIDE family is comprised of three naturally occurring bioactive peptides, pancreatic polypeptide (PP), neuropeptide Y (NPY), and peptide YY (PYY), that are found in the gut and brain. PYY is released from endocrine L cells of the distal digestive tract by indirect stimulation from the proximal gut through neural and humoral pathways and by direct stimulation of L cells by luminal contents (7, 16). Two endogenous forms of PYY, PYY-(1–36) and PYY-(3–36), are released into the circulation by a meal (15). Proposed gastrointestinal actions of PYY are inhibition of gastric secretion, inhibition of pancreatic secretion, inhibition of intestinal secretion, and inhibition of gastrointestinal motility (1, 4, 8, 26, 27, 29).

PYY binds and activates at least three receptor subtypes (Y_1, Y_2, and Y_5) in rats and humans, and it may interact with a postulated fourth subtype, the peripheral Y_2-like receptor. Here we use the nomenclature for Y receptors suggested by the International Union of Pharmacology (5). These Y receptor subtypes display different patterns of affinity and activation for the two endogenous ligands PYY and PYY-(3–36) and for the two synthetic analogs [Pro^{34}]PYY and [D-Trp^{32}]PYY. In general, the Y_1 receptor subtype has high affinity for PYY and [Pro^{34}]PYY, the Y_2 has high affinity for PYY and PYY-(3–36), and the Y_5 subtype binds PYY, PYY-(3–36), [Pro^{34}]PYY, and [D-Trp^{32}]PYY with high affinity. Figure 1 shows the primary structures of the agonists used in our studies. These different patterns of receptor selectivity could be caused by differences in primary structure, differences in tertiary structure due to altered conformations of the ligands in solution, or both.

Early descriptions of the structure of avian PP were derived from analysis of X-ray crystallography data (6), which led to modeling of potential structures of mammalian PP, NPY, and PYY by computer analysis (14). The solution structures of PP and of NPY have been studied by circular dichroism (CD) and nuclear magnetic resonance (NMR) (11, 12, 19, 23, 24, 28). All analyses of PP have consistently found evidence for a folded structure (the "PP fold") stabilized by hydrophobic interactions among residues in the NH_2- and COOH-terminal portions. A similar structure has been assumed to exist for NPY and PYY because of their high sequence homologies to PP.

Such results have led to hypotheses that this stable structure of PP family peptides is critical for binding

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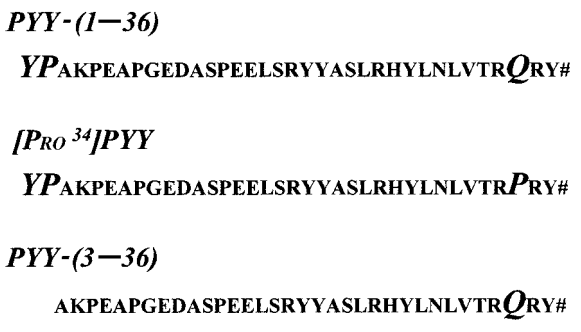

Fig. 1. The primary sequences of rat peptide YY (PYY) and the receptor subtype-selective PYY analogs [Pro^{34}]PYY and PYY-(3–36). PYY binds with similar affinity at both Y_1 and Y_2 receptors. In contrast, the synthetic [Pro^{34}]PYY analog binds the Y_1 but not the Y_2 receptor and the endogenous PYY-(3–36) analog binds the Y_2 but not the Y_1 receptor.

and activation of PP/NPY/PYY-specific receptors and that receptor selectivity depends in part on differences in solution structure produced by amino acid deletions or substitutions in naturally occurring or synthetic Y receptor agonists. The purpose of this study was to determine whether removing amino acids from the NH_2 terminus of PYY [to form PYY-(3–36)] or substitution of glutamine at position 34 with a proline (to form [Pro^{34}]PYY) causes secondary and tertiary structure changes in the peptide that could contribute to Y receptor selectivity.

