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Functional consequences of neuropeptide Y Y_2 receptor knockout and Y_2 antagonism in mouse and human colonic tissues

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1 Neuropeptide Y (NPY), peptide YY (PYY) and pancreatic polypeptide (PP) differentially activate three Y receptors (Y_1, Y_2 and Y_4) in mouse and human isolated colon.

2 The aim of this study was to characterise Y_2 receptor-mediated responses in colon mucosa and longitudinal smooth muscle preparations from wild type ($Y_2+/+$) and knockout ($Y_2-/-$) mice and to compare the former with human mucosal Y agonist responses. Inhibition of mucosal short-circuit current and increases in muscle tone were monitored in colonic tissues from $Y_2+/+$ and $Y_2-/-$ mice $\pm$ Y_1 ((*R*)-*N*-[[4-(aminocarbonylaminomethyl)phenyl)methyl]-N^2-(diphenylacetyl)-argininamide-trifluoroacetate (BIBO3304) or Y_2 (*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 (BIIE0246) antagonists.

3 Predictably, $Y_2-/-$ tissues were insensitive to Y_2-preferred agonist PYY(3-36) ($\leqslant$100 nM), but unexpectedly Y_4-preferred PP responses were right-shifted probably as a consequence of elevated circulating PP levels, particularly in male $Y_2-/-$ mice (Sainsbury *et al*., 2002).

4 BIBO3304 and BIIE0246 elevated mucosal ion transport, indicating blockade of inhibitory mucosal tone in $Y_2+/+$ tissue. While BIBO3304 effects were unchanged, those to BIIE0246 were absent in $Y_2-/-$ mucosae. Neither antagonist altered muscle tone; however, BIIE0246 blocked NPY and PYY(3-36) increases in $Y_2+/+$ basal tone. BIBO3304 abolished residual Y_1-mediated NPY responses in $Y_2-/-$ smooth muscle.

5 Tetrodotoxin significantly reduced BIIE0246 and PYY(3-36) effects in $Y_2+/+$ mouse and human mucosae, but had no effect upon Y-agonist contractile responses, indicating that Y_2 receptors are located on submucosal, but not myenteric neurones.

6 Tonic activation of submucosal Y_2 receptors by endogenous NPY, PYY or PYY(3-36) could indirectly reduce mucosal ion transport in murine and human colon, while direct activation of Y_2 receptors on longitudinal muscle results in contraction.
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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; BIBP3435, [(*S*)-N^2-diphenylacetyl)-*N*-[(4-hydroxyphenyl)methyl]-argininamide] (acetate salt); 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; CCh, carbachol; CYN-154806, Ac-4NO_2-Phe-c(D-Cys-Tyr-D-Trp-Lys-Thr-Cys)-D-Tyr-NH_2; hPP, human pancreatic polypeptide; KH, Krebs Henseleit; NPY, neuropeptide Y; Pro^{34}PYY, human [Leu^{31}, Pro^{34}]PYY; PYY, peptide YY; PYY(3-36), peptide YY(3-36); sst, somatostatin 14-28; TTX, tetrodotoxin; UK14,304, 5-bromo-*N*-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine; VIP, vasoactive intestinal polypeptide

Introduction

Neuropeptide Y (NPY) suppresses synaptic excitation in the central and peripheral nervous systems (King *et al*., 2000; Weiser *et al*., 2000; Silva *et al*., 2001) by activating distinct Y receptors, most commonly postsynaptic or postjunctional Y_1 receptors and presynaptic Y_2 receptors (Dumont *et al*., 1998; Weiser *et al*., 2000; Kaga *et al*., 2001; Bahn *et al*., 2002). In the hypothalamus, activation of Y_2 receptors inhibits NPY-mediated tonic inhibition of adjacent pro-opiomelanocortin (POMC) neurones leading to satiety in mouse and man (Batterham *et al*., 2002). In the peripheral nervous system, Y_2 receptors also provide significant presynaptic (or prejunctional) inhibition that can either be autoinhibitory (Malmström *et al*., 2002), inhibitory upon noradrenergic (Cunningham *et al*., 1994) or cholinergic neurotransmission (Smith-White *et al*., 2002), but little is known about Y_2 receptor modulation of nonadrenergic, noncholinergic (NANC) neurotransmission.

