Ser[13]-phosphorylated PYY from porcine intestine with a potent biological activity

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Abstract We have isolated a posttranslationally modified form of peptide YY (PYY) from porcine intestine and shown by MALDI-TOF and electrospray tandem mass spectrometry that it is phosphorylated at Ser[13]. Phospho-PYY exhibits high affinity for binding to neuropeptide Y (NPY) receptors Y1, Y2 and Y5. The IC_{50} values with the Y1, Y2, and Y5 receptor subtypes were for NPY 2.4, 3.1, and 3.3 nM, for PYY 2.3, 0.94, and 3.2 nM, and for phospho-PYY 4.6, 2.2, and 5.5 nM, respectively. Phospho-PYY potently inhibits forskolin-stimulated cAMP accumulation in SK-N-MC cells with an IC_{50} value of 0.5 nM compared to 0.15 nM for non-phosphorylated PYY. The finding of phosphorylation of PYY is unusual among hormonal peptides, and emphasizes the importance of direct protein analysis of gene products.

Key words: Peptide hormone phosphorylation; Casein kinase; cAMP production; Receptor binding; Mass spectrometry

1. Introduction

Peptide YY (PYY) is a 36 residue peptide originally isolated from porcine upper small intestine using a chemical method to detect the C-terminal Tyr-amide structure [1,2]. PYY, pancreatic polypeptide (PP) [3], and neuropeptide Y (NPY) [4] are structurally similar and form a peptide family. However, they are expressed in different tissues and have distinct functions [5,6]. While PYY and PP are mainly found in gastrointestinal mucosa and pancreas and are involved in the control of digestive functions, NPY is the most abundant neuropeptide present in the nervous systems [7,8]. PYY is present in the endocrine cells of both the intestine and the pancreas (A-cells) [9]. PYY mRNA is also expressed in a few neuronal cell populations of the rat [10,11], and PYY-like immunoreactivity has been observed in gut neurons of several mammals [12]. PYY is released in response to meals and inhibits gastric and pancreatic secretion as well as gastric and intestinal mobility [5].

Several receptors have been identified that bind all three peptides, but with different affinities [8,13–15]. The receptors belong to the large family of G protein-coupled receptors and are denoted as Y receptor subtypes (Y1, Y2, Y4, Y5, y_6). PYY and NPY bind with almost equal affinity to Y1 and Y2 receptors, but PP has significantly lower affinity towards these. The Y5 receptor is believed to play a role in orexigenic properties of NPY [16].

In the present study we have isolated a novel form of PYY. The new form is phosphorylated at Ser[13], defining a class of gastrointestinal peptide hormones conjugated with a phosphate group.

2. Materials and methods

2.1. Purification of phospho-PYY

A concentrate of thermostable intestinal peptides (CTIP) was prepared from porcine intestines, and an aqueous solution of CTIP was fractionated with ethanol [17], and a fraction precipitating at pH 7.2 ± 0.1 prepared as described [18].

The first HPLC purification step was performed under the same conditions as reported earlier [18], but the purification now followed measurements of cAMP production in SK-N-MC cells. An active fraction eluting between 150 and 160 ml was collected and used in the subsequent purification.

The second cation exchange HPLC step was carried out using the same column at a lower pH. The column was equilibrated with 20 mM Na-phosphate (pH 2.5) containing 20% acetonitrile (buffer A), the sample was diluted 10 times with water, the pH adjusted to 2.5, and loaded onto the column. The peptides were eluted with a gradient of 9–23.5% buffer B, which consisted of the equilibration buffer containing 2 M NaCl. The elution was completed in 30 column volumes (CV) at a flow rate of 20 ml/min.

The third step was a reverse phase (RP) HPLC on a YMC ODS-AP, 10×100 mm column (5 μm, 300 Å). Eluent A was 0.1% trifluoroacetic acid (TFA)/water, and eluent B was 0.1% TFA/95% acetonitrile/water, the gradient was 20–40% B in 30 CV. The peak fraction with cAMP producing activity was further purified through another step of RP-HPLC.

The final purification of phospho-PYY by RP-HPLC was carried out on a μRPC C2/C18 column (4.6×100 mm, Amersham Pharmacia Biotech) (Fig. 1) eluted with the solvent system consisting of eluent A, 0.1% TFA/water, and eluent B, 0.1% TFA/95% acetonitrile/water. A gradient of 30–45% B in 15 CV was used. The peak fraction was analyzed.

2.2. Sequence analysis

Edman degradation was carried out using a Procise HT instrument

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(Applied Biosystems). Samples were applied to Biobrene-treated glass fiber filter discs.

2.3. Tryptic cleavage and desalting

Trypsin cleavage was carried out in 0.1 M ammonium bicarbonate at 37°C using modified enzyme from Promega (0.2 μg). The peptides in the digest were either separated by RP-HPLC or desalted on a Poros R3 resin (Perseptive Biosystems), where the peptides were eluted with 60% acetonitrile containing 1% acetic acid.

