British Journal of Pharmacology (2001) 135, 1505–1512

www.nature.com/bjp

Neuropeptide Y, Y_1, Y_2 and Y_4 receptors mediate Y agonist responses in isolated human colon mucosa

*,[1]Helen M. Cox & [1]Iain R. Tough

[1]Centre for Neuroscience Research, King's College London, GKT School of Biomedical Sciences, Hodgkin Building, Guy's Campus, London SE1 9RT

1 The aim of this study was to provide a pharmacological characterization of the Y receptor types responsible for neuropeptide Y (NPY), peptide YY (PYY) and pancreatic polypeptide (PP) effects upon electrogenic ion transport in isolated human colonic mucosa.

2 Preparations of descending colon were voltage-clamped at 0 mV in Ussing chambers and changes in short-circuit current (I_{sc}) continuously recorded. Basolateral PYY, NPY, human PP (hPP), PYY(3–36), [Leu[31], Pro[34]]PYY (Pro[34]PYY) and [Leu[31], Pro[34]]-NPY (Pro[34]NPY) all reduced basal I_{sc} in untreated colon. Of all the Y agonists tested PYY(3–36) responses were most sensitive to tetrodotoxin (TTX) pretreatment, indicating that Y_2-receptors are located on intrinsic neurones as well as epithelia in this tissue.

3 The EC_{50} values for Pro[34]PYY, PYY(3–36) and hPP were 9.7 nM (4.0–23.5), 11.4 nM (7.6–17.0) and 14.5 nM (10.2–20.5) and response curves exhibited similar efficacies. The novel Y_5 agonist [Ala[31], Aib[32]]-NPY had no effect at 100 nM.

4 Y_1 receptor antagonists, BIBP3226 and BIBO3304 both increased basal I_{sc} levels *per se* and inhibited subsequent PYY and Pro[34]PYY but not hPP or PYY(3–36) responses. The Y_2 antagonist, BIIE0246 also raised basal I_{sc} levels and attenuated subsequent PYY(3–36) but not Pro[34]PYY or hPP responses.

5 We conclude that Y_1 and Y_2 receptor-mediated inhibitory tone exists in human colon mucosa. PYY and NPY exert their effects via both Y_1 and Y_2 receptors, but the insensitivity of hPP responses to either Y_1 or Y_2 antagonism, or to TTX, indicates that Y_4 receptors are involved and that they are predominantly post-junctional in human colon.
British Journal of Pharmacology (2001) **135,** 1505–1512

Keywords: Pancreatic polypeptides; neuropeptide Y receptors; human colon mucosa; electrogenic ion transport

Abbreviations: BIBO3304 ((R)-N-[[4-(Aminocarbonylaminomethyl)phenyl)methyl]-N[2]-(diphenylacetyl)-argininamide-trifluoroacetate; BIBP3226; (N[2]-(diphenylacetyl)-N-[(4-hydroxy-phenyl)methyl]-D-arginine amide) and BIIE0246, ((S)-N[2]-[[1-[2-[4-[(R,S)-5,11-Dihydro-6(6H)-oxodibenz[b,e]azepin-11-yl]-1-piperazinyl]-2-oxoethyl]cyclopentyl]acetyl]-N-[2-[1,2-dihydro-3,5(4H)-dioxo-1,2-diphenyl-3H-1,2,4-triazol-4-yl]ethyl]-argininamide); hPP, human pancreatic polypeptide; KH, Krebs-Henseleit; NPY, neuropeptide Y; Pro[34]NPY, porcine [Leu[31], Pro[34]]NPY; Pro[34]PYY, human [Leu[31], Pro[34]]PYY; PYY, peptide YY; SRIF, somatostatin 14-28; TTX, tetrodotoxin; UK14,304, 5-bromo-N-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine.

Introduction

Peptide YY (PYY), neuropeptide Y (NPY) and pancreatic polypeptide (PP) constitute a group of structurally related 36 amino acid long peptides, known collectively as the pancreatic polypeptides (Larhammar, 1996). NPY is a neurotransmitter in the periphery, including the enteric nervous system where it is extensively expressed in myenteric and submucous neurones, along the length of the intestine (Ekblad *et al*., 1987, 1988) in most mammalian species investigated to date. PYY in contrast is an endocrine peptide found predominantly in the lower bowel (Böttcher *et al*., 1984) while PP was initially located in pancreatic acini (Larsson *et al*., 1975) and subsequently found in discrete gastrointestinal endocrine cells in certain species (Sundler *et al*., 1993). The presence of these three peptides in different intestinal cell types stimulated a plethora of studies (for review see Cox, 1998) however, few investigations have been performed either with human tissue discarded at the time of bowel resection or in human subjects (Playford *et al*.,1990; Holzer-Petsche *et al*., 1991). In healthy volunteers, *i.v.* PYY prolonged small bowel transit time and attenuated ileal fluid hypersecretion pre-stimulated by vasoactive intestinal polypeptide (VIP, Playford *et al*., 1990) and a similar anti-secretory effect was observed following *i.v.* NPY after stimulation with prostaglandin E_2 (Holzer-Petsche *et al*., 1991).

Since the publication of these two human studies four new types of NPY receptor (designated Y receptors) have been characterized (for review see, Michel *et al*., 1998). Of the six different Y receptor types known to exist, three are most frequently identified in the mammalian intestine i.e. the Y_1, Y_2 and Y_4 receptors. Y_1 receptor mRNA has been shown by *in situ* hybridization to be located in the mucosa, basal glands and in submucous and myenteric ganglia of the human colon (Wharton *et al*., 1993). Improved cellular resolution using a Y_1 specific antibody has shown immunoreactivity (IR) within intrinsic ganglia, varicose fibres in the muscularis mucosa and

*Author for correspondence; E-mail: helen.m.cox@kcl.ac.uk

notably, within scattered cells in the mucosa and basal glands of human sigmoid colon (Peaire *et al*., 1997). In Henle's plexus co-localization of NPY and Y_1-IR was observed, a combination also seen in rat intestine submucous ganglia (Jackerott & Larsson, 1997) but mismatches were more frequent, prompting Peaire *et al*. (1997) to suggest that other Y receptors are probably present in human colon. Indeed Northern analyses show low levels of Y_2 receptor mRNA (Gehlert *et al*., 1996) in the human small intestine and Y_4 receptor mRNA in human small and large bowel (Lundell *et al*., 1995) but their respective cellular localization was not addressed. Despite its truncation the y_6 receptor is also apparently expressed in human small intestine and colon (Matsumoto *et al*., 1996) but the functional significance of this pseudogene remains unknown.

