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Localization and characterization of neuropeptide Y binding sites in porcine and human colon

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1 We have used quantitative receptor autoradiography to investigate the localization and characteristics of binding sites for ^{125}Iodine-Bolton Hunter-labelled human neuropeptide Y ([^{125}I]-BH-NPY) in porcine and human colon, and compared the binding characteristics with those found in porcine spleen.

2 Saturable, specific, high affinity [^{125}I]-BH-NPY binding was localized to myenteric ganglia in porcine and human colons, and to submucosal ganglia in porcine colon.

3 Specific [^{125}I]-BH-NPY binding to porcine myenteric ganglia was reversible in the presence of guanosine 5'-O-(3-thiotriphosphate) and was inhibited by related peptides with the rank order of potency; porcine NPY = human NPY = peptide tyrosine tyrosine (PYY) >> pancreatic polypeptide.

4 The Y_2 selective analogue, NPY(13–36), competed for [^{125}I]-BH-NPY binding to porcine myenteric ganglia with greater potency than the Y_1 selective analogue, [Leu^{31}, Pro^{34}]NPY, the difference being small, but significant.

5 The characteristics of [^{125}I]-BH-NPY binding to porcine myenteric ganglia were similar to those observed concurrently to porcine splenic red pulp.

6 The small difference in inhibitory potencies between NPY(13–36) and [Leu^{31}, Pro^{34}]NPY observed in this study in comparison with previous studies was not explained by differential ligand depletion during incubations, but may be due to differences in methodology between binding studies performed on tissue sections and on membranes.

7 We conclude that specific [^{25}I]-BH-NPY binding sites are present in the myenteric and submucosal ganglia of the colon and that these sites may act as functional receptors by which NPY and PYY modulate colonic motility and electrolyte transport.

Keywords: Neuropeptide Y; myenteric plexus; colon; receptors; peptide YY; autoradiography; spleen

Introduction

Neuropeptide Y (NPY) is a 36 amino acid regulatory peptide initially isolated from brain as a consequence of its C-terminal amide residue (Tatemoto *et al.*, 1982). NPY-like immunoreactivity (NPY-LI) is widely distributed in peripheral tissues. In human and porcine colon, NPY-LI is present in sympathetic nerves, particularly around blood vessels, and also in a proportion of intrinsic nerves in the myenteric and submucosal plexi (Ferri *et al.*, 1984). In the spleen, NPY-LI has similarly been demonstrated in perivascular sympathetic nerves (Lundberg *et al.*, 1988a).

NPY is a member of a family of regulatory peptides which includes the structurally related pancreatic polypeptide (PP) and peptide tyrosine tyrosine (PYY). The peptides NPY and PYY share many biological actions (Sheikh, 1991), but have markedly different distributions in peripheral tissues. PYY-like immunoreactivity (PYY-LI) has been demonstrated in endocrine cells in both human and porcine colon, but not in sympathetic or intrinsic neurones (Lundberg *et al.*, 1982), whereas NPY-LI has not been demonstrated in intestinal endocrine cells.

PYY-LI is released into the systemic circulation in mammals following feeding (Adrian *et al.*, 1985; Taylor, 1985), and may therefore act as a circulating hormone. In addition, local release of NPY and PYY may serve paracrine, autocrine or neurotransmitter functions. NPY and PYY each affect intestinal smooth muscle motility, probably by modifying neurotransmitter release from myenteric neurones rather than by a direct effect on intestinal smooth muscle (Lundberg *et al.*, 1982; Hellstrom *et al.*, 1985; 1989; Hellstrom, 1987; Cadieux *et al.*, 1990). NPY and PYY inhibit secretion in both the small and large intestines (Okuno *et al.*, 1992).

NPY and PYY act through specific cell surface, G-protein-linked receptors. Pharmacological and molecular biological techniques have indicated the existence of at least 3 subclasses of NPY receptor, referred to as Y_1, Y_2 and Y_3 (Wahlestedt *et al.*, 1990; Rimland *et al.*, 1991). The analogue [Leu^{31}, Pro^{34}]NPY has been proposed as a specific ligand and agonist at the Y_1 receptor (Fuhlendorff *et al.*, 1990), while Y_2 receptors bind and can be activated by long C-terminal fragments of NPY, including the fragment NPY(13–36). The recently cloned Y_3 receptor demonstrates a higher affinity for NPY than PYY, by contrast to the Y_1 and Y_2 receptors which exhibit equal affinities for these two endogenous peptides, or a slightly higher affinity for PYY (Rimland *et al.*, 1991).

Quantitative *in vitro* receptor autoradiography allows the pharmacological characterization of anatomically localized binding sites (Palacios & Dietl, 1989). We have now used this technique to investigate the localization and characterization of binding sites for ^{125}Iodine-Bolton Hunter-labelled human NPY([^{125}I]-BH-NPY) in human and porcine colon in an attempt to characterize NPY receptors involved in the modulation of colonic motility and secretion. We have, furthermore, compared the characteristics of [^{125}I]-BH-NPY binding in porcine colon with that in porcine spleen, the latter having been extensively characterized previously in membrane preparations as a Y_2 subclass of receptor (Lundberg *et al.*, 1988a,b; Modin *et al.*, 1991).

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Methods

Tissues

Porcine spleens ($n = 12$) and colons ($n = 12$) were obtained fresh from a local abattoir within 15 min postmortem. Normal human colonic tissues ($n = 7$) were obtained from the resection edge of colonic carcinomata and human spleens ($n = 6$) were obtained post-mortem or at operative splenectomy. Rat spleens ($n = 6$) were obtained from adult Wistar rats immediately after they had been killed by an overdose of phenobarbitone. All tissues were snap frozen in melting dichlorodifluoromethane (Arcton-12, ICI), and stored at −70°C until cut to 10 μm sections and thaw-mounted on Vectabond treated slides (Vector Laboratories, Peterborough, UK). Mounted sections were dried at 4°C for 2 h then used immediately or stored with desiccant at −20°C until use.

Binding conditions

For binding experiments, sections were preincubated twice for 15 min in buffer A (10 mM HEPES, 130 mM NaCl, 4.7 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, pH 7.4). Excess buffer was removed and consecutive sections incubated for 2 h in 45 μl (colons) or 70 μl (spleens) buffer B (buffer A plus 1% bovine serum albumin) containing 0.2 nM [^{125}I]-BH-NPY alone (total binding), or with an excess (1 μM) of unlabelled human NPY (hNPY) (non-specific binding). Sections were washed twice for 5 min in buffer A then rinsed in distilled water and dried in a stream of cold air. Incubations, were performed in humid chambers at room temperature, which was 22°C except for the duration of analogue rebinding experiments, when the ambient room temperature was 27 to 29°C. In addition, association time course experiments were also undertaken at 37°C.