In previous work, models of the conformation of NPY were confounded by the presence of molecular dimerization under the solution conditions used to generate CD and NMR data, requiring cautious interpretation of results when attempting to extend the structure model to receptor binding. Thus, although the proposed role of stable solution structure of Y receptor agonists in binding, activation, and receptor subtype selectivity is an attractive possibility that could be a model for understanding the contribution of tertiary structure to bioactivity of peptide ligands, it is supported only by indirect evidence. The PP fold that has been used as a model for PYY and NPY tertiary structure is based on conformations that have been determined for the tertiary structures of avian and bovine PP (6, 19). Modeling based on the PP fold (rather than on directly determined NPY structures) was necessary for NPY because three groups found that the peptide formed a head-to-toe dimer between amphipathic α-helices formed from residues 13-36 (the NH_2-terminal residues were flexible in solution) (11, 24, 28). However, Darbon et al. (12) reported a monomeric NPY structure with a tertiary fold similar to the PP fold. The solution structures of the monomeric forms of these peptides are important because NPY and PYY occur as monomers under physiological concentrations, i.e., <1 nM, in the circulation.

Only limited structural studies have been performed on PYY and on NPY and PYY agonists. Analyses of the secondary structures of PYY and its analogs by CD have revealed that two analogs with NH_2-terminal changes had greater helicity and more potent bioactivity, suggesting that tertiary structure could influence activity (22). Furthermore, other studies showed that NPY helicity decreased as NH_2-terminal amino acids were removed (17) and that helical content could be disrupted by single D-amino acid substitutions for natural L-forms (18) or by substitution of alanine for natural amino acids (3).

In this study, we demonstrate with CD and one-dimensional (1-D) NMR spectra that altering the primary structure of PYY significantly changes the secondary and tertiary structure of these analogs. These findings are consistent with the proposed role of three-dimensional (3-D) conformation in determining receptor subtype selectivity.

METHODS

Peptides. Porcine PYY-(1–36), PYY-(3–36), and [Pro^{34}]-PYY-(1–36) were synthesized in the UCLA Peptide Synthesis Facility using 9-fluorenylmethyloxycarbonyl (FMOC) strategy on an Advanced Chem Tech 396 Peptide Synthesizer. The peptides were cleaved from the resin, protecting groups were removed, and the peptides were then purified to >90% as evaluated by high-performance capillary electrophoresis.

Analytical ultracentrifugation. The sedimentation equilibrium analyses for PYY analogs were performed at UCLA in the laboratory of Prof. V. Shumaker on a Beckman Optima XLA analytical ultracentrifuge. Three samples of each peptide at 0.1, 0.7, and 2 mM (in 150 mM KCl, pH 5.0, at 21°C) were centrifuged at 40,000 and 32,000 rpm using a 12-mm six-channel cell until equilibrium was established. Approximate molecular weights were determined by assuming a single species and fitting the absorbance at 290 nm vs. radius data by nonlinear regression analysis. A molecular weight higher than the calculated peptide mass indicates self-association and can be fit to a monomer-dimer (or higher order) model.

CD and NMR. The CD experiments were performed on a Jasco J-600 spectropolarimeter (Easton, MD). A 0.1-mm cell was used with micromolar concentrations of peptide, and ellipticity was measured over a wavelength range of 180–300 nm. The 1-D NMR studies were conducted on a Varian Unity Plus 500 MHz spectrometer (Varian Associates, Palo Alto, CA). Typically, 128 transients were coadded with a sweep width of 6,000 Hz, an acquisition time of 0.5 s, and a presaturation delay of 1.5 s. Both the presaturation of water and acquisition of data were performed using the transmitter channel. The NMR data were processed with zero filling to 65,536 points and 1 Hz of line broadening using the Varian NMR software.