Functional studies have identified Y receptor heterogeneity in different intestinal regions from the same species, as well as between species. We and others have shown Y_2 receptors exclusively bind and mediate PYY/NPY anti-secretory effects in rat jejunum (Servin *et al*., 1989; Cox & Cuthbert, 1990; Cox & Krstenansky, 1991; Cox & Tough, 2000) while in rat colon mucosa a combination of Y_1 and Y_2 receptors are responsible (Tough & Cox, 1996; Cox & Tough, 2000). An RT–PCR study identified multiple Y receptors in rat jejunal crypt epithelia (Goumain *et al*., 1998) though Y_2 mRNA predominated and subsequently the full-length Y_2 receptor was cloned from crypts in this region (Goumain *et al*., 2001). In addition Y_4 and Y_5 receptor mRNA were observed in jejunal crypts, while colonic epithelia co-expressed only Y_2 and Y_4 receptors. A combination of Y_1, Y_4 and Y_5 receptor mRNA were however found in 'non-epithelial' tissue from rat colon (Goumain *et al*., 1998). While the functional significance of neuronal Y_1 and epithelial Y_2 receptors is becoming clear in the rat gastrointestinal tract, that of mucosal Y_4 and Y_5 receptors has yet to be identified. It is noteworthy however, that we recently discovered a unique Y_4-mediated activity stimulated by PP in monolayers of a human colonic adenocarcinoma cell line (Cox *et al*., 2001b).

Our aim was therefore to establish the pharmacology of PYY, NPY (both of which have affinity for Y_1, Y_2 and Y_5 receptors; Michel *et al*., 1998) and hPP (which is the preferred endogenous agonist at Y_4 receptors) inhibitory responses in isolated preparations of human colon mucosa. The selective blockade of PYY, NPY responses by either Y_1 antagonists (BIBP3226; Wieland *et al*., 1995 and BIBO3304; Wieland *et al*., 1998), or the Y_2 receptor antagonist, BIIE0246 (Doods *et al*., 1999) provides the first evidence of a functional role for each of these Y receptors in human colon. Antagonist-insensitive responses to hPP additionally indicate a Y_4-inhibitory mechanism that could provide a novel therapeutic target in the future.

Methods

Tissue specimens and their preparation

Specimens of human distal colon were obtained anonymously from 45 patients undergoing bowel resection for primary intestinal carcinoma. Pieces of colon obtained fresh at the time of surgery, were macroscopically normal and were taken no less than 15 cm from the edge of the tumour. Circumferential 2–3 cm wide strips of tissue were placed immediately in fresh Krebs Henseleit (KH) solution with the following composition (in mM): NaCl 118, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, glucose 11.1 (pH 7.4) and were usually sufficient for at least four mucosal 1 cm^2 preparations, plus an adjacent piece for routine histology. Mucosae were prepared by removing the overlying smooth muscle to leave an intact mucosa with muscularis mucosae attached (verified by histological examination). The tissues used in this study were non-pathological, although in four out of 45 specimens minor inflammation of the lamina propria was observed. This study was approved by the Guy's and St Thomas' Hospitals Research Ethics Committee and from April 2001 onwards, tissue was also obtained with the patients' consent.

Measurement of electrogenic ion transport across human colonic mucosal preparations

At least four mucosal preparations (obtained from each colon) were placed in individual Ussing chambers (exposed area, 0.64 cm^2) bathing both sides with 5 ml oxygenated (95% O_2/5% CO_2) KH maintained at 37°C. Tissues were voltage-clamped at 0 mV (using a DVC 1000 automatic voltage clamp, World Precision Instruments, Stevenage, U.K.) as described in detail previously (Cox *et al*., 1988), and the resultant basal short-circuit current (I_{sc}) allowed to stabilize, normally within 30–45 min. Once a stable I_{sc} was obtained additions of either peptides, nonpeptide antagonists (either 1 μM BIBP3226, 300 nM BIBO3304 or 1 μM BIIE0246, throughout) or tetrodotoxin (TTX, 100 nM throughout) were made to the basolateral reservoir alone and the resultant changes in I_{sc} were recorded continuously. Tissues were not paired in this study and the response means ± s.e.mean were calculated from pooled data (converted to $\mu A.cm^{-2}$) from different specimens. Differences between data groups were determined using unpaired Student's *t*-test with a threshold for statistical significance of $P<0.05$. EC_{50} values (with 95% confidence limits) were calculated from pooled concentration-response relationships using the curve-fitting programme Graphpad Prism (v. 3.0, Graphpad Software Inc., California, U.S.A.).

Materials

All peptides were purchased from Bachem UK Ltd (Merseyside, U.K.) and aliquots were frozen and stored at −20°C undergoing a single freeze-thaw cycle only. The peptides used in this study were the porcine sequences of PYY, NPY, Pro^{34}NPY, and human PP (hPP), PYY(3–36) and Pro^{34}PYY. BIBP3226 (N^2-(diphenylacetyl)-N-[(4-hydroxy-phenyl)methyl]-D-argininamide), BIBO3304 ((R)-N-[[4-(Aminocarbonylaminomethyl)phenyl)methyl]-N^2-(diphenylacetyl)- argininamide-trifluoroacetate, and BIIE0246 ((S)-N^2-[[1-[2-[4-[(R,S)-5,11-Dihydro-6(6H)-oxodibenz[b,e]azepin-11-yl]-1-piperazinyl]-2-oxoethyl] cyclopentyl] acetyl]-N-[2-[1,2-dihydro-3,5(4H)-dioxo-1,2-diphenyl-3H-1,2,4-triazol-4-yl]ethyl]-argininamide) were gifts from Boehringer Ingelheim Pharma KG (Biberach an der Riss, Germany). UK14,304 (5-bromo-N-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine) was from Research Biochemical International (Natick, MA, U.S.A.) and TTX was purchased from Sigma (Poole, U.K.).

Results

Descending colon tissue was obtained from 45 patients with an average age of 65.7±2.0 year (31 men, 14 women) and provided 178 mucosal preparations in this study. The basal resistance and I_{sc} values were 103.0±2.8 Ω cm^2 and 67.4±2.7 μA cm^{-2} ($n=178$) respectively and there were no significant changes in resistance over the course of the experimental period (data not shown). Basolateral TTX (100 nM, Figure 1) reduced basal I_{sc} by 29.1±4.6 μA cm^{-2} ($n=47$) and the toxin was subsequently used to inhibit intrinsic neurogenic activity. Single additions of the Y agonists, PYY (100 nM), hPP (100 nM, Figures 1 and 2) and NPY to naive tissues, each produced prolonged reductions in basal I_{sc}. However, the rate of onset and time to maxima for PYY responses (and NPY, data not shown) were different to those stimulated by hPP. The pooled time-courses (Figure 2) show a significantly slower onset of hPP responses that reached a maximum within 10 min whereas PYY responses were larger and maximal by 20 min. Subsequent addition of somatostatin 14–28 (SRIF,