Binding conditions were optimised by comparing different buffers, incubation and wash times and ligand concentrations. Association time courses were obtained with 0.2 nM [^{125}I]-BH-NPY and dissociation time course experiments were performed following 2 h incubation with 0.2 nM [^{125}I]-BH-NPY, by the addition of unlabelled hNPY to a final concentration of 1 μM, or by transfer of sections to a bath containing 300 ml of buffer A without NPY. Saturation studies were performed with 0.05 nM to 0.8 nM [^{125}I]-BH-NPY, and also by incubation with 0.2 nM [^{125}I]-BH-NPY plus increasing concentrations of unlabelled human NPY in the range 31 pM to 4000 pM. Binding inhibition studies used 2 h incubations with 0.2 nM [^{125}I]-BH-NPY plus various concentrations of unlabelled ligand. Binding inhibition with [Leu^{31}, Pro^{34}]NPY was performed on 2 groups of 6 cases for each of porcine spleen and colon, using [Leu^{31}, Pro^{34}]NPY from 2 different sources (see below). The inhibitory potencies of the 2 batches of [Leu^{31}, Pro^{34}]NPY were directly compared on paired samples from 6 porcine spleens.

Tissue and ligand stability

The stability of [^{125}I]-BH-NPY binding sites in tissue sections, and of [^{125}I]-BH-NPY and competing ligands in supernatants, during the incubations, were assessed in order to help validate calculated kinetic, equilibrium and inhibition constants.

Possible loss of specific [^{125}I]-BH-NPY binding in tissue sections during incubations was assessed with extended preincubations. Sections of porcine colon were preincubated in buffer A for 0.5 to 5 h before incubation with 0.2 nM [^{125}I]-BH-NPY for a further 2 h.

Ligand stability was initially assessed by rebinding experiments. [^{125}I]-BH-NPY (100 μl, 0.2 nM) in buffer B, with or without the addition of peptidase inhibitors, was incubated for 0, 2 or 4 h on 10 μm sections of porcine colon. Supernatant (70 μl) was then transferred to fresh, preincubated consecutive sections of porcine colon and incubated for a further 2 h, before washing and exposure of sections to film. Separate experiments demonstrated that increasing incubation volumes from 70 μl to 100 μl did not significantly affect binding.

Possible causes of depletion of [^{125}I]-BH-NPY binding activity in supernatants during incubation with porcine colon were further investigated. Sections of porcine colon (10 μm) were incubated with 100 μl 0.2 nM [^{125}I]-BH-NPY and 25 μl aliquots of supernatant were collected before and after 2 and 4 h incubation. Aliquots were then diluted 1:19 (v/v) in incubation buffer (B).

To assess possible adherence of ligand to tissue sections or slides, 200 μl of each sample ($n = 6$ paired samples before and after incubation) was further diluted to 2 ml in acetic acid:water (5:95 v/v), and counted for radioactivity in a NE1600 multiwell counter (Nuclear Enterprises, Edinburgh).

Reverse-phase, high performance liquid chromatography (h.p.l.c.) was used to assess possible degradation of ligand; h.p.l.c. was performed on 200 μl aliquots of 6 samples (3 following preincubation with porcine colon, and 3 control samples of [^{125}I]-BH-NPY in buffer B, without preincubation) obtained as above, using a Waters dual pump system with a μBondapack C_{18} column (30 × 0.8 cm, Waters Ass., Chester, U.K.). Elution was carried out isocratically at 2 ml min^{-1} in acetic acid:water (5:95 v/v) followed by a 15 min linear gradient to propan-1-ol:acetic acid:water (40:3:57 v/v/v). One minute fractions were counted for radioactivity. Percentage yields of radioactivity are expressed as means of 3 samples. The albumin peak was identified by difference in ultraviolet absorption at 280 nm between eluates from incubation buffer (B) and preincubation buffer (A).

The major peak of radioactivity in each of 4 samples (2 before and 2 following 2 h incubation with porcine colon) were reanalysed by h.p.l.c. Solvent was removed under vacuum and each sample dissolved in acetonitrile:water:HFBA (20:100:0.08 v/v/v) and subjected to h.p.l.c. on a μBondapak C_{18} column (30 × 0.8 cm). Elution was carried out isocratically at 2 ml min^{-1} in acetonitrile:water:HFBA (20:100:0.08 v/v/v) followed by a 15 min linear gradient to acetonitrile:water:HFBA (70:100:0.08 v/v/v). One minute fractions were again counted for radioactivity.

Attempts were made to reduce ligand depletion by adding the following peptidase inhibitors to the incubation buffer; captopril (1 μM), phosphoramidon (1 μM), bestatin (20 μM), a combination of leupeptin (10 μM), bacitracin (1.5 mM) and chymostatin (5 mg l^{-1}), or all 6 antipeptidases.

Depletion of inhibitory potency of the analogues NPY (13–36) and [Leu^{31}, Pro^{34}]NPY by porcine colon and spleen, and of unlabelled hNPY by porcine colon was assessed by a modification of the method used in rebinding experiments. Buffer B (70 μl) containing unlabelled analogue (0.1 μM for colon, 0.2 μM for spleen) or hNPY (2 nM) was incubated with each tissue section for 2 h at room temperature. Supernatant from each section was then mixed with an equal volume of 0.4 nM [^{125}I]-BH-NPY and transferred to fresh, consecutive 10 μm sections of the corresponding tissue and further incubated for 2 h at room temperature. In parallel, further consecutive sections were incubated with 0.4 nM [^{125}I]-BH-NPY mixed with an equal volume of buffer B, 1 μM unlabelled hNPY, either of the analogues without prior incubation with tissue sections (final concentrations 0.5 μM and 0.1 μM for colons and spleens respectively), or unlabelled NPY (final concentration 1 nM). Sections were then washed and apposed to film as described below. The concentrations of hNPY and analogues were selected to give approximately 50% inhibition of [^{125}I]-BH-NPY binding.

Quantitation

Unfixed sections were apposed to Hyperfilm-^{3}H (Amersham, UK) and exposed for 3 days at 4°C. Films were subsequently developed in D-19 developer (Kodak) for 3 min at 20°C and fixed in Amfix (Amersham, UK). Autoradiograms were

quantified on an IBAS 2000 image analysis system (Kontron, Watford, UK). Standard curves relating grey values to log concentration of bound [^{125}I]-BH-NPY (amol mm^{-2}) were obtained for each film using sections of radiolabelled polymer standards (American Radiolabelled Chemicals Inc., St. Louis, U.S.A.). Specific binding was calculated as total minus non-specific binding. Each experiment was performed on tissue from 5 to 12 cases.

The kinetic constants for association (k_{+1}) and dissociation (k_{-1}) were derived from association and dissociation time course experiments. The rate of loss of specific [^{125}I]-BH-NPY bind-ings in tissue sections was estimated as a monoexponential decay constant from specific binding data following extended preincubations.