Radioligand binding. F-12, DMEM, and BSA were purchased from Sigma (St. Louis, MO), fetal bovine serum from Atlanta Biologicals (Atlanta, GA), and geneticin from Calbiochem (La Jolla, CA). CHO-A1 cells containing Y_1 receptors (21) were grown in F-12 medium containing 10% fetal bovine serum, 1% streptomycin and penicillin, and 500 μg/ml geneticin; Ngp37 cells containing Y_2 receptors (gift of Dr. Anil Rustgi) were grown in DMEM containing 10% fetal bovine serum and 1% streptomycin and penicillin. Both lines were maintained at 37°C and 5% CO_2. For binding studies, cells were seeded (50,000 cells/well) in 12-well plates and grown to 90% confluence. Cells were washed once with 1 ml of DMEM containing 25 mM HEPES (pH 7.4) and 10 mM $NaHCO_3$ and were then incubated with the same buffer containing 100 pM ^{125}I-PYY (for description of radiolabel preparation, see Ref. 20) and various concentrations of unlabeled PYY forms. After

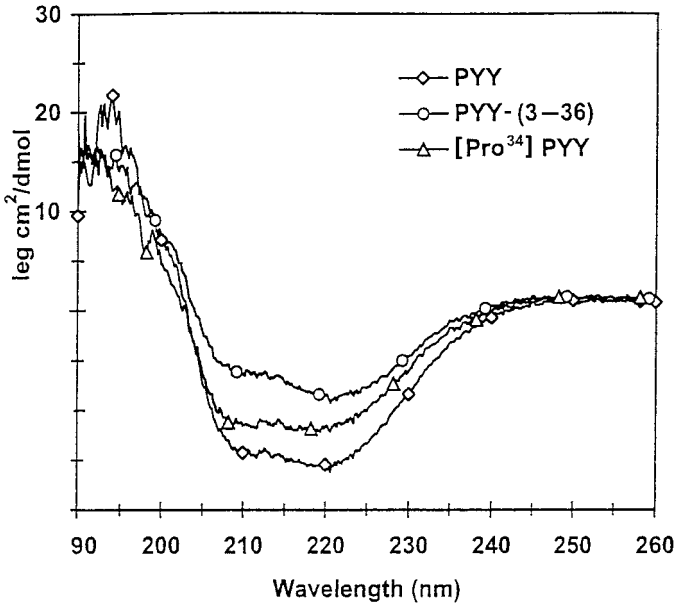

Fig. 2. A plot of the circular dichroism (CD) molecular ellipticity from 190–260 nm of 0.27 mM PYY, PYY-(3–36), and [Pro^{34}]PYY at pH 6 in H_2O with 150 mM NaCl. A more negative ellipticity at 222 nm is indicative of a greater number of residues in a helical structure (9). The calculated helicity for PYY is 42%, for [Pro^{34}]PYY is 32%, and for PYY-(3–36) is 23%.

1 h at 37°C, plates were washed 4 times with 1 ml ice-cold PBS and the cells were dissolved in 1 ml 0.5 N NaOH. Radioactivity in the lysates was counted in a gamma counter. Saturation binding curves were analyzed at equilibrium to determine concentrations of PYY peptides that inhibited 50% of saturable ^{125}I-PYY binding (IC_{50}). Radioligand equilibrium binding data were fit by nonlinear least-squares regression analysis (LIGAND) to estimate the dissociation constant (K_d) for PYY and the IC_{50} for the PYY analogs. The inhibition constant (K_i) estimates were calculated with the Cheng and Prusoff equation (10).

Molecular modeling. Model building, molecular dynamics (MD), and energy minimization were performed with Molecular Simulations Insight and Discover modules (MSI, San Diego, CA) on a Silicon Graphics Indigo R4000 UNIX workstation (Mountain View, CA). The MSI cvff force field was used. PYY-(1–36) was assembled stepwise from the NH_2 terminus using the primary sequence. The residues 15–36 were then made to adopt a helical conformation, and the backbone dihedral angles around residues 12–14 were adjusted to bring the NH_2- and COOH-terminal residues in close proximity (a PP fold). For [Pro^{34}]PYY, the PYY-(1–36) model was used and Glu^{34} was substituted with a proline residue. For PYY-(3–36), the two NH_2-terminal residues were deleted and the backbone dihedral angles of residues 12–14 were adjusted so the NH_2 and COOH termini were not in close proximity. These models were then molecule energy minimized via the steepest descent method for 200 iterations, and 1,000 steps of MD at 300 K (1 fs/step) were performed. After the MD was complete, the molecule was again energy minimized for 200 iterations. A distance-dependent dielectric was used to simulate the presence of water, and the charge state of the basic and acidic groups on the peptide were those found at pH 7. The resulting structure was fit with a ribbon representation of the backbone residues to more easily identify structural alterations.