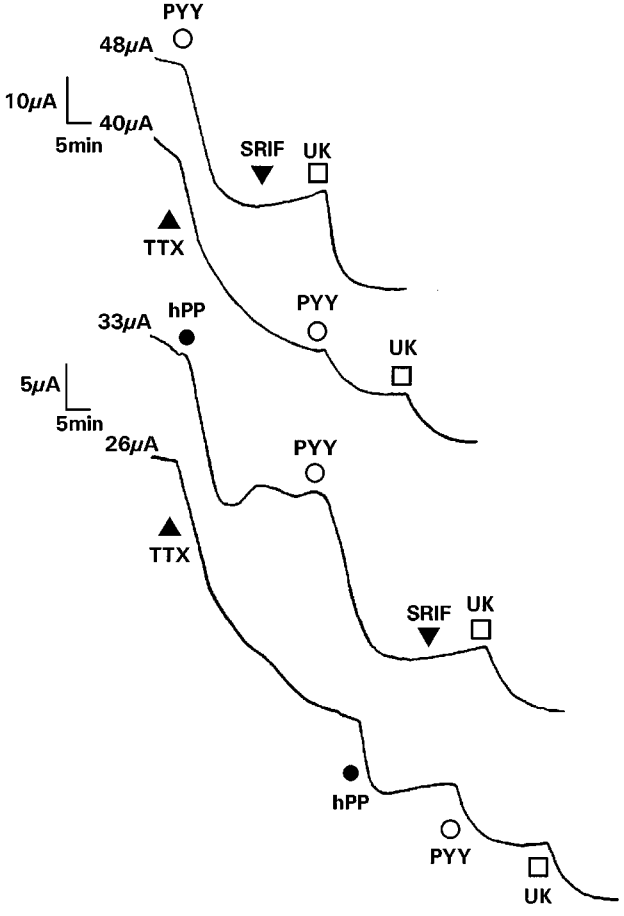

Figure 1 Representative responses to PYY and PP in control and TTX-pretreated colonic preparations. TTX (100 nM) was added to the lower of each pair of traces only. The basal I_{sc} values are given to the left of each record. Note the different μA scales for each pair of traces and that the tissue area exposed was 0.64 cm^2. All additions were made to the basolateral reservoir and the final concentrations are as follows: PYY (100 nM) hPP (100 nM) SRIF (100 nM) and the α_2-agonist, UK14,304 (1 μM).

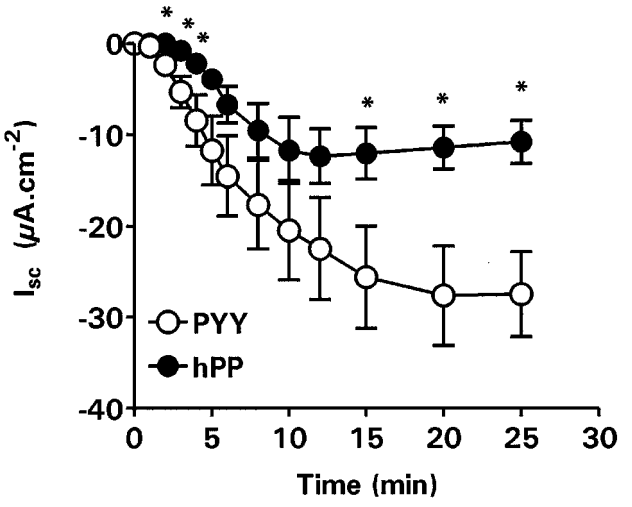

Figure 2 Pooled time-course responses to single additions of PYY or hPP (both at 100 nM). Peptide additions were made to the basolateral reservoir of untreated tissues and the inhibition of basal I_{sc} was recorded as described in the methods. Each point is the mean±s.e.mean from at least four observations in each group. Levels of significance are indicated as follows; $^*P<0.05$.

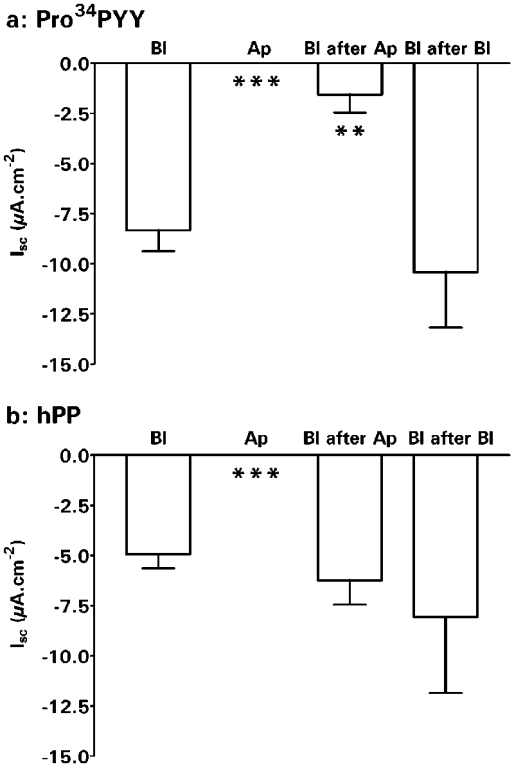

Figure 3 The sidedness of electrogenic responses to (a) Pro^{34}PYY and (b) hPP. All peptide concentrations were 100 nM and additions were made cumulatively to either the basolateral (Bl) or apical (Ap) reservoir as indicated. The I_{sc} was allowed to stabilize before a second peptide addition was made (between 20–50 min). The changes in basal I_{sc} were pooled and each bar represents the mean−s.e.mean from three observations in each group. Significant differences between data groups and respective basolateral controls were $P<0.01$ throughout.