Equilibrium dissociation constants (K_D) and maximal binding to myenteric ganglia (B_{max}) for each of 5 porcine colons were derived from saturation experiments by non-linear regression. In addition, values were also calculated from data from kinetic experiments, K_D being k_{-1}/k_{+1}, and $B_{max} = B_{eq}$ $(K_D + L)/L$, where B_{eq} is the equilibrium binding with 0.2 nM [^{125}I]-BH-NPY, and L is the free concentration of [^{125}I]-BH-NPY (= 0.2 nM).

The concentrations of unlabelled ligands producing 50% inhibition of binding of 0.2 nM [^{125}I]-BH-NPY to myenteric ganglia (IC_{50}) were calculated for each case from binding inhibition experiments. Inhibition constant (K_i) values were derived from IC_{50} values according to the formula; $K_i = IC_{50}/(1 + L/K_D)$. For porcine colon, the K_D value used was that derived from saturation experiments. For porcine spleen, K_D was estimated from binding inhibition experiments using unlabelled NPY and the formula $K_D = IC_{50} - L$.

Curve fitting and experimental derivation of constants were performed by iterative non-linear regression using GraphPAD Inplot 3.1 (GraphPAD, San Diego). Single-site models were used for saturation and kinetic data, and a multisite model for fitting sigmoid curves to binding inhibition data. Values are expressed as arithmetic or geometric means as appropriate with 95% confidence intervals. Between groups comparisons were made on the original geometric data by paired or unpaired Student's t test or by ANOVA, as appropriate. Values of $P<0.05$ were taken as significant.

Histochemistry and microautoradiography

For microscopic localization of binding, sections with bound [^{125}I]-BH-NPY were fixed in Bouin's fixative for 60 min at 22°C, washed in distilled water and dried in cold air, then dipped in photographic emulsion (Ilford K5) and exposed for 7 days at 4°C. Dipped sections were then developed as for films (above) and counterstained with haematoxylin and eosin. In addition, for each case of porcine colon, consecutive sections to those used for the production of autoradiographic images on film were fixed in formol saline and stained for acetylcholinesterase according to the method of El-Badawi & Schenk (1967). Autoradiograms were then compared with acetylcholinesterase stained sections to establish the localization of binding of [^{125}I]-BH-NPY to cholinesterase positive ganglia.

Chemicals

[^{125}I]-BH-NPY was obtained from Amersham International plc, UK, with a specific activity of 2000 Ci mmol^{-1}. [^{125}I]-PYY (porcine) was obtained from Du Pont (UK) Ltd., Stevenage, specific activity 2200 Ci mmol^{-1}. NPY (13–36) (porcine) and [Leu^{31}, Pro^{34}]NPY (porcine) were obtained from Peninsula Laboratories plc, U.K. A further supply of [Leu^{31}, Pro^{34}]NPY was obtained from Sigma Chemical Co., Poole, UK. Human and porcine NPY, porcine peptide YY, human pancreatic polypeptide, β-cyclic calcitonin gene-related peptide, substance P, neurokinin A, guanosine 5'-O-(3-thiotriphosphate) (GTP-γ-S), bacitracin, bestatin, captopril, chymostatin, leupeptin and phosphoramidon, and enzyme-free bovine serum albumin were each obtained from Sigma Chemical Co., Poole, UK. H.p.l.c. solvents were obtained from Rathburn Chemicals, Peebleshire, Scotland.

Results

Specific [^{125}I]-BH-NPY binding sites were detected in porcine colon, localized on autoradiography films to the region of the myenteric plexus and on similar structures in the submucosal region (Figure 1a,b). [^{125}I]-PYY gave an identical distribution of specific binding. Low density non-specific binding was demonstrated in other regions of smooth muscle, submucosa and mucosa, and high density, non-specific binding at the luminal surface. Light microscopic examination of counterstained, emulsion-dipped sections of porcine colon confirmed specific binding of [^{125}I]-BH-NPY to ganglia in the myenteric and submucosal plexuses (Figure 2). Silver grains were densely distributed over a majority of ganglion cells. Comparison of autoradiography films with light microscopy of corresponding sections histochemically stained for acetylcholinesterase confirmed that specific [^{125}I]-BH-NPY binding corresponded to acetylcholinesterase-positive ganglia in both the myenteric and submucosal plexi. Specific binding of [^{125}I]-BH-NPY was not identified on vascular smooth muscle under these conditions. In 5 of 7 cases of normal human

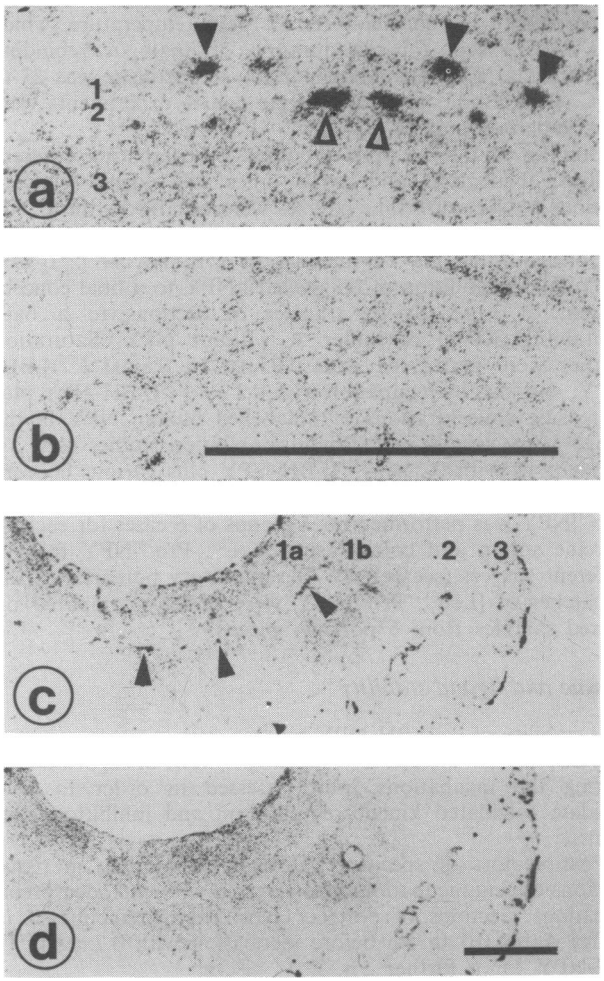

Figure 1 Film autoradiographs of [^{125}I]-Bolton Hunter-labelled human neuropeptide Y binding to porcine (a,b) and human (c,d) colon: total binding (a,c); non-specific binding (b,d); closed arrow-heads myenteric ganglia; open arrow-heads submucosal ganglia; (1) muscle ((1a) longitudinal muscle, (1b) circular muscle); (2) submucosa; (3) mucosa. Bars = 2 mm.