Y_2 receptors are not, however, always presynaptic, nor are Y_1 receptors exclusively postsynaptic, or postjunctional (Cox & Cuthbert, 1990; McAuley & Westfall, 1992; Mannon *et al*., 1999; for reviews see, Cox, 1998; Michel *et al*., 1998). For

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example, epithelial Y_2 receptors alone mediate NPY-, PYY- and PYY(3-36)-induced inhibition of Cl^- secretion across rat jejunum mucosa (Cox *et al*., 1988; Cox & Cuthbert, 1990; Cox & Tough, 2000). In the rat colon mucosa, Y_2 receptors are coactivated with Y_1 receptors to inhibit anion secretion and in this tissue each receptor type exhibits pre- and postjunctional responses (Tough & Cox, 1996). The selective nonpeptide antagonists for Y_1 (BIBO3304, Wieland *et al*., 1998) and Y_2 receptors (BIIE0246, Doods *et al*., 1999; Dumont *et al*., 2000) have been crucial for the pharmacological characterisation of NPY, PYY and PYY(3-36) effects, especially in tissues coexpressing multiple Y receptor types. The two antagonists have enabled determination of the functional significance of Y_1 and Y_2 receptors in many isolated tissues and *in vivo* systems. For example in human isolated colon where NPY, and its hormone analogues, peptide YY (PYY), PYY(3-36) and pancreatic polypeptide (PP) are all antisecretory (and each agonist was equi-effective, Cox & Tough, 2002), only PP effects (i.e. Y_4 receptor-mediated effects) were insensitive to treatment with a combination of a Y_1 and Y_2 receptor antagonist. The same was also true of PP responses in the 129Sv mouse colon mucosa where all four peptides were inhibitory (but Y_1 responses predominated, Cox *et al*., 2001). Thus, in isolated human and murine colon mucosae, Y_1, Y_2 and Y_4 receptors have been shown to differentially mediate Y agonist inhibition of ion transport, and Y_5 receptors play no functional role in either tissue (Cox *et al*., 2001; Cox & Tough, 2002).

In the intestine, NPY released locally from enteric submucous secretomotor neurones innervating the mucosa (Ekblad *et al*., 1987; 1988; Sang & Young, 1996; see also review by Furness, 2000) can be antisecretory by activating either Y_2 or Y_1 receptors, or both. In contrast, the endocrine peptides PYY, its fragment PYY(3-36) and PP, which are expressed and released from intestinal or pancreatic type F endocrine cells, respectively (Sundler *et al*., 1993; Arantes & Nogueira, 1997), will differentially activate Y_1, Y_2 or Y_4 receptors in murine and human large bowel to provide further antisecretory, or proabsorptive, influences following ingestion of a meal. How these effects are coordinated with changes in the patterns of smooth muscle contraction and the modulatory roles that each Y receptor plays in the final integrated intestinal response, remains to be elucidated. Few studies of Y-agonist effects upon intestinal smooth muscle have actually included selective Y antagonists. However, from the agonist orders of potency, we may predict that tonic contractions stimulated by NPY and PYY (Pheng *et al*., 1999; Ferrier *et al*., 2000) are Y_2-mediated, while those to PP are most likely Y_4-mediated (in rat, Pheng *et al*., 1999; Ferrier *et al*., 2000; and rabbit intestine, Feletou *et al*., 1999). Neither the Y_1 receptor (Feletou *et al*., 1999; Ferrier *et al*., 2000) nor the Y_5 receptor (Feletou *et al*., 1999) appear to have a significant direct role on normal rodent intestine smooth muscle.

Thus, our initial aim was to elucidate the functional role(s) of Y_2 receptors in isolated smooth muscle and mucosal preparations using the Y_2 antagonist, BIIE0246 (Doods *et al*., 1999; Dumont *et al*., 2000) in wild-type ($Y_2+/+$) murine tissue. The functional phenotype exhibited by these preparations were compared with those from germline Y_2 receptor knockout ($Y_2-/-$) mice and preliminary investigations have already been presented to the British Pharmacological Society (Hyland *et al*., 2002). Our second aim was to establish whether Y_2 receptors were pre- or postjunctional and these studies were performed with murine and human colon mucosae. Thirdly, recent studies by Sainsbury *et al*. (2002) using the same germline $Y_2-/-$ mouse, described an unexpected gender-specific increase in circulating PP in male $Y_2-/-$ mice (together with complex changes in several parameters of energy homeostasis). How elevated circulating PP levels alter peripheral tissue sensitivity to Y agonists or to blockade of such responses by Y receptor antagonists, was of particular interest to us. We therefore set out to establish whether gender-specific differences in Y receptor responses were apparent in isolated intestinal tissue from germline $Y_2-/-$ in comparison with $Y_2+/+$ mice.

Methods

Tissue preparation

Germline $Y_2-/-$ and $Y_2+/+$ mice were generated and maintained on a mixed C57BL/6–129/SvJ background as described previously (Sainsbury *et al*., 2002). Age-matched adult mice (both sexes, 12 weeks or more) were asphyxiated with CO_2, weighed, the ascending and descending colon taken and 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). Luminal contents were removed by washing gently with KH.