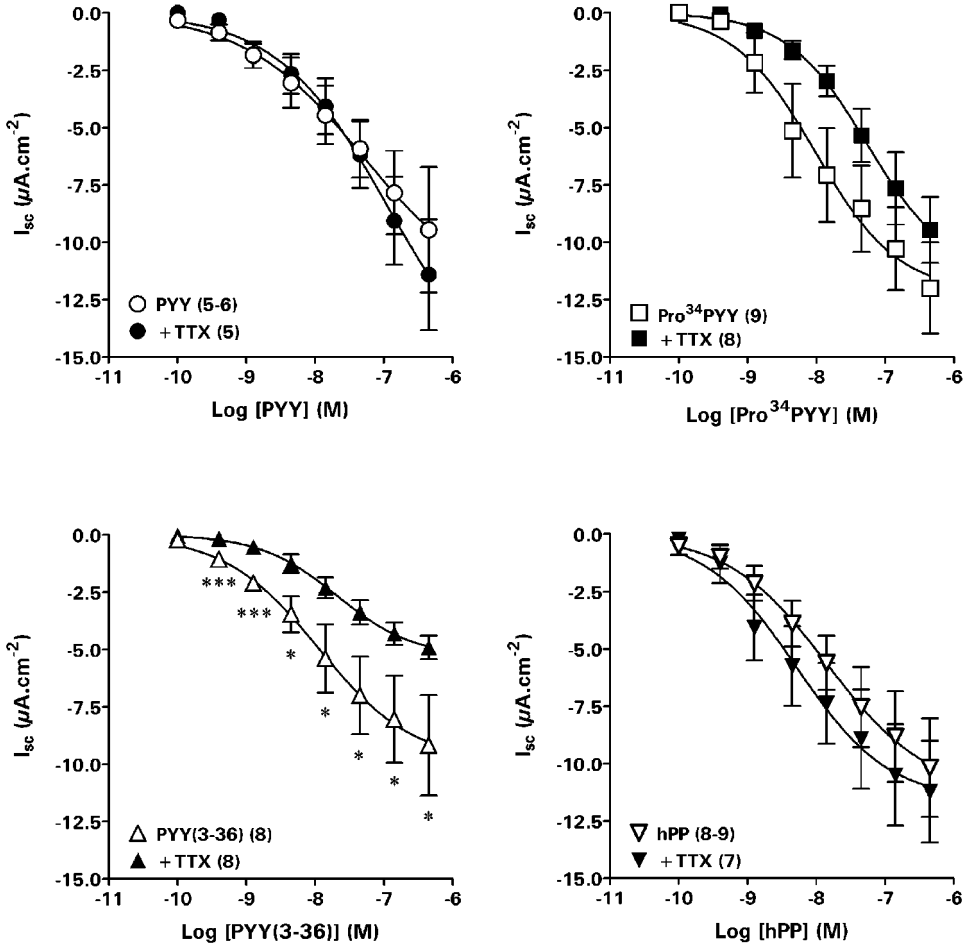

Figure 4 Concentration-response relationships for four Y-agonists; PYY, Pro^{34}PYY, PYY(3–36) and hPP in the presence or absence of TTX (100 nM). Curves were generated from pooled cumulative responses for each peptide and between 5–9 preparations from different patients were used in each case. The EC_{50} values (and confidence limits) for Pro^{34}PYY, PYY(3–36) and hPP response curves are quoted in Table 1.

Table 1 The EC_{50} values (with 95% confidence limits) for concentration-response curves to hPP, Pro^{34}PYY and PYY(3-36) in the presence or absence of TTX (100 nM)

	EC_{50} values (nM)	
Peptide	*Control*	*+ TTX*
Pro^{34}PYY	9.7 (4.0–23.5)	79.2 (48.2–130.3)
PYY(3-36)	11.4 (7.6–17.0)	19.1 (16.2–22.5)
hPP	14.5 (10.2–20.5)	5.2 (2.3–12.0)

Data was pooled from between five and nine specimens from different patients and the resultant curves shown in Figure 4 were analysed to give the EC_{50} values above (with confidence limits in parenthesis).

100 nM, Figure 1) was not effective, while the α_2-agonist, UK14,304 (1 μM) consistently produced further reductions in I_{sc} (Figure 1, -35.7 ± 1.4 μA cm^{-2} ($n=159$) that were partially sensitive to TTX (-29.9 ± 2.1 μA cm^{-2}, $n=47$, $P<0.05$).

Following apical addition of either the Y_1-preferred, Pro^{34}PYY (100 nM) or Y_4-preferring hPP (100 nM) there was no change in basal I_{sc} (Figure 3A,B). Addition of hPP to the basolateral reservoir subsequent to apical hPP (without intermediate washout) stimulated reductions in I_{sc} that were not significantly different from controls (Figure 3A). There was no further increase in the response size following a second basolateral addition of hPP (100 nM). This was true also for duplicate additions of basolateral Pro^{34}PYY (100 nM, Figure 3B). However basolateral responses to Pro^{34}PYY (100 nM) were attenuated significantly following apical pretreatment of this peptide.

Cumulative concentration-response relationships for PYY (which stimulates, Y_1, Y_2, $Y_5 >> Y_4$ receptors), Pro^{34}PYY ($Y_1 > Y_5 > Y_4$), PYY(3–36) ($Y_2 > Y_5$) and hPP ($Y_4 > Y_1 \geq Y_5$ receptors) in the absence and presence of TTX are shown in Figure 4 and the EC_{50} values for the latter three peptides are given in Table 1. The responses to PYY(3–36) (between 1–300 nM) were significantly inhibited by pretreatment with TTX, indicating a partial neurogenic contribution. Pro^{34}PYY responses at lower concentrations (i.e. 3 and 10 nM) appeared to be sensitive to TTX but not significantly so. Neither hPP nor PYY concentration-response relationships exhibited TTX-sensitivity. The latter peptide responses also showed little sign of saturating, due probably to the fact that PYY can co-stimulate several Y receptors, e.g. Y_1, Y_2 and Y_5 receptors at nM concentrations. It is notable that in naive tissues Pro^{34}PYY, PYY(3–36) and hPP response curves exhibited similar maxima. In contrast the recently described

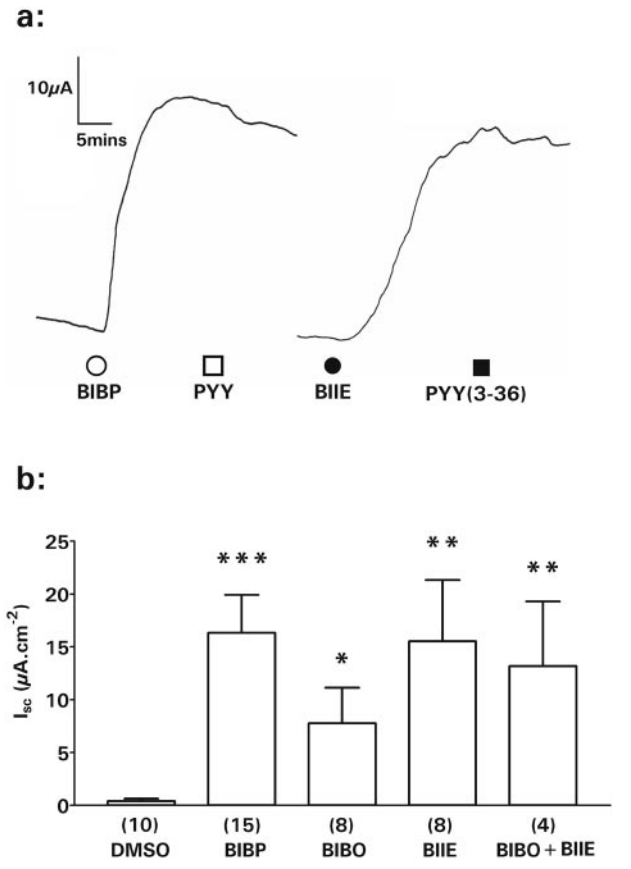

Figure 5 The effects of Y_1 and Y_2 antagonists upon basal I_{sc} levels. (a) Representative traces of the effects of BIBP3226 and BIIE0246 (both at 1 μM) upon untreated colon mucosa. (b) Pooled increases in I_{sc} and comparison with vehicle controls (0.01% DMSO). Each bar represents the mean + s.e.mean where the number of observations shown in parenthesis: $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