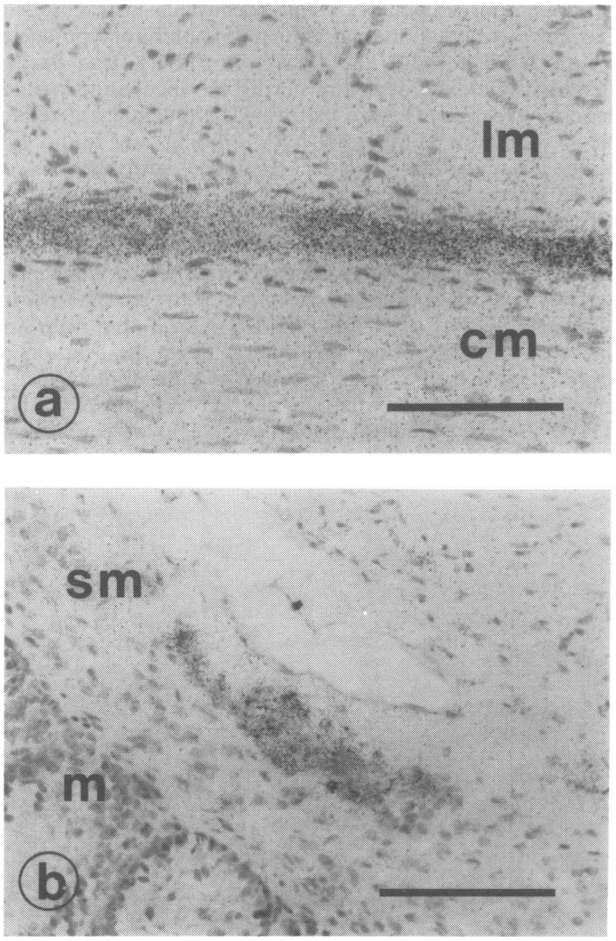

Figure 2 Photomicrographs of emulsion-dipped sections of porcine colon: myenteric ganglion (a); submucosal ganglion (b); (lm) longitudinal muscle, (cm) circular muscle, (sm) submucosa, (m) mucosa. Haematoxylin and eosin counterstained. Bars = 100 μm.

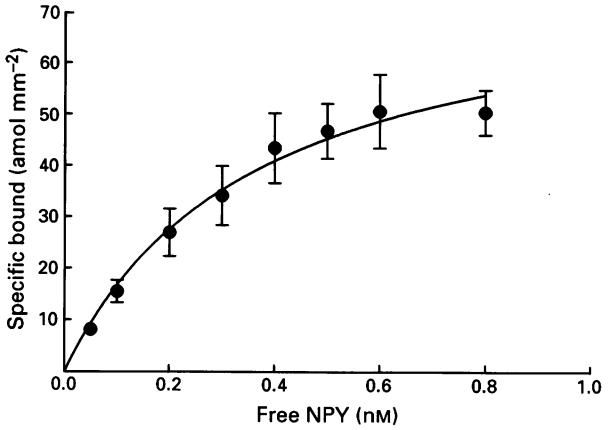

Figure 3 Saturation of specific [^{125}I]-Bolton Hunter-labelled neuropeptide Y binding to porcine colonic myenteric ganglia. Each point represents the mean (s.e.mean, vertical bars) of 5 cases.

colon similar specific binding of [^{125}I-BH-NPY was localized to ganglia in the myenteric plexus (Figure 1c,d).

Equilibrium studies indicated that binding of [^{125}I]-BH-NPY to porcine colonic myenteric ganglia was saturable, and of high affinity (Table 1, Figure 3). High affinity binding was confirmed in kinetic studies (Table 1), which revealed that specific binding of 0.2 nM [^{125}I]-BH-NPY to myenteric ganglia reached a maximum within 2 h at room temperature (Figure 4). At 37°C specific binding reached a maximum by 30 min but rapidly declined with increasing incubation times beyond 50 min. All subsequent experiments were therefore performed at room temperature.

Dissociation of ligand from the myenteric plexus following the addition of 1 μM unlabelled pNPY was slow, with > 50% of equilibrium binding remaining after 5 h (Figure 4). Similar results were obtained by transferring labelled sections to a bath containing 300 ml buffer A without NPY. The addition of the non-hydrolysable GTP analogue, GTP-γ-S to the dissociation buffer greatly increased dissociation of specific [^{125}I]-BH-NPY binding from the myenteric plexus. After 2 h in the presence of GTP-γ-S (2 μM) specific binding was reduced to 9% (7–12%) of equilibrium binding, as compared with 77% (69–85%) in the absence of GTP-γ-S.

There was a gradual decline in specific binding of [^{125}I]-BH-NPY to myenteric ganglia with increasing preincubation times. The rate of decline in specific binding was similar to that observed following the addition of unlabelled NPY in the absence of GTP-γ-S (decay constant = 5.5 (2.5–12.0) $\times 10^{-5}$ s^{-1}). Furthermore, a similar decline in specific binding to myenteric ganglia was observed with prolonged incubations with [^{125}I]-BH-NPY beyond 2 h.

The binding activity of 0.2 nM [^{125}I]-BH-NPY was reduced following a 2 h incubation with sections of porcine colon when compared with that of fresh ligand. Specific binding to porcine colon myenteric ganglia was reduced from 104 (83–130) amol mm^{-2} to 54 (48 to 61) amol mm^{-2} ($P < 0.0005$). Addition of phosphoramidon, captopril, bestatin, leupeptin, chymostatin or bacitracin, alone or in combination, to the incubation buffer did not significantly increase specific binding to porcine myenteric ganglia, nor did it prevent the decline in NPY binding activity in the supernatants.

Radioactivity counts in supernatants from this experiment, representing total ^{125}I not bound to the solid phase, did not reveal any significant decline during the 2 h incubation, from 1740 (1588 to 1892) c.p.m. to 1588 (1524 to 1654) c.p.m. ($P > 0.05$).

H.p.l.c. on a propan-1-ol:acetic acid gradient of control, standard samples not preincubated with tissue sections (Figure 5a) resulted in a single peak of radioactivity with a retention time of 12 min (h.p.l.c. fractions 15 and 16) appearing shortly after the albumin peak (retention time 11 min, fraction 14). Radioactivity in this peak accounted for 91% of that injected.

Table 1 Kinetic and equilibrium constants for [^{125}I]-Bolton Hunter-labelled neuropeptide Y binding to porcine colon myenteric ganglia

Experimental design	k_{+1} ($M^{-1}s^{-1}$)	k_{-1} (s^{-1})	K_D (pM)	B_{max} (amol mm^{-2})
Saturation	–	–	409 (220–760)	81 (70–93)
Kinetic	9 (4–20) $\times 10^5$	3.2 (1.9–5.5) $\times 10^{-5}$	60 (20–230)	128 (60–279)

Values are expressed as geometric means (95% CI), $n = 5$.

Following 2 h incubation with porcine colon, h.p.l.c. again produced a major peak of radioactivity with a retention time of 12 min, accounting for 83% of that injected, as well a minor peak with a retention time of 9 min, (h.p.l.c. fractions 12 and 13) accounting for a further 6% of injected radioactivity (Figure 5b). Further h.p.l.c. of the major peaks from 2 samples with and 2 without incubation with porcine colon using an acetonitrile gradient revealed a single peak of radioactivity in all cases with a retention time of 10 min (fractions 13 and 14) and no minor peaks, with a yield of 88%.