Murine and human descending colon mucosal preparations

Human colon was obtained with the consent of patients undergoing elective bowel resection for primary intestinal carcinoma (the study was approved by the Guy's and St Thomas' Hospitals Research Ethics Committee; Cox & Tough, 2002). Mucosae were prepared by removing overlying circular and longitudinal smooth muscle layers by dissection under a microscope. Mouse and human colon routinely provided six or eight adjacent mucosal pieces, respectively. Either were placed between two halves of Perspex Ussing chambers (exposed area 0.2 cm^2 for mouse or 0.6 cm^2 for human tissues), bathed both sides with 5 ml of oxygenated (95% O_2/5% CO_2) KH and maintained at 37°C. Mucosae were voltage-clamped at 0 mV (using a DVC 1000 automatic voltage clamp, World Precision Instruments, Stevenage, U.K.) as previously described (Cox *et al*., 1988; 2001). Once a stable short-circuit current (I_{sc}) was obtained, agonists or antagonists were added to the basolateral reservoir only and the resultant changes in I_{sc} were recorded continuously. Murine descending colon mucosae were pretreated with vasoactive intestinal polypeptide (VIP) (30 nM) and 15 min later with either a single concentration of a Y agonist or an appropriate Y receptor antagonist was added (for a further 10–15 min) prior to agonist addition. Tetrodotoxin (TTX) pretreatment was for 15 min prior to VIP addition in mouse mucosa and for 30 min once a stable basal I_{sc} had been achieved with human mucosae. Maximal agonist-induced changes in I_{sc} were pooled (and quoted as $\mu A\,cm^{-2}$) and means $\pm$ 1 s.e.m. were calculated, where each mucosal or smooth muscle preparation provided a single n value.

Ascending colon longitudinal smooth muscle preparation

Each section of ascending colon provided two adjacent segments of longitudinal smooth muscle (each 1 cm long), which were cut distal to the caecal junction. Segments were washed with KH, attached with thread and suspended in an organ bath (10 ml) in oxygenated (95% O_2/5% CO_2) KH, maintained at 37°C. Tissues were stretched to a basal tension of 1 g and were allowed to equilibrate (for 45 min) with three intermittent KH washes. Isometric changes in basal tension were recorded in response to Y agonists in the absence or presence of specific Y antagonists (added 15 min prior to the agonist). Agonist-induced maximum increases in basal tone (within 5 min of agonist addition) were pooled and are quoted as increases in g tension throughout (mean ± 1 s.e.m.). Carbachol (CCh, 10 μM) was added as an internal contractile control at the end of each assay.

Statistical analysis

Each mucosal and smooth muscle preparation provided single observations, which were not paired but were pooled to provide means ± 1 s.e.m. For agonist–response curves, EC_{50} values (with 95% confidence limits) were calculated from pooled single agonist additions using GraphPad Prism (v. 3.0, GraphPad Software Inc., CA, U.S.A.). Student's unpaired *t*-test was used to compare responses ± antagonist. Multiple comparisons between data groups were evaluated using one-way ANOVA with either Bonferroni's or Dunnett's post-test, where appropriate. In each case, a *P*-value of less than 0.05 was considered statistically significant.

Materials

Peptides were purchased from Bachem U.K. Ltd (Merseyside, U.K.) unless otherwise stated and aliquots were frozen and stored at −20°C, only undergoing a single freeze–thaw cycle. The porcine (p) sequences of PYY, NPY and PP (the latter from Eli Lilly Inc., IN, U.S.A.), plus the human (h) sequences of PP, PYY(3-36) and Pro^{34}PYY were used as indicated. [(*S*)-N^2-diphenylacetyl)-*N*-[(4-hydroxyphenyl)methyl]-argininamide] (acetate salt) (BIBP3435), ((*R*)-*N*-[[4-(aminocarbonylaminomethyl)-phenyl)methyl]-N^2-(diphenyl acetyl)-argininamide-trifluoroacetate (BIBO3304) and (*S*)-N^2-[[1-[2-[4-(*R*,*S*)-5,11-dihydro-6(6H)-oxidobenz[b,e]azepin-11-y1]-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 (BIIE0246) were all obtained from Boehringer Ingelheim Pharma KG (Biberach an der Riss, Germany). [Ac-4NO_2-Phe-c(D-Cys-Tyr-D-Trp-Lys-Thr-Cys)-D-Tyr-NH_2 (CYN-154806) was a gift from Glaxo Institute of Applied Pharmacology (Cambridge, U.K.). 5-bromo-*N*-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine (UK14,304) was purchased from Research Biochemical International (Natick, MA, U.S.A.) and CCh and TTX were from Sigma (Poole, U.K.).