RESULTS

Analytical ultracentrifugation. Sedimentation equilibrium analyses were performed for PYY, [Pro^{34}]PYY, and PYY-(3–36). Monomer-dimer K_d (K = [monomer][monomer]/[dimer]) were found as follows: PYY, $(2.2 \pm 0.3) \times 10^{-2}$ M; [Pro^{34}]PYY, $(5.7 \pm 0.2) \times 10^{-3}$ M; and PYY-(3–36), $(2.0 \pm 0.3) \times 10^{-2}$ M at 21°C, pH 6, and 0.15 M NaCl. Thus all three forms are present as 80–90% monomers at 1 mM aqueous solution concentrations. Because the NMR and CD studies of PYY and PYY analogs described below were performed under similar solution conditions, the major contributor to spectra was the monomeric form.

CD data. The CD molecular ellipticities at particular wavelengths can be used to calculate the percentage of the total number of residues of a peptide in helical, β-sheet, and random coil conformations (9). The specific sequence of residues involved in these secondary structures cannot be established by this method. Peptide amino acids arranged in these conformations differentially absorb right- and left-handed circularly polarized light (expressed as ellipticity) at characteristic wavelengths (e.g., an α-helix has a positive peak at +195 nm and negative peaks at −208 and −222 nm). If more than one conformation is present, the spectrum represents a concentration-weighted average of the various bands.

CD spectra collected on PYY, [Pro^{34}]PYY, and PYY-(3–36) display helical character with minima at 208 and 222 nm (Fig. 2). There were marked differences among the three PYY forms, and the helical content of both [Pro^{34}]PYY and PYY-(3–36) is decreased relative

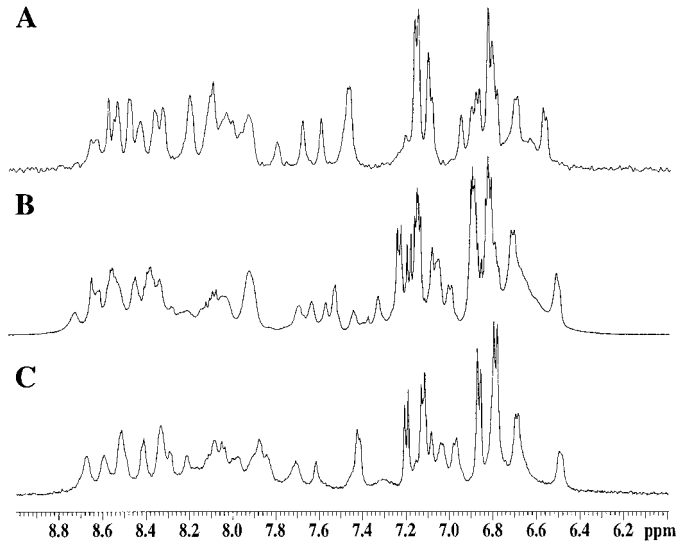

Fig. 3. The amide and aromatic proton region of the 500 MHz ^{1}H-nuclear magnetic resonance (NMR) spectra in 90% H_2O-10% D_2O with 150 mM NaCl (25°C) of 0.27 mM PYY-(3–36) at pH 5 (*A*), 0.51 mM [Pro^{34}]PYY at pH 5 (*B*), and 0.84 mM PYY at pH 6 (*C*). The differences observed in the pattern of amide and aromatic proton signals (with only minor modifications in the primary sequence of the analogs) indicate significant alteration of the secondary and tertiary structure of the three forms. The intensities of *spectra A* and *B* were adjusted so that the aromatic proton signal at 6.8 parts per million (ppm) was similar to this signal in the PYY spectrum.