Binding inhibition analysis on 6 porcine colons demonstrated relative affinities for the [^{125}I]-BH-NPY binding sites in myenteric ganglia of pNPY = hNPY = pYY >> NPY (13–36) ≥ [Leu^{31}, Pro^{34}] NPY >> PP (Table 2, Figure 6). The unrelated peptides substance P (1 μM), neurokinin A (1 μM) and calcitonin gene-related peptide (1 μM) did not significantly inhibit [^{125}I]-BH-NPY binding to myenteric ganglia.

To investigate further the relative potencies of the selective Y_1 and Y_2 ligands, [Leu^{31}, Pro^{34}]NPY and NPY (13–36) in inhibiting binding of [^{125}I]-HB-NPY to porcine colon, binding inhibition studies were performed on samples from an additional 6 pigs using [Leu^{31}, Pro^{34}]NPY obtained from Sigma Chemical Co., Poole, UK, as well as NPY (13–36). Results from this experiment were closely similar to those obtained previously, and combining the results from colons of all 12 pigs revealed NPY (13–36) to be slightly, but significantly, more potent at inhibiting specific [^{125}I]-BH-NPY binding to porcine colonic myenteric ganglia (Table 2) with a mean difference in log IC_{50} = 0.4 (0.04 to 0.75, P = 0.032). None the less, NPY (13–36) and [Leu^{31}, Pro^{34}]NPY were each able to inhibit completely specific [^{125}I]-BH-NPY binding to porcine myenteric ganglia, with Hill coefficients near unity.

To assess whether the closely similar inhibitory potencies of both NPY (13–36) and [Leu^{31}, Pro^{34}]NPY was due to differential analogue depletion during the incubation period, rebinding experiments were performed. No significant reduction in inhibition of [^{125}I]-BH-NPY binding was found following preincubation of either analogue with sections of porcine colon.

Specific [^{125}I]-BH-NPY binding sites were also demonstrated on porcine spleen, located over red pulp. Only non-specific binding could be demonstrated either to white pulp arterioles, or to splenic arteries. As with porcine colon, equilibrium binding of 0.2 nM [^{125}I]-BH-NPY to red pulp was achieved within 2 h. There was no significant dissociation of specific [^{125}I]-BH-NPY binding in red pulp following 2 h incubation in buffer containing neither [^{125}I]-BH-NPY nor GTP-γ-S (specific binding 103% (85–123%) of equilibrium binding). Addition of 2 μM GTP-γ-S to the dissociation

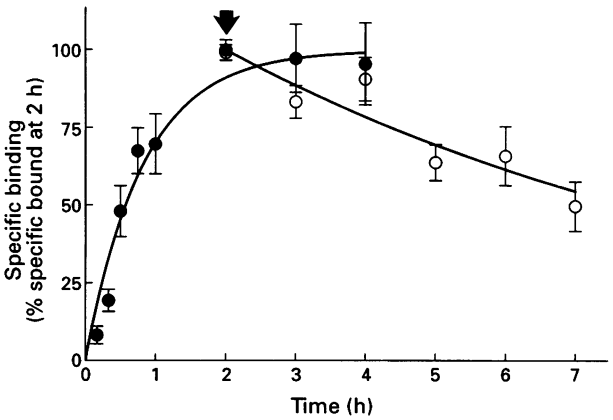

Figure 4 Association (●) and dissociation (○) time courses of specific binding of 0.2 nM [^{125}I]-Bolton Hunter-labelled neuropeptide Y to myenteric ganglia in porcine colon at room temperature. Arrow; addition of 1 μM unlabelled NPY for dissociation time course. Each point represents the mean (± s.e.mean, vertical bars) of 5 cases.

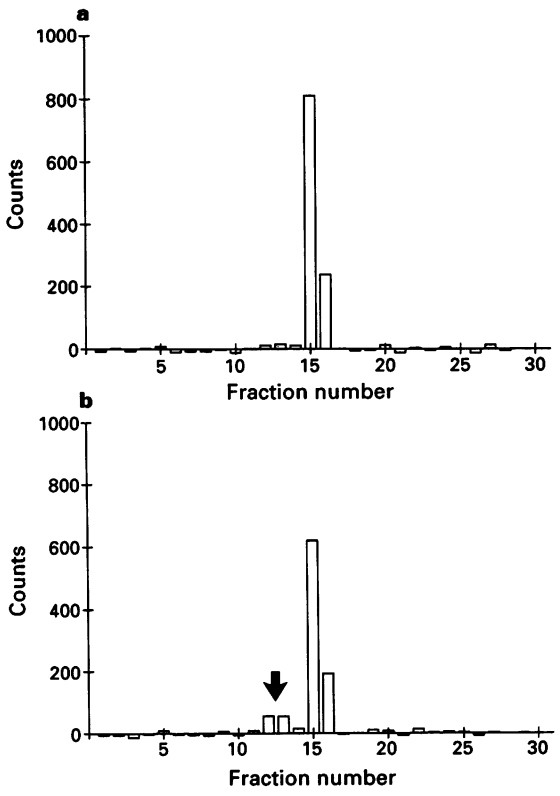

Figure 5 High performance liquid chromatography of [^{125}I]-Bolton Hunter-labelled neuropeptide Y on a propanol/acetic acid gradient (a) before, and (b) after 2 h incubation with sections of porcine colon. One minute samples of eluent were counted for 30 s and background subtracted. Arrow; second peak of radioactivity appearing after incubation.

Table 2 Inhibition of binding of 0.2 nM [^{125}I]-Bolton Hunter-labelled neuropeptide Y ([^{125}I]-BH-NPY) to porcine colon by unlabelled peptides

Ligand	*IC_{50}* (nM)		*K_i* (nM)		*Hill coefficient*	
pNPY	0.29	(0.19–0.44)	0.19	(0.12–0.30)	1.29	(0.75–2.22)
hNPY	0.35	(0.21–0.60)	0.24	(0.14–0.40)	1.56	(1.16–2.08)
PYY	0.44	(0.27–0.74)	0.30	(0.18–0.50)	1.65	(1.29–2.10)
[Leu^{31}, Pro^{34}]NPY	27*	(18–42)	18	(12–28)	1.14	(1.03–1.26)
NPY (13–36)	11*	(6–21)	7	(4–14)	1.13	(0.88–1.46)
PP	670	(410–1060)	450	(275–712)		

Values are expressed as geometric means (95% CI). *NPY (13–36) more potent than [Leu^{31}, Pro^{34}]NPY, P = 0.032, paired t test, n = 12. p = porcine; h = human; PYY = peptide tyrosine tyrosine; PP = pancreatic polypeptide