2.4. Mass spectrometry

Mass spectrometric data were acquired on a hybrid quadrupole time-of-flight (Q-TOF) mass spectrometer (MS) (Micromass, Manchester, UK), a Voyager DE Pro (Perseptive Biosystems, Framingham, MA, USA), and a Finnigan Lasermat 2000 (Thermo Bioanalysis, UK) as described [19,20].

2.5. Receptor binding assay

Cells used in the binding experiments with NPY receptor subtypes were SK-N-MC for the NPY1, KAN-Ts for the NPY2, and HEK293 transfected with human NPY5 cDNA for the NPY5 receptor. Cells were grown to confluence on 150 cm^2 tissue culture plates, washed with phosphate-buffered saline (PBS), and scraped into 50 ml tubes. After centrifugation, the supernatant was aspirated, and the pellets frozen and stored at −80°C. Thawed pellets were homogenized with a tissue grinder for 8 s in 20 mM Tris–HCl, 2 mM EDTA. The homogenate was centrifuged at $800\times g$ for 5 min and the collected supernatant recentrifuged at $25\,000\times g$ for 25 min. The resulting pellet was resuspended in binding buffer (20 mM HEPES, 120 mM NaCl, 0.22 mM KH_2PO_4, 1.3 mM $CaCl_2$, 0.8 mM $MgSO_4$). Membranes were incubated with [^{125}I]PYY (300 pM) in the presence or absence of test compound for 1 h at room temperature. The reaction was stopped by centrifugation and subsequent removal of the supernatant. The pellets were washed once with PBS and the radioactivity was counted in a Packard Cobra gamma counter. Specific binding to the NPY receptor subtypes was determined by radioactivity that was bound in the presence of 300 nM NPY.

2.6. cAMP accumulation assay

SK-N-MC human neuroblastoma cells that express NPY1 receptors were plated in 96 well plates. When confluent, DMEM–F12 medium was replaced with 100 μl/well fresh medium supplemented with 2 mM isobutylmethylxanthine. After 20 min, cells were exposed to PYY or phospho-PYY at various concentrations. After 10 min, forskolin was added to a final concentration of 1 μM. After an additional 20 min, 0.2 vol 0.5 N HCl was added to each well. Cells were kept frozen overnight, thawed, and a 20 μl aliquot of the medium was transferred to a cAMP Flashplate (NEN, Boston, MA, USA). [^{125}I]cAMP was added and the subsequent radioimmunoassay (RIA) was measured using a Packard Topcount microplate reader after overnight incubation at 4°C.

2.7. Assay for insulin release from pancreatic islets

The effect of peptide fractions on glucose-induced insulin release was monitored in a system of isolated rat pancreatic islets [21]. After incubation, samples of the medium were analyzed with RIA of insulin [21].

3. Results and discussion

3.1. Purification

Fractions from pig intestinal extracts were screened for peptides which increased cAMP production in SK-N-MC cells. Further purification separated phospho-PYY from these and other contaminating peptides (Fig. 1), and the pure fraction was subjected to structural analysis.

3.2. Structural analysis

The purified peptide was analyzed by Edman degradation that revealed a sequence corresponding to porcine PYY.

The molecular mass of the peptide was determined by matrix-assisted laser desorption ionization (MALDI)-TOF and electrospray (ES) mass spectrometries (MS), and a monoisotopic mass value of 4318.3 Da was obtained. The result indicates that the peptide has an about 80 Da higher mass than predicted from the amino acid sequence of PYY (4238.13 Da) suggesting that the peptide is phosphorylated or sulfated.

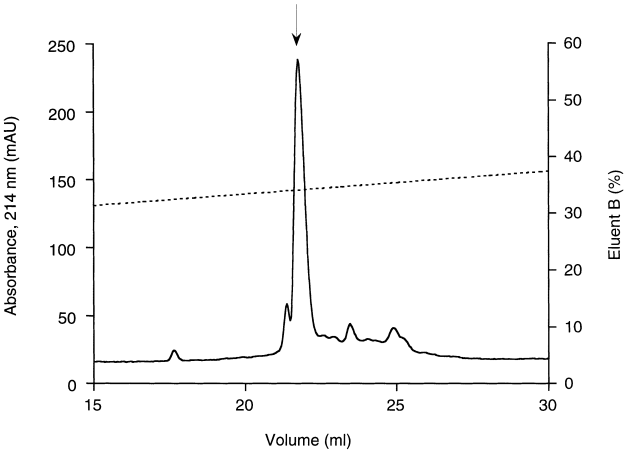

Fig. 1. Final purification of phospho-PYY from porcine intestine by RP-HPLC. Column, μRPC C2/C18 (4.6×100 mm); mobile phase, 0.1% TFA/water (eluent A), 0.1% TFA/95% acetonitrile/water (eluent B); gradient 30–45% eluent B in 15 column volumes. The fraction marked with the arrow was analyzed.