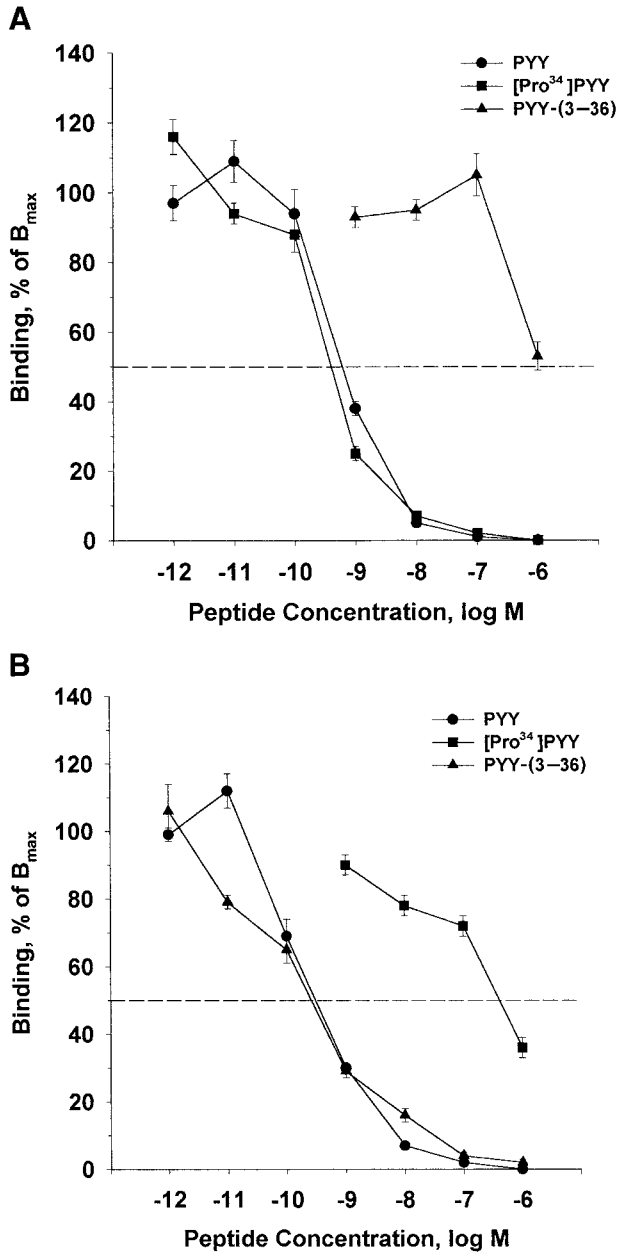

Fig. 4. Displacement of ^{125}I-radiolabeled PYY by PYY and its analogs, PYY-(3–36) and [Pro^{34}]PYY, to Y_1 and Y_2 receptor-containing cell lines. *A*: ^{125}I-PYY is displaced by [Pro^{34}]PYY equivalently to PYY and >2,500-fold less avidly by PYY-(3–36) from Y_1 receptors on CHO-A1 cells. *B*: ^{125}I-PYY is displaced by PYY-(3–36) equivalently to PYY and >24,000-fold less avidly by [Pro^{34}]PYY from Ngp37 cells containing Y_2 receptors. B_{max}, maximum binding.

to PYY. The percent helicity of these analogs was calculated from the spectra shown in Fig. 2 using the molecular ellipticity at 222 nm (9): PYY, [Pro^{34}]PYY, and PYY-(3–36) had 42, 31, and 24% helicity, respectively. It should be noted that any alteration in the secondary structure of these forms must be accompanied by changes in tertiary structure.

NMR data. 1-D ^{1}H-NMR data were used to verify that PYY, [Pro^{34}]PYY, and PYY-(3–36) have altered secondary and tertiary structure. The dispersion of the NMR amide proton chemical shifts provides information about the chemical environment of probe nuclei. For example, the amide protons of the various amino acid types in a random coil conformation (i.e., flexible in solution) have chemical shift values in the 8.09–8.44 parts per million (ppm) range (30). In the presence of secondary and tertiary structure, these protons show a much wider range of chemical shift values. Differences in the line widths and patterns of the amide protons between peptides with nearly identical sequences also indicate an altered tertiary structure for these protons (Fig. 3). In this work, the ^{1}H-NMR spectra of the three PYY peptides display different patterns and a nonrandom-coil-like chemical shift range (7.16–8.72 ppm). The altered amide proton resonance patterns and the range of amide proton chemical shifts are consistent with the differing secondary and tertiary structure in PYY, [Pro^{34}]PYY, and PYY-(3–36) observed in the CD data.