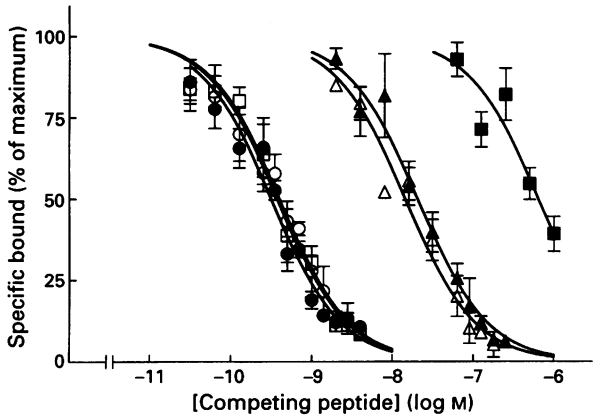

Figure 6 Competitive inhibition of 0.2 nM [^{125}I]-Bolton Hunter-labelled neuropeptide Y ([^{125}I]-BH-NPY) binding to porcine colon myenteric plexus by unlabelled peptides: (●) porcine NPY; (○) human NPY; (□) porcine peptide tyrosine tyrosine; (▲) [Leu^{31}, Pro^{34}] NPY; (△) NPY (13–36); (■) pancreatic polypeptide. Each point represents the mean (± s.e. mean, vertical bars) of 6 cases.

buffer significantly decreased specific binding to 15% (10–24%) of equilibrium binding after 2 h.

Binding inhibition studies on 12 porcine spleens indicated that, as with porcine colon, NPY (13–36) was slightly but significantly, more potent than [Leu^{31}, Pro^{34}]NPY at inhibiting specific [^{125}I]-BH-NPY binding to porcine splenic red pulp (Table 3, mean difference in log IC_{50} values = 0.53 (0.09–0.96), $P = 0.02$). Two separate batches of [Leu^{31}, Pro^{34}]NPY were tested on consecutive sections of 6 porcine spleens. Only small differences in inhibitory potencies were observed between batches with IC_{50} values of 1.0 (0.8–1.4) $\times 10^{-7}$ M and 3.5 (1.6–7.4) $\times 10^{-7}$ M respectively.

Rebinding experiments were performed on sections of porcine spleen to assess possible differential analogue depletion during incubations. No significant diminution in inhibitory potency was observed for either [Leu^{31}, Pro^{34}]NPY or NPY (13–36) following 2 h incubation with porcine spleen.

In contrast to the porcine spleen, specific [^{125}I]-BH-NPY binding was not demonstrable in human spleens, nor in normal rat spleen under the conditions tested.

Discussion

We have demonstrated specific, saturable, high affinity binding sites for [^{125}I]-BH-NPY in ganglia of the myenteric and submucosal plexuses of normal porcine colon. Specific binding showed similar high affinity for both NPY and PYY suggesting that these sites may represent shared NPY/PYY receptors. NPY-like immunoreactivity (NPY-LI) is present in nerve fibres and occasional ganglion cells of both myenteric and submucosal plexi in the colon of most mammalian species so far studied, including pig and man (Furness *et al.*, 1983; Ferri *et al.*, 1984). PYY-LI is localized to mucosal endocrine cells in mammalian colon (Lundberg *et al.*, 1982) and is released into the circulation following the ingestion of a meal (Taylor, 1985; Greeley *et al.*, 1989).

Both NPY and PYY have pharmacological actions in mammalian colon. Both were shown in the cat and guinea-pig to reduce colonic motility, induce colonic relaxation, and also to reduce tone in precontracted colon (Lundberg *et al.*, 1982; Hellstrom *et al.*, 1985; Hellstrom, 1987; Wiley & Owyang 1987). Subsequent studies have shown that NPY and PYY increase rectal tone in the cat and, similarly, increase basal tone in the rat distal colon (Hellstrom *et al.*, 1989; Cadieux *et al.*, 1990). Each of these effects of NPY may be mediated by actions on myenteric neurones, rather than by a direct action on colonic smooth muscle (Hellstrom, 1987; Wiley & Owyang, 1987; Cadieux *et al.*, 1990). NPY inhibits calcium currents in rat cultured myenteric plexus neurones, providing further evidence of functional NPY receptors in the myenteric plexus (Hirning *et al.*, 1990). The specific binding sites for [^{125}I]-BH-NPY and [^{125}I]-PYY observed in this study in myenteric ganglia may represent functional receptors through which locally released NPY and PYY may regulate colonic motility.

NPY and PYY inhibit electrolyte secretion by colonic mucosa (Cox *et al.*, 1988; Okuno *et al.*, 1991). In contrast to binding studies in small intestinal mucosal membranes in rats (Nguyen *et al.*, 1990), we were unable to demonstrate specific binding of either [^{125}I]-BH-NPY or [^{125}I]-PYY to porcine colonic mucosa. However, specific binding of both ligands was observed to ganglia in the colonic submucosal plexus, suggesting that effects of NPY on colonic secretion may, at least in part, be indirectly mediated through submucosal nerves.

In addition to effects on intestinal motility and secretion, NPY induces mesenteric vasoconstriction and potentiates noradrenaline-induced vasoconstriction, both probably post-junctional effects on vascular smooth musle (Hellstrom *et al.*, 1985; Andriantsitohaina & Stoclet, 1990) as well as reducing noradrenaline release by a prejunctional action (Westfall *et al.*, 1987). Despite these known pharmacological effects of NPY on mesenteric vasculature, we were unable to detect specific binding to blood vessels in any of our tissues. Specific binding of [^{125}I]-BH-NPY to colonic vessels may be present, but at a low density by comparison with non-specific binding. Modin *et al.* (1991) found that specific binding of iodinated NPY to splenic arteries represented only 10% of total binding, despite demonstrating splenic vasoconstriction by NPY. Such low specific to non-specific ratios are not amenable to detailed study by quantitative *in vitro* receptor autoradiography. Other possibilities include blocking of binding by endogenous ligand, perhaps released when the animals were killed, or differences in affinity for Bolton Hunter-labelled NPY of vascular smooth muscle as compared with myenteric ganglia and splenic red pulp.

H.p.l.c. revealed only a small degree of ligand degradation following 2 h incubation of [^{125}I]-BH-NPY with porcine colon. The significant loss of [^{125}I]-BH-NPY binding ability during the 2 h incubation period therefore appears to be multifactorial, and only partially explained by ligand degradation. Other possibilities include the release of inhibitory factors such as GTP or endogenous NPY from tissue sections, or the inactivation of components of buffer B required

Table 3 Binding inhibition of 0.2 nM [^{125}I]-Bolton Hunter-labelled neuropeptide Y ([^{125}I]-BH-NPY) to porcine spleen by unlabelled peptides

Ligand	*IC_{50} (nM)*	*K_i (nM)*	*Hill coefficient*
pNPY	1.5 (0.6–3.5)		1.9 (1.5–2.4)
[Leu^{31}, Pro^{34}]NPY	112* (42–291)	97 (37–252)	1.1 (0.8–1.5)
NPY (13–36)	34* (27–42)	29 (23–36)	1.7 (1.3–2.0)

Values are expressed as geometric means (95% CI). *NPY (13–36) more potent than [Leu^{31}, Pro^{34}]NPY at inhibiting binding of [^{125}I]-BH-NPY to porcine splenic red pulp ($P = 0.02$, paired t test, $n = 12$)

for binding, such as the albumin.