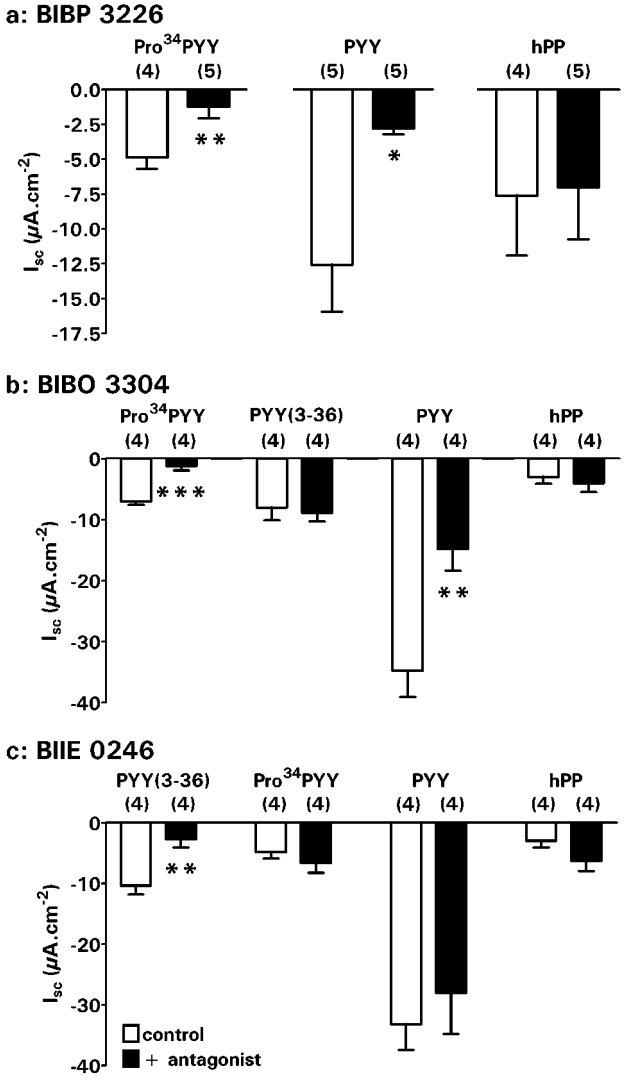

Figure 6 The effects of Y_1-antagonists in (a); BIBP3226 (1 μM) and (b); BIBO3304 (300 nM), and in (c); the novel Y_2-antagonist BIIE0246 (1 μM) upon selected Y-agonist responses. All values are the mean − s.e.mean with n numbers shown in parenthesis above either vehicle control agonist responses (open bars) or those following antagonist pretreatment (black bars). (a) Responses to Pro^{34}PYY, hPP and PYY (all 10 nM) were obtained in each case 20 min after either controls or after BIBP3226. (b) and (c) Agonist additions were made sequentially at 20 min intervals and final concentrations were 10 nM for Pro^{34}PYY and PYY(3 − 36) with PYY (100 nM) added last. Significant selective antagonism was observed as indicated; $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

selective Y_5 agonist [Ala^{31}, Aib^{32}]NPY (100 nM, Cabrele *et al*., 2000) had no effect upon basal I_{sc} (0.0 ± 0.0 μA cm^{-2}, $n=4$) but subsequent addition of PYY (100 nM) reduced the current by -7.6 ± 1.3 μA cm^{-2} ($n=4$). In addition, [D-Trp^{32}]NPY (which has previously been suggested as a Y_5 receptor agonist) also had no significant effect upon basal I_{sc} up to a concentration of 300 nM (data not shown).

In order to definitively establish which Y receptors were responsible for the Y agonist responses, three selective Y antagonists were investigated; two Y_1 antagonists BIBP3226 and BIBO3304, and the novel Y_2 antagonist BIIE0246. Currently there are no Y_4 receptor antagonists while selective, high affinity Y_5 antagonists are not yet commercially available. Each nonpeptide blocker was applied to the basolateral reservoir 20 min prior to either Pro^{34}PYY, PYY(3 − 36), PYYor hPP. Unexpectedly, all three antagonists stimulated prolonged increases in basal I_{sc} levels (Figure 5A,B) although there was no apparent additive effect of BIBO3304 and BIIE0246 (Figure 5B). Subsequent to addition of BIBP3226 (1 μM) a significant inhibition of Pro^{34}PYY and PYY responses (Figure 6A) was observed. Responses to hPP were unaffected by BIBP3226 (at 10 nM, Figure 6A; and at 110 nM, where control hPP responses were -13.7 ± 5.9 μA cm^{-2}, $n=4$; and plus BIBP3226, -12.5 ± 4.2 μA cm^{-2}, $n=5$, $P=0.9$). BIBO3304 (300 nM) virtually abolished Pro^{34}PYY responses (10 nM), partially inhibited subsequent PYY (100 nM) responses but the Y_1 antagonist had no significant effect upon either PYY(3 − 36) or hPP responses (Figure 6B). The Y_2 antagonist, BIIE0246 (1 μM) only reduced PYY(3 − 36) responses (by 74%) leaving subsequent Pro^{34}PYY, PYY and hPP effects unchanged (Figure 6C). Following the pretreatment of colonic mucosa with a combination of BIBO3304 and BIIE0246 (both at 1 μM) PYY responses were reduced 43% (at 100 nM from controls of -10.8 ± 1.2 μA cm^{-2}, $n=4$ to -6.2 ± 1.6 μA cm^{-2}, $n=4$, $P=0.058$). The two antagonists did not however alter UK14,304 responses either alone or in combination (the latter controls being -31.7 ± 9.4 μA cm^{-2}, $n=4$ and plus BIBO3304 and BIIE0246, -48.5 ± 6.0 μA cm^{-2}, $n=4$; $P<0.05$).

Discussion

The values of resistance and I_{sc} obtained in the present study for left colon mucosae are comparable with those published previously for similar muscle-stripped preparations under voltage-clamp conditions (Keely *et al*., 1995; Stack *et al*., 1996). Basolateral addition of all four native Y agonists, NPY, PYY, PYY(3–36) and hPP, produced prolonged reductions in basal I_{sc} with similar efficacies while the unrelated peptide, SRIF was not effective in any of the tissues tested. The time-courses of electrogenic responses to PYY and hPP were subtly different; PYY responses were more rapid in onset but reached their maximum later than hPP. The difference between these maximal Y agonist responses may reflect differing kinetics of peptide–Y receptor(s) activation e.g. Pro^{34}PYY may stimulate pre- and post-junctional Y_1 receptors while hPP may activate a single population of epithelial Y_4 receptors. The absence of electrogenic responses following apical addition of either Pro^{34}PYY or hPP indicates that basolateral targeting of Y receptors (probably Y_1 and Y_4) occurs in human colonic epithelia. This is true for most, if not all other Y receptor studies in mucosal preparations to date, including rat jejunum (Cox *et al*., 1988), rat descending colon (Tough & Cox, 1996) as well as polarized layers of human colonic adenocarcinoma cells (Cox & Tough, 1995; Cox *et al*., 2001b). The blunting of basolateral Pro^{34}PYY responses following apical peptide treatment (but not so for hPP) may indicate that Pro^{34}PYY is able to pass across the epithelium to initiate desensitization (though not significant receptor activation) while hPP is retained in the apical reservoir. In addition, substrate-specific peptidases on apical membranes may also differentially degrade hPP compared with Pro^{34}PYY.