Binding of PYY analogs to Y_1 and Y_2 receptors. The data (Fig. 4*A*) for Y_1 binding show equivalent displacement of ^{125}I-PYY by PYY and [Pro^{34}]PYY, whereas PYY-(3–36) was <0.1% as effective as PYY for displacing label from these receptors. Scatchard analysis yielded a K_d for PYY of 0.42 nM and K_i values (calculated from the IC_{50} values with the Cheng and Prusoff equation) of 0.21 nM for [Pro^{34}]PYY and 1,050 nM for PYY-(3–36). For Y_2 receptor binding, the data (Fig. 4*B*) show nearly equivalent binding for PYY and PYY-(3–36), whereas [Pro^{34}]PYY was <0.01% as efficient in displacing label. Scatchard analysis indicated a K_d of 0.03 nM for PYY and K_i values for [Pro^{34}]PYY of 710 nM and for PYY-(3–36) of 0.11 nM.

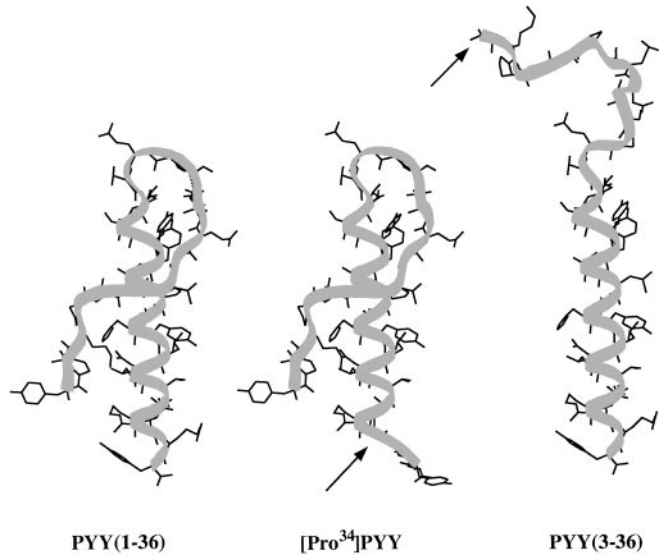

Fig. 5. Molecular models of PYY (*left*), [Pro^{34}]PYY (*middle*), and PYY-(3–36) (*right*) showing differences in the juxtaposition of the NH_2 and COOH termini and changes in the COOH-terminal helix that lead to changes in the receptor subtype selectivity. These models were constructed as described in METHODS. The arrows on the [Pro^{34}]PYY and PYY-(3–36) models indicate the critical regions of the PYY sequence that are important for Y receptor subtype selectivity.

DISCUSSION

The current study provides the first comprehensive analysis of the solution characteristics of PYY, including aggregation, secondary and tertiary structure, and their relationship to receptor binding properties. It is also the first to provide comparative analyses of these factors for PYY itself, the naturally occurring Y_2 selective agonist PYY-(3–36), and the synthetic Y_1 selective agonist [Pro^{34}]PYY. Our results indicate that there are clear differences in the solution structures of these forms of PYY and in their selectivity for Y_1 and Y_2 receptors. This provides strong support for the hypothesis that stable tertiary structures of Y receptor agonists in solution are important determinants of their selectivity.

Previous studies on the structure of the related peptide NPY have been hampered by formation of aggregates at the micromolar to millimolar peptide concentrations required for NMR and CD studies. Three NMR studies have shown that NPY exists as a dimer under such conditions (11, 24, 28), a structure unlikely to occur in more physiological settings. Dimer formation thus limits the usefulness of these NPY structure analyses for any attempt to correlate 3-D conformation with biological functions. For PYY and its analogs, our sedimentation equilibrium data indicate that these peptides exist predominantly as monomers under the conditions used in the NMR and CD experiments of this study.