Nonetheless, even a 50% depletion of binding activity during the incubation, would not be expected to affect substantially calculated K_D and K_i values. It is apparent, therefore, that, in the absence of GTP, [^{125}I]-BH-NPY binds to myenteric ganglia with high affinity. The markedly increased dissociation observed in the presence of GTP-γ-S indicates the presence of a second (low) affinity state for these binding sites which is characteristic of G protein-linked receptors.

Kinetic studies and competition with unlabelled ligands indicate that, under identical conditions, porcine myenteric ganglion and splenic red pulp binding sites for [^{125}I]-BH-NPY share many characteristics. In particular, both are sensitive to GTP-γ-S, and both [Leu^{31}, Pro^{34}]NPY and NPY (13–36) completely inhibited specific binding with similar potencies, NPY (13–36) being slightly, but significantly more potent than [Leu^{31}, Pro^{34}]NPY. These findings suggest that porcine colonic myenteric ganglia and splenic red pulp each bear the same subclass of NPY receptor. The closely similar potencies of the two analogues observed in this study contrast, however, with results from membrane preparations of porcine spleen described by Modin *et al.* (1991), who found NPY (13–36) to be approximately 100 times more potent than [Leu^{31}, Pro^{34}]NPY in inhibiting specific binding of iodinated NPY. However, the same authors also found that *in vivo*, both analogues were approximately equipotent at increasing porcine splenic vascular resistance, each being one tenth as potent as intact NPY. The relative potencies of NPY and NPY (13–36) at inhibiting [^{125}I]-BH-NPY binding to myenteric ganglia observed in our study are consistent with studies *in vitro* on intestinal muscle strips which indicate that long C-terminal fragments of NPY inhibit muscle tone, but are less active than the intact peptide (Allen *et al.*, 1987).

We considered whether a differential depletion of the two analogues could explain their apparently similar inhibitory potencies in porcine colon and spleen, but no significant loss of inhibitory potency was observed for either NPY (13–36), or [Leu^{31}, Pro^{34}]NPY following preincubation with porcine spleen or colon.

It remains possible that the process of membrane preparation can affect binding characteristics of membrane G protein-receptor complexes, or that specificity may be influenced by the use of different incubation conditions. Such factors may be particularly important in studies using receptor agonists, the binding of which to receptors is dependent on the integrity of receptor-G protein interactions. We have demonstrated two affinity states of [^{125}I]-BH-NPY binding sites in porcine colon and spleen, depending on the presence of GTP analogues. Y_1 and Y_3 receptor cDNA sequences have recently been cloned (Rimland *et al.*, 1991; Herzog *et al.*, 1992; Larhammer *et al.*, 1992), and it is hoped that hybridization techniques using specific nucleotide probes will provide a means of distinguishing the expression of receptor subtypes not dependent on relative differences in binding of agonists.

Specific binding sites for [^{125}I]-BH-NPY were identified on the myenteric ganglia of normal human colon in 5 of 7 cases, suggesting that NPY may be a modulator of human colonic motility, and that porcine colon may be an appropriate model for studying the colonic actions of NPY and PYY. By contrast to the red pulp of porcine spleen, however, specific [^{125}I]-BH-NPY binding sites were not identified in any of 6 specimens of normal human spleen, or in normal rat spleens. These findings may represent interspecies differences in the distribution of NPY receptors, and are similar to comparative studies performed on the kidney (Leys *et al.*, 1987).

It remains to be determined whether abnormalities of NPY release or receptor expression underlie diseases characterized by disordered gut motility, such as the irritable bowel syndrome and idiopathic megacolon. Similarly, abnormalities of NPY/PYY systems in disorders of colonic secretion such as inflammatory bowel disease await full investigation.

In conclusion, we have demonstrated specific [^{125}I]-BH-NPY binding sites in human and porcine colonic myenteric ganglia, and in porcine colonic submucosal ganglia. These binding sites have the characteristics of high affinity, G protein-linked receptors and show similar kinetic and specificity characteristics to [^{125}I]-BH-NPY binding sites in porcine splenic red pulp, when studied under identical conditions. This distribution of [^{125}I]-BH-NPY binding sites suggests that NPY/PYY may modulate colonic motility and secretion by regulating colonic neuronal activity. NPY and PYY have been implicated in a variety of disorders of the human gastrointestinal tract (Sheikh, 1991) and the development of metabolically stable compounds which interact with colonic NPY/PYY receptors may offer novel therapeutic approaches to these diseases.

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References

ADRIAN, T.E., FERRI, G.-L., BACARESE-HAMILTON, A.J., FUESSL, H.S., POLAK, J.M. & BLOOM, S.R. (1985). Human distribution and release of a putative new gut hormone, peptide YY. *Gastroenterology*, **89**, 1070–1077.

ALLEN, J., HUGHES, J. & BLOOM, S.R. (1987). Presence, distribution, and pharmacological effects of neuropeptide Y in mammalian gastrointestinal tract. *Dig. Dis. Sci.*, **32**, 506–512.

ANDRIANTSITOHAINA, R. & STOCLET, J.C. (1990). Enhancement by neuropeptide Y (NPY) of the dihydropyridine-sensitive component of the response to α_1-adrenoceptor stimulation in rat isolated mesenteric arterioles. *Br. J. Pharmacol.*, **99**, 389–395.

CADIEUX, A., T-BENCHEKROUN, M., FOURNIER, A. & ST-PIERRE, S. (1990). Pharmacological actions of neuropeptide Y and peptide YY in rat colon. *Ann. N. Y. Acad. Sci.*, **611**, 372–375.

COX, H.M., CUTHBERT, A.W., HAKANSON, R. & WAHLESTEDT, C. (1988). The effect of neuropeptide Y and peptide YY on electrogenic ion transport in rat intestinal epithelia. *J. Physiol.*, **398**, 65–80.

EL-BADAWI, A. & SCHENK, E.A. (1967). Histochemical methods for separate consecutive and simultaneous demonstration of acetylcholinesterase and norepinephrine in cryostate sections. *J. Histochem. Cytochem.*, **15**, 580–588.

FERRI, G.-L., ALI-RACHEDI, A., TATEMOTO, K., BLOOM, S.R. & POLAK, J.M. (1984). Immunocytochemical localization of neuropeptide Y-like immunoreactivity in extrinsic noradrenergic and intrinsic gut neurons. *Front. Horm. Res.*, **12**, 81–84.