The sensitivity of human colon mucosa to the four Y agonists (Figure 4) implicate the presence of three Y receptor types, i.e. Y_1 receptors (stimulated by Pro^{34}PYY and PYY) Y_2 receptors (activated by PYY(3–36) and PYY) and Y_4 receptors (which are sensitive to hPP, higher concentrations of Pro^{34}PYY, but not to PYY). The selective inhibition of Pro^{34}PYY and PYY following competitive Y_1 antagonists, BIBP3226 or BIBO3304, and the specific blockade of PYY(3–36) responses by BIIE0246 confirm the involvement of both Y_1 and Y_2 receptors in both PYY and its circulating fragment PYY(3–36) in human colon mucosa. The resistance of hPP responses to Y_1 and Y_2 receptor antagonism indicates the presence of a further Y receptor type, most likely the Y_4 receptor in human colon. The inability of the new Y_5 selective agonist, [Ala^{31},Aib^{32}]NPY (Cabrele *et al*., 2000) to inhibit basal I_{sc} levels, indicates this receptor type is not involved in modulating ion transport across human descending colon mucosa. PP-mediated Y_4 responses are rare in mammalian mucosae, however inhibitory responses to nM concentrations of hPP have recently been observed in three human colonic cell lines (Cox & Tough, 1995; Holliday *et al*., 1997; Cox *et al*., 2001b) and mouse descending colon mucosa pretreated with VIP (Holliday *et al*., 2000; Cox *et al*., 2001a). Subsequently, RT–PCR analysis has shown constitutive Y_4 receptor expression in the human colonic cell lines (Cox *et al*., 2001b) but the absence of specific Y_4 antagonists precludes any further clarification of this receptor type's functional significance.

It is worth noting at this juncture that an unrelated mechanism has been suggested to contribute to Y agonist effects in both human and murine intestine. PYY and NPY are anti-secretory in isolated mouse jejunum but Riviere *et al*. (1993) proposed that these effects were mediated via haloperidol-sensitive σ sites, a feature also observed in healthy human volunteers, where PYY attenuated prostaglandin E_2-induced jejunal secretion with haloperidol sensitivity (Roze *et al*., 1997). However, our own *in vitro* investigations with human and murine colon mucosae do not support this thesis. We observed that haloperidol pretreatment (1 μM, for either 20 or 15 min in human or mouse tissue respectively) had no effect *per se*, no significant effect upon subsequent hPP, PYY or UK14,304 responses, but it did attenuate dopamine responses significantly (Cox *et al*., 1999). Thus we concluded that mucosal responses to Y agonists and the α_2-adrenoceptor agonist UK14,304, did not involve either dopamine or endogenous activators of putative σ sites.

The prolonged elevations in basal I_{sc} observed following BIBP3226, BIBO3304 and BIIE0246 implicate a significant level of Y_1- and Y_2-mediated inhibitory tone in human colon mucosae. None of the three antagonists tested previously in rat (Tough & Cox, 1996; Cox & Tough, 2000; Goumain *et al*., 2001) or mouse mucosae (Cox *et al*., 2001a), or in human epithelial monolayers (Cox *et al*., 2001b) have any effect upon basal or stimulated I_{sc} levels. Competitive blockade of Y_1 and Y_2 receptors with consequent alleviation of human colonic Y receptor-mediated inhibitory tone will be clarified in future studies by monitoring the release of NPY (or other candidate inhibitory enteric neurotransmitters) and PYY (from colonic endocrine cells) from superfused pieces of human mucosa. BIIE0246 has recently been shown to inhibit basal and NPY(13–36)-stimulated NPY release from rat hypothalamic slices, suggestive of a pre-synaptic role for Y_2 receptors in this tissue (King *et al*., 2000) and blocks PYY anti-secretory responses in rat jejunum mucosa (Cox & Tough, 2000; Goumain *et al*., 2001) and colon mucosa (Cox & Tough, 2000) proving the presence of Y_2 receptors in these intestinal preparations. Two elegant immunohistochemical studies of human colon (Peaire *et al*., 1997; Mannon *et al*., 1999) have shown extensive Y_1-specific IR in myenteric, submucous and Henle's plexi, in nerve endings in the muscularis mucosae and throughout the colonic epithelium, often along the basolateral surface (Mannon *et al*., 1999). Thus Y_1 receptors are located both pre- and post-junctionally in human colon. The absence of primary antisera selective for either Y_2 or Y_4 receptors currently precludes similar detailed study of the patterns of these receptors' localizations. Tissue-specific differences in Y receptor expression patterns will have functional consequences, as observed for PYY sensitivity, which varies significantly along the length of the mouse intestine. Basolateral PYY, Pro^{34}PYY, PYY(3–36) and hPP have no effect upon electrogenic ion transport across mucosal sheets of murine jejunum and only small inhibitory responses to the three PYY analogues in duodenum and ascending colon, in contrast with the pronounced inhibitory effects of all four agonists in mouse descending colon (Cox *et al*., 2001a).

The attenuation of basal I_{sc} by TTX indicates a significant spontaneous neuronal excitatory component in human colon mucosa, in keeping with the observations of Stack *et al*. (1996) but in contrast with those of Kuwahara *et al*. (1989)

who found no effect of the neurotoxin (at 200 nM). *In vitro* studies with rodent intestine mucosae have often exhibited TTX-insensitive NPY and PYY responses (Hubel & Renquist, 1986; Cox *et al*., 1988; Strabel & Diener, 1995; Tough & Cox, 1996). Our observations in this investigation, that TTX pretreatment significantly attenuated but did not abolish responses to Y_2-preferred, PYY(3–36) implicate a neurogenic as well as direct epithelial components mediated by Y_2 receptors. In contrast the absence of significant TTX-sensitivity for Pro^{34}PYY responses, indicates that Y_1-mediated inhibition of ion transport occurs predominately *via* post-junctional targets, i.e. epithelial cells. The TTX insensitivity of hPP responses also discounts neuronal Y_4 mediation in human colon mucosa. We conclude therefore that functionally significant Y_1 and Y_4 receptors are located on basolateral epithelial surfaces together with PYY(3–36)-activated Y_2 receptors and that the latter receptor type are also expressed on intrinsic neurones.