To identify the modification and its position, the purified peptide was digested with trypsin. Analysis of the resulting peptides by MALDI-TOF MS indicated that the modified residue is in the N-terminal tryptic fragment, PYY (residues 1–19; YPAKPEAPGEDASPEELSR). This fragment was investigated using ES-MS/MS. The triply charged form of the peptide (*m/z* 708.31) was subjected to collision-induced dissociation (CID) in the positive ion mode. Series of y- and b-type ions were detected and the y-ions following y_7^+ (at *m/z* 897.40) were found to be shifted (Fig. 2). Interpretation of the product ion spectrum showed that the 80 Da modification, $-HPO_3$ (79.9663 Da) or $-SO_3$ (79.9568 Da) was located to Ser^{13}. To further ascertain the identity of the modification the peptide was analyzed in the negative ion mode. The doubly deprotonated parent ion (*m/z* 1059.95) was subjected to CID. The product ion spectrum showed an intense peak at 78.9664, characteristic of PO_3^-, indicating the presence of a phosphate group in the peptide. From these results we conclude that the native PYY is phosphorylated at Ser^{13}.

Ser^{13}-phospho-PYY was synthesized and analyzed by ES-MS/MS. The mass spectra of the synthetic peptide were identical to those of the peptides isolated from the intestinal material, confirming the structure.

Phosphorylation in PYY is probably catalyzed by a protein kinase, most likely casein kinase [22], which recognizes the sequence –Ser/Thr-X-Glu/Asp–, where X is any amino acid. Indeed, PYY contains such a consensus sequence near Ser^{13}, where X = Pro.

The peptides in the PP/NPY/PYY family are highly conserved among different animal species. Comparison of these three porcine peptides with PYY from several other species indicates that within PYY, the serine residue at position 13 is conserved throughout all characterized mammalian species (Fig. 3). It is therefore probable that phosphorylation of PYY at Ser^{13} may also occur in other species, including humans. However, the other two family members, PP and NPY,

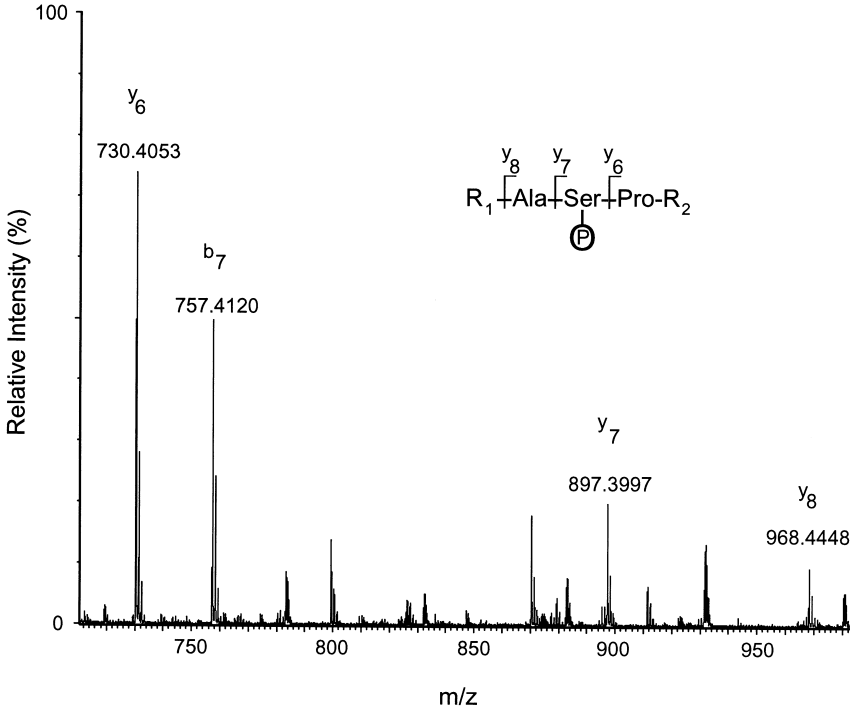

Fig. 2. Tandem MS analysis of phospho-PYY. CID spectrum of the triply charged ion at *m/z* 708.31 of the N-terminal tryptic peptide. The modified product ions y_7 and y_8 are shifted by 79.97 Da. The samples were infused from a nanospray needle at 900–1700 V with cone voltage at 40 V, and argon was used as the collision gas.

are different at just this position. Peptide PP has Thr at position 13. Although Thr may also be phosphorylated in biological systems, this often appears less decisive, and it is therefore possible that the PP molecule is quite different in sensitivity to phosphorylation. More differently, the NPY sequence has a Pro at position 13, which excludes the existence of an NPY phosphopeptide involving that position.