FUHLENDORFF, J., GETHER, U., AADERLUND, L., LANGELAND-JOHANSEN, N., THOGERSEN, H., MELBERG, S.G., OLSEN, U.B., THASTRUP, O. & SCHWARTZ, T.W. (1990). [Leu^{31}, Pro^{34}] Neuropeptide Y: a specific Y_1 receptor agonist. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 182–186.

FURNESS, J.B., COSTA, M., EMSON, P.C., HADANSON, R., MOGHIMZADEH, E., SUNDLER, F., TAYLOR, I.L. & CHANCE, R.E. (1983). Distribution, pathways and reactions to drug treatment of nerves with neuropeptide Y- and pancreatic polypeptide-like immunoreactivity in the guinea-pig digestive tract. *Cell Tissue Res.*, **234**, 71–92.

GREELEY, G.H., JENG, Y.-J., GOMEZ, G., HASHIMOTO, T., HILL, F.L.C., KERN, K., KUROSKY, T., CHUO, H.-F. & THOMPSON, J.C. (1989). Evidence for regulation of peptide-YY release by the proximal gut. *Endocrinol.*, **124**, 1438–1443.

HELLSTROM, P.M. (1987). Mechanisms involved in colonic vasoconstriction and inhibition of motility induced by neuropeptide Y. *Acta Physiol. Scand.*, **129**, 549–556.

HELLSTROM, P.M., LUNDBERG, J.M., HOKFELT, T. & GOLDSTEIN, M. (1989). Neuropeptide Y, peptide YY, and sympathetic control of rectal tone and anal canal pressure in the cat. *Scand. J. Gastroenterol.*, **24,** 231–243.

HELLSTROM, P.M., OLERUP, O. & TATEMOTO, K. (1985). Neuropeptide Y may mediate effects of sympathetic nerve stimulations on colonic motility and blood flow in the cat. *Acta Physiol. Scand.*, **124,** 613–624.

HERZOG, H., HORT, Y.J., BALL, H.J., HAYES, G., SHINE, J. & SELBIE, L.A. (1992). Cloned human neuropeptide Y receptor couples to two different second messenger systems. *Proc. Natl. Acad. Sci. U.S.A.*, **89,** 5794–5798.

HIRNING, L.D., FOX, A.P. & MILLER, R.J. (1990). Inhibition of calcium currents in cultured myenteric neurons by neuropeptide Y: evidence for direct receptor/channel coupling. *Brain Res.*, **532,** 120–130.

LARHAMMAR, D., BLOMQVIST, A.G., YEE, F., JAZIN, E., YOO, H. & WAHLESTEDT, C. (1992). Cloning and functional expression of a human neuropeptide Y/peptide YY receptor of the Y1 type. *J. Biol. Chem.*, **267,** 10935–10938.

LEYS, K., SCHACHTER, M. & SEVER, P. (1987). Autoradiographic localisation of NPY receptors in rabbit kidney: comparison with rat, guinea-pig and human. *Eur. J. Pharmacol.*, **134,** 233–237.

LUNDBERG, J.M., HEMSEN, A., RUDEHILL, A., HARFSTRAND, A., LARSSON, O., SOLLEVI, A., SARIA, A., HOKFELT, T., FUXE, K. & FREDHOLM, B.B. (1988a). Neuropeptide Y- and alpha-adrenergic receptors in pig spleen: localization, binding characteristics, cyclic AMP effects and functional responses in control and denervated animals. *Neuroscience*, **24,** 659–672.

LUNDBERG, J.M., HEMSEN, A., LARSSON, O., RUDEHILL, A., SARIA, A., FREDHOLM, B.B. (1988b). Neuropeptide Y receptor in pig spleen: binding characteristics, reduction of cyclic AMP formation and calcium antagonist inhibition of vasoconstriction. *Eur. J. Pharmacol.*, **145,** 21–29.

LUNDBERG, J.M., TATEMOTO, K., TERENIUS, L., HELLSTROM, P.M., MUTT, V., HOKFELT, T. & HAMBERGER, B. (1982). Localisation of Peptide YY (PYY) in gastrointestinal endocrine cells and effects on intestinal blood flow and motility. *Proc. Natl. Acad. Sci. U.S.A.*, **79,** 4471–4475.

MODIN, A., PERNOW, J. & LUNDBERG, J.M. (1991). Evidence for two neuropeptide Y receptors mediating vasoconstriction. *Eur. J. Pharmacol.*, **203,** 165–171.

NGUYEN, T.D., HEINTZ, G.G., KAISER, L.M., STALEY, C.A. & TAYLOR, I.L. (1990). Neuropeptide Y; differential binding to rat intestinal laterobasal membranes. *J. Biol. Chem.*, **265,** 6416–6422.

OKUNO, M., NAKANISHI, T., SHINOMURA, Y., KIYOHARA, T., ISHIKAWA, H. & TARUI, S. (1992). Peptide YY enhances NaCl and water absorption in the rat colon in vivo. *Experientia*, **48,** 47–50.

PALACIOS, J.M. & DIETL, M.M. (1989). Regulatory peptide receptors: visualization by autoradiography. In *Regulatory Peptides.* ed. Polak, J.M. pp. 70–97. Basel: Birkhauser Verlag.

RIMLAND, J., XIN, W., SWEETNAM, P., SAIJOH, K., NESTLER, E.J. & DUMAN, R.S. (1991). Sequence and expression of a neuropeptide Y receptor cDNA. *Mol. Pharmacol.*, **40,** 869–875.

SHEIKH, S.P. (1991). Neuropeptide Y and peptide YY: major modulators of gastrointestinal blood flow and function. *Am. J. Physiol.*, **261,** G701–715.

TATEMOTO, K., CARLQUIST, M. & MUTT, V. (1982). Neuropeptide Y–a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. *Nature*, **296,** 659–660.

TAYLOR, I.L. (1985). Distribution and release of peptide YY in dog measured by specific radioimmunoassay. *Gastroenterology*, **88,** 731–747.

WAHLESTEDT, C., GRUNDEMAR, L., HAKANSON, R., HEILIG, M., SHEN, G.H., ZUKOWSKA-GROJEC, Z. & REIS, D.J. (1990). Neuropeptide Y receptor subtypes, Y1 and Y2. *Ann. N. Y. Acad. Sci.*, **611,** 7–26.

WESTFALL, T.C., CARPENTIER, S., CHEN, X., BEINFELD, M.C., NAES, L. & MELDRUM M.J. (1987). Prejunctional and postjunctional effects of neuropeptide Y at the noradrenergic neuroeffector junction of the perfused mesenteric arterial bed of the rat. *J. Cardiovasc. Pharmacol.*, **10,** 716–722.

WILEY, J. & OWYANG, C. Neuropeptide Y inhibits cholinergic transmission in the isolated guinea pig colon: Mediation through α-adrenergic receptors. *Proc. Natl. Acad. Sci. U.S.A.*, **84,** 2047–2051.

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