In conclusion, we have established that human descending colon mucosa expresses three Y receptor types whose activation will result in inhibition of electrogenic ion transport. The existence of Y_1 and Y_2 receptors and their stimulation to initiate prolonged reductions in basal I_{sc} have been proven using competitive antagonists, namely BIBP3226, BIBO3304 and BIIE0246. The Y_1 and Y_2 antagonist insensitivity of hPP-mediated responses indicates the presence of a third Y receptor type, most probably the Y_4 receptor in human colon mucosa. Mucosal Y_4 receptor-mediated inhibitory ion transport effects could indicate a novel therapeutic target, Y_4 selective agonists potentially acting as antidiarrhoeals while Y_4 antagonists might be anti-constipatory. This is a viable prospect since relatively few human peripheral tissues appear to express Y_4 receptors (Lundell *et al*., 1995; Bard *et al*., 1995) and side-effects should therefore be limited.

We thank the surgical teams in the Department of General Surgery, St Thomas' Hospital for their help in obtaining the specimens used in this study. We also thank Dr Henri Doods and Boehringer Ingelheim Pharma KG for kindly providing BIBP3226, BIBO3304 and BIIE0246. This research was funded by the Trustees of Guy's Hospital, the Kimmel Cancer Foundation and most recently by The Wellcome Trust.

References

BARD, J.A., WALKER, M.W., BRANCHEK, T.A. & WEINSHANK, R.L. (1995). Cloning and functional expression of a human Y_4 subtype receptor for pancreatic polypeptide, neuropeptide Y and peptide YY. *J. Biol. Chem.,* **270,** 26762–26765.

BÖTTCHER, G., SJÖLUND, K., EKBLAD, E., HÅKANSON, R., SCHWARTZ, T.W. & SUNDLER, F. (1984). Co-existence of PYY and glicentin in endocrine cells of the gut. *Regul. Pept.,* **8,** 261–266.

CABRELE, C., LANGER, M., BADER, R., WIELAND, H.A., DOODS, H.N., ZERBE, O. & BECK-SICKINGER, A. (2000). The first selective agonist for the Neuropeptide Y Y_5 receptor increases food intake in rats. *J. Biol. Chem.,* **275,** 36043–36048.

COX, H.M. (1998). Peptidergic regulation of intestinal ion transport: a major role for pancreatic polypeptide family. *Digestion,* **59,** 395–399.

COX, H.M. & CUTHBERT, A.W. (1990). The effects of NPY and its fragments upon basal and electrically stimulated ion secretion in rat jejunum mucosa. *Br. J. Pharmacol.,* **101,** 247–252.

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

COX, H.M. & KRSTENANSKY, J.L. (1991). The effects of selective amino acid substitution upon NPY antisecretory potency in rat jejunum mucosa. *Peptides,* **12,** 323–327.

COX, H.M., POLLOCK, E.L. & TOUGH, I.R. (1999). Responses to Y receptor agonists are insensitive to haloperidol pretreatment in human and mouse colonic mucosae *in vitro*. *Br. J. Pharmacol.,* **128,** 102P.

COX, H.M., POLLOCK, E.L., TOUGH, I.R., & HERZOG, H. (2001a). Multiple Y receptors mediate pancreatic polypeptide responses in mouse colon mucosa. *Peptides,* **22,** 445–452.

COX, H.M. & TOUGH, I.R. (1995). Functional characterisation of receptors with affinity for PYY, NPY, [Leu^{31},Pro^{34}]NPY and PP in a human colonic epithelial cell line. *Br. J. Pharmacol.,* **116,** 2673–2678.

COX, H.M. & TOUGH, I.R. (2000). Functional studies with a novel neuropeptide Y Y_2 receptor antagonist, BIIE0246 in isolated preparations from the rat gastrointestinal tract. *Br. J. Pharmacol.,* **129,** 89P.

COX, H.M., TOUGH, I.R., ZANDVLIET, D.W.J. & HOLLIDAY, N.D. (2001b). Constitutive expression of neuropeptide Y Y_4-like receptors in a human adenocarcinoma cell line. *Br. J. Pharmacol.,* **132,** 345–353.

DOODS, H., GAIDA, W., WIELAND, H.A., DOLLOINGER, H., SCHNORRENBERG, G., ESSER, F., ENGEL, W., EBERLEIN, W. & RUDOLF, K. (1999). BIIE0246: A selective and high affinity neuropeptide Y Y_2 receptor antagonist. *Eur. J. Pharmacol.,* **384,** R3–R5.

EKBLAD, E., EKMAN, R., HÅKANSON, R. & SUNDLER, F. (1988). Projections of peptide-containing neurons in rat colon. *Neuroscience,* **27,** 655–674.

EKBLAD, E., WINTER, C., EKMAN, E., HÅKANSON, R, & SUNDLER, F. (1987). Projections of peptide-containing neurons in rat small intestine. *Neuroscience,* **20,** 169–188.

GEHLERT, D.R., BEAVERS, L.S., JOHNSON, D., GACKENHEIMER, S.L., SCHOBER, D.A. & GADSKI, R.A. (1996). Expression cloning of a human brain Y_2 receptor. *Mol. Pharmacol.,* **49,** 224–228.

GOUMAIN, M., VOISIN, T., LORINET, A-M., DUROC, R., TSOCAS, A., ROZE, C., ROUET-BENZINEB, P., HERZOG, H., BALASUBRAMANIAM, A. & LABURTHE, M. (2001). The peptide YY-preferring receptor mediating inhibition of small intestinal secretion is a peripheral Y2 receptor: pharmacological evidence and molecular cloning. *Mol. Pharmacol.,* **60,** 124–134.

GOUMAIN, M., VOISIN, T., LORINET, A-M. & LABURTHE, M. (1998). Identification and distribution of mRNA encoding the Y_1, Y_2, Y_4 and Y_5 receptors for peptides of the PP-fold family in the rat intestine and colon. *Biochem. Biophys. Res. Comm.,* **247,** 52–56.

HOLLIDAY, N.D., POLLOCK, E.L., TOUGH, I.R. & COX, H.M. (2000). PYY preference is a common characteristic of neuropeptide Y receptors in human, rat and mouse gastrointestinal epithelia. *Can. J. Physiol. Pharmacol.,* **78,** 126–133.

HOLLIDAY, N.D., TOUGH, I.R. & COX, H.M. (1997). Inhibition of cAMP-dependent chloride secretion by PP receptors and α_2-adrenoceptors in a human colonic epithelial cell line. *Naunyn-Schmiedeberg's. Arch. Pharmacol.,* **355,** 183–189.