Our receptor binding data confirmed that PYY is a potent agonist at both Y_1 and Y_2 receptors, whereas [Pro^{34}]PYY and PYY-(3–36) exhibited significantly different binding affinities at these subtypes: the Y_1/Y_2 selectivity ratio (the ratio of binding affinities at the Y_1 and Y_2 receptor) was 14 for PYY, 0.0003 for [Pro^{34}]PYY, and 9,500 for PYY-(3–36). These marked differences in selectivity ratios could be caused by altered primary structure (e.g., substitution of proline for Glu^{34} or deletion of two amino acids) on specific residue-to-residue interactions between ligand and receptor. They could also be caused by changes in secondary and tertiary conformation of the ligand that modify spatial interactions with receptor subtypes. Further studies with analogs designed to have altered structure but minimal alteration in primary structure could substantiate this concept.

We found that the different receptor binding affinities of PYY, [Pro^{34}]PYY, and PYY-(3–36) were accompanied by three different percent helicities calculated from CD data. We found no evidence for the presence of other secondary structural features (such as β-sheets) in any of the PYY forms. It has been reported that PYY and the Y_2-selective agonist PYY-(13–36) also differ in helical content and activation of Y_2 receptors (22). This group found the same value for helical content of PYY as reported here and nearly the same values for the Y_2-specific agonist PYY-(13–36) as we did for PYY-(3–36). PYY-(3–36) and PYY-(13–36) are both highly specific Y_2 agonists, although they differ markedly in potency. The similarities in structure (i.e., helicity) of PYY-(3–36) and PYY-(13–36) suggest an important relationship between peptide structure and receptor selectivity. Our results also show that the Y_1-selective analog [Pro^{34}]PYY has a helical content that differs from both PYY and PYY-(3–36). This finding is also consistent with a recent report that NPY differs in helicity from [Pro^{34}]NPY (25).

Further evidence for different tertiary structures in the PYY forms we studied is found in the NMR data. NMR chemical shifts are very sensitive to local changes in secondary and tertiary structure. Although these chemical shifts cannot be reliably translated into tertiary structure, they do provide a qualitative measure of structural alterations. The primary structure of PYY, [Pro^{34}]PYY, and PYY-(3–36) are very similar. If their 3-D conformations were the same, only small changes in the pattern of aromatic and amide proton signals would occur. We observed marked changes in the pattern of these signals (Fig. 3), consistent with significant changes in the tertiary structure of the peptides.

It cannot be directly established from our data whether the observed differences in secondary and tertiary structures of PYY forms are responsible for Y receptor subtype selectivity. However, such a mechanism for the three PYY peptides analyzed here is consistent with the NMR and CD spectra and receptor binding data. A plausible model to explain our findings (on the basis of the PP fold structure) is shown in Fig. 5. In this model, we postulate that the three analogs exist in three different tertiary structures produced by their slightly altered primary structures. For PYY, the structural element that confers its ability to bind to Y_1 is the juxtaposition of its NH_2 and COOH termini; for Y_2 binding, the selective element is the COOH-terminal helix. PYY contains both of these structural elements and binds potently to both receptor subtypes. The proline in [Pro^{34}]PYY disrupts the COOH-terminal helix, thereby diminishing the binding to Y_2 receptor subtype. The removal of the NH_2-terminal dipeptide from PYY to form PYY-(3–36) eliminates the juxtaposition of the two termini, thus reducing binding to the Y_1 receptor subtype. This model is supported by similar proposals by others for Y_1- and Y_2-selective NPY analogs (2, 13).

In summary, a model for the 3-D conformation of PYY and its receptor subtype selective analogs has been generated from direct structural studies of monomeric peptides in solution. This model provides a concrete picture of how subtle changes in the primary amino acid sequences in PYY may result in dramatic alterations in tertiary structure that lead to receptor subtype selectivity.

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