3.3. Biological activity

Phospho-PYY was assayed in SK-N-MC cells for cAMP production and for inhibition of forskolin-stimulated cAMP production (Fig. 4). We found that PYY and phospho-PYY had no effect on basal cAMP production in SK-N-MC cells up to micromolar concentrations. Phospho-PYY strongly inhibited forskolin-stimulated cAMP production in these cells similarly to PYY. The IC_{50} value for phospho-PYY was 0.5 nM, and for non-phosphorylated PYY it was 0.15 nM. We also tested the effects of phospho-PYY on several NPY receptor subtypes and found that it had high affinity for Y1, Y2, and Y5 receptors as determined by displacement of [^{125}I]PYY. The IC_{50} values with the Y1, Y2, and Y5 receptor subtypes were for NPY 2.4, 3.1, and 3.3 nM, for PYY 2.3, 0.94, and 3.2 nM, and for phospho-PYY 4.6, 2.2, and 5.5 nM, respectively.

The effect of phospho-PYY on insulin secretion was tested in isolated pancreatic islets from rats at different concentrations and was compared to that of unmodified PYY. No effect was observed either with synthetic unmodified PYY or with synthetic phospho-PYY. Similarly, PYY was without effect on basal and glucose-induced insulin release when infused in fed anesthetized rats [23].

Phosphorylation is a general regulatory mechanism that occurs in cellular proteins. However, phosphorylation of bioactive peptides or their precursors has been described earlier only in a few cases, such as ACTH, proenkephalin A, chromogranin A, and a few others [24–28]. Proenkephalin A-derived peptides have been shown to acquire antibacterial properties upon phosphorylation [29].

The exact amount of phosphorylated PYY could not be determined from mass spectrometry but phospho-PYY is a minor form, compared to its non-phosphorylated counterpart.

The finding of phospho-PYY is unusual, since it represents phosphorylation in a secreted hormonal peptide. Given the slightly lower receptor binding and inhibitory activities of cAMP production of the phosphorylated PYY than of the non-phosphorylated peptide, phosphorylation is not a mechanism to switch the product on or off, but could participate in fine regulation of PYY activity. Moreover, the peptide may specifically recognize a yet undiscovered receptor subtype, or be involved in a yet unknown biological function for this peptide. It is also possible that phosphorylation may alter the stability of the peptide in its biological environment.

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PYY	pig	YPAKPEAPGEDA**S**PEELSRYYASLRHYLNLVTRQRY
NPY	pig	YPSKPDNPGEDA**P**AEDLARYYSALRHYINLITRQRY
PP	pig	APLEPVYPGDDA**T**PEQMAQYAAELRRYINMLTRPRY
PYY	human	YPIKPEAPGEDA**S**PEELNRYYASLRHYLNLVTRQRY
PYY	rat	YPAKPEAPGEDA**S**PEELSRYYASLRHYLNLVTRQRY
PYY	bovine	YPAKPQAPGEHA**S**PDELNRYYTSLRHYLNLVTRQRF
PYY	chicken	AYPPKPESPGDAA**S**PEEIAQYFSALRHYINLVTRQRY

Fig. 3. Alignment of the amino acid sequences of PYY from different species with those of porcine NPY and PP. Position 13 is highlighted by bold type and underlined.

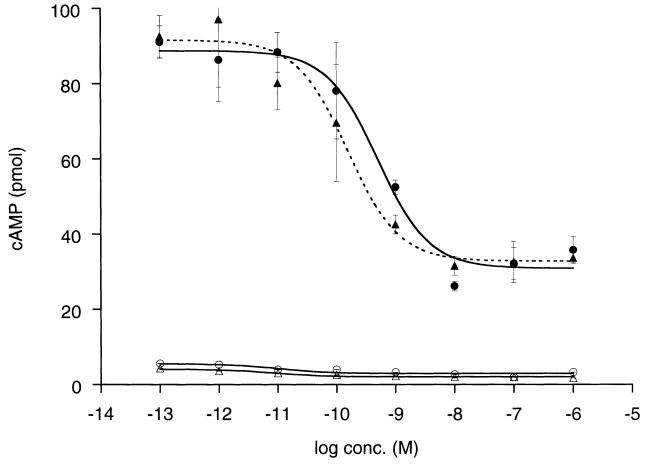

Fig. 4. Interaction of phospho-PYY with NPY subtypes. Effect of PYY (triangles) and phospho-PYY (circles) on basal (empty symbols) and forskolin-stimulated cAMP accumulation (filled symbols) in SK-N-MC cells. The cells were incubated with peptides in the presence and absence of forskolin and the accumulated cAMP was measured using the Flashplate technique.

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