HOLZER-PETSCHE, U., PETRITSCH, W., HINTERLEITNER, T., EHERER, A., SPERK, G. & KREJS, G.J. (1991). Effect of NPY on jejunal water and ion transport in humans. *Gastroenterology,* **101,** 325–330.

HUBEL, K.A. & RENQUIST, K.S. (1986). Effect of neuropeptide Y on ion transport by the rabbit ileum. *J. Pharmacol. Exp. Ther.,* **238,** 167–169.

JACKEROTT, M. & LARSSON, L-I. (1997). Immunocytochemical localisation of the Y_1 receptor in enteric neurones, endothelial cells, and endocrine-like cells of the rat intestinal tract. *J. Histochem. Cytochem.,* **45,** 1643–1650.

KEELY, S.J., STACK, W.A., O'DONOGHUE, D.P. & BAIRD, A.W. (1995). Regulation of ion transport by histamine in human colon. *Eur. J. Pharmacol.,* **279,** 203 – 209.

KING, P.J., WILLIAMS, G., DOODS, H. & WIDDOWSON, P.S. (2000). Effect of a selective neuropeptide Y Y_2 receptor antagonist, BIIE0246 on neuropeptide Y release. *Eur. J. Pharmacol.,* **396,** R1 – R3.

KUWAHARA, A., COOKE, H.J., CAREY, H.V., MEKHJIAN, M.D., ELLISON, E.C. & MCGREGOR, M.D. (1989). Effects of enteric neural stimulation on chloride transport in human left colon *in vitro*. *Dig. Dis. Sci.,* **34,** 206 – 213.

LARHAMMAR, D. (1996). Evolution of neuropeptide Y, peptide YY and pancreatic polypeptide. *Regul. Pept.,* **62,** 1 – 11.

LARSSON, L-I., SUNDLER, F. & HÅKANSON, R. (1975). Immunohistochemical localisation of hPP to a population of islet cells. *Cell Tissue Res.,* **156,** 167 – 171.

LUNDELL, I., BLOMQVIST, A., BERGLUND, M., SCHOBER, D. A., JOHNSON, D., STATNICK, M.A., GADSKI, R.A., GEHLERT, D.R. & LARHAMMAR, D. (1995). Cloning of a human receptor of the NPY family with high affinity for PP and PYY. *J. Biol. Chem.,* **270,** 29123 – 29128.

MANNON, P.J., KANUNGO, A., MANNON, R.B. & LUDWIG, K.A. (1999). Peptide YY/NPY Y_1 receptor expression in the epithelium and mucosal nerves of the human colon. *Regul. Pept.,* **83,** 11 – 19.

MATSUMOTO, M., NOMURA, T., MOMOSE, K., IKEDA, Y., KONDUO, Y., AKIHO, H., TOGAMI, J., KIMURA, Y., OKADA, M. & YAMAGUCHI, T. (1996). Inactivation of a novel NPY/PYY receptor gene in primate species. *J. Biol. Chem.,* **271,** 27217 – 27220.

MICHEL, M.C., BECK-SICKINGER, A., COX, H.M., DOODS, H.N., HERZOG, H., LARHAMMAR, D., QUIRION, R., SCHWARTZ, T.W. & WESTFALL, T. (1998). XVI. IUPHAR Recommendations for the nomenclature of neuropeptide Y, peptide YY and pancreatic polypeptide receptors. *Pharmacol. Rev.,* **50,** 143 – 150.

PEAIRE, A.E., KRANTIS, A. & STAINES, W.A. (1997). Distribution of the NPY receptor Y_1 within human colon. *J. Auton. Nerv. Syst,* **67,** 168 – 175.

PLAYFORD, R.J., DOMIN, J., BEECHAM, J., PARMAR, K.B., TATEMOTO, K., BLOOM, S.R. & CALAM, J. (1990). Peptide YY: a natural defence against diarrhoea. *Lancet,* **335,** 1555 – 1557.

RIVIERE, P.J.M., ROA, R.K., PASCAUD, X., JUNIEN, J.L. & PORRECA, F. (1993). Effects of NPY, PYY and sigma ligands on ion transport in mouse jejunum. *J. Pharmacol. Exp. Ther.,* **264,** 1268 – 1274.

ROZE, C., MOLIS, C., FU-CHENG, X., ROPERT, A., GENEVE, J. & GALMICHE, J.M. (1997). PYY inhibition of prostaglandin-induced intestinal secretion is haloperidol-sensitive in humans. *Gastroenterology,* **112,** 1520 – 1528.

SERVIN, A.L., ROUYER-FESSARD, C., BALASUBRAMANIAM, A., SAINT PIERRE, S. & LABURTHE, M. (1989). PYY and NPY inhibit VIP-stimulated cAMP production in rat small intestine. *Endocrinology,* **124,** 692 – 700.

STACK, W.A., FILIPOWICZ, B. & HAWKEY, C.J. (1996). Nitric oxide donating compounds stimulate human colonic ion transport in vitro. *Gut,* **39,** 93 – 99.

STRABEL, D. & DIENER, M. (1995). The effect of neuropeptide Y on sodium, chloride and potassium transport across the rat distal colon. *Br. J. Pharmacol.,* **115,** 1071 – 1079.

SUNDLER, F., BÖTTCHER, G., EKBLAD, E. & HÅKANSON, R. (1993). PP, PYY and NPY, occurrence and distribution in the periphery. From: *The Biology of Neuropeptide Y and Related Peptides*; Ed: Colmers WF, Wahlestedt C. Humana Press Inc. Totowa NJ.

TOUGH, I.R. & COX, H.M. (1996). Selective inhibition of NPY Y_1 receptors by BIBP3226 in rat and human epithelial preparations. *Eur. J. Pharmacol.,* **310,** 55 – 60.

WHARTON, J., GORDON, L., BYRNE, J., HERZOG, H., SELBIE, L., MOORE, K., SULLIVAN, M.H.F., ELDER, M.G., MOSCOSO, G., TAYLOR, K.M., SHINE, J. & POLAK, J.M. (1993). Expression of the human NPY Y_1 receptor. *Proc. Natl. Acad. Sci.,* **90,** 687 – 691.

WIELAND, H.A., ENGEL, W., EBERLEIN, W., RUDOLF, K. & DOODS, H.N. (1998). Subtype selectivity of the novel nonpeptide neuropeptide Y Y_1 receptor antagonist BIBO3304 and its effect on feeding in rodents. *Br. J. Pharmacol.,* **125,** 549 – 555.

WIELAND, H.A., WILLIM, K.D., ENTZEROTH, M., WIENEN, W., RUDOLF, K., EBERLEIN, W., ENGEL, W. & DOODS, H.N. (1995). Subtype selectivity and antagonistic profile of the nonpeptide Y_1 receptor antagonist BIBP3226. *J. Pharmacol. Exp. Ther.,* **275,** 143 – 149.

(Received October 11, 2001
Revised January 8, 2002
Accepted January 8, 2002)