Results

General features of $Y_2+/+$ and $Y_2-/-$ mice and their descending colon mucosa

Age-matched female $Y_2+/+$ and $Y_2-/-$ mice weighed significantly less than their male counterparts of similar age, and $Y_2-/-$ mice were leaner than $Y_2+/+$ mice of a similar age (*P*-values ranging from 0.05 to 0.001, Table 1) as observed previously by Baldock *et al*. (2002). Since Sainsbury *et al*. (2002) described significantly elevated circulating PP levels in male germline $Y_2-/-$ (~15 nM) compared with male $Y_2+/+$ mice (~5 nM), and lower PP levels in female mice of either genotype (~2 nM in $Y_2-/-$ and 1 nM in $Y_2+/+$ mice), we segregated our data accordingly.

Mucosal resistance and basal I_{sc} levels were not significantly different in preparations from male and female $Y_2+/+$ and $Y_2-/-$ mice (Table 1). Electrogenic responses to basolateral VIP (30 nM, which stimulates cAMP-mediated epithelial Cl^- secretion and consequently elevates I_{sc}) were unchanged. Neither the maxima (Table 1) nor the time courses (data not shown) of VIP responses were different between mucosae from $Y_2+/+$ and $Y_2-/-$ tissue of either gender.

Mucosal I_{sc} responses to the Y_2 preferred agonist PYY(3-36) and to PYY± the Y_1 antagonist, BIBO3304 or the Y_2 antagonist, BIIE0246

In $Y_2+/+$ colon mucosae, PYY(3-36) attenuated VIP-elevated I_{sc} in a concentration-dependent manner with similar potency in wild-type male and female tissue (Figure 1a, b; Table 1). No PYY(3-36) responses were observed (up to 100 nM, Figure 1b) in $Y_2-/-$ tissue from either gender and the small decrease in I_{sc} observed with 300 nM PYY(3-36) (Figure 1b, male data only) was abolished by BIBO3304

Table 1 A comparison of basal current, basal resistance, in I_{sc} following addition of 30 nM VIP and weight between $Y_2+/+$ and $Y_2-/-$ mice

	$Y_2+/+$		$Y_2-/-$	
	Male	*Female*	*Male*	*Female*
Body weight (g)	31.9 ± 0.4 (25)	25.6 ± 0.4 (22)***	28.6 ± 0.6 (37)$^{+++}$	22.6 ± 0.5 (32)***$^{+}$
Age (weeks)	17.4 ± 1.4 (16)	18.8 ± 1.0 (20)	18.7 ± 1.0 (31)	19.4 ± 1.6 (26)
Resistance (Ω cm^2)	31.9 ± 1.7 (85)	37.6 ± 2.9 (65)	34.3 ± 1.9 (72)	34.7 ± 1.4 (88)
Basal current (μA cm^{-2})	52.7 ± 4.3 (138)	41.0 ± 2.3 (103)	44.6 ± 2.8 (167)	36.6 ± 3.0 (152)
VIP (30 nM) (μA cm^{-2})	66.9 ± 5.1 (105)	67.8 ± 4.4 (109)	65.9 ± 4.1 (173)	70.4 ± 4.2 (155)
EC_{50} PYY(3–36) (nM)	10.1 (6.7–15.4)	10.2 (4.3–23.9)	No response	No response
EC_{50} Pro ^{34}PYY (nM)	17.6 (9.2–33.8)	6.2 (1.9–20.1)	39.2 (25.0–61.4)	18.3 (7.3–46.2)
EC_{50} hPP (nM)	3.7 (0.3–41.7)	9.9 (3.2–30.1)	58.3 (43.0–79.2)	16.3 (5.7–46.8)

Values are ± 1 s.e.m. with *n* values in parenthesis. All EC_{50} values (with 95% confidence limits) are calculated from the pooled agonist concentration–response curves. No responses were recorded with PYY(3–36) ⩽100 nM. $^{+}P \leqslant 0.05$, $^{+++}P \leqslant 0.001$ comparisons between $Y_2+/+$ and $Y_2-/-$ mice and, ***$P \leqslant 0.001$ for comparisons between males and females within each group.

(300 nM, data not shown, Hyland *et al*., 2002). PYY (10 nM) responses following PYY(3-36) (10 nM) addition were not significantly different in $Y_2-/-$ tissues ($-25.8\pm6.6\,\mu A\ cm^{-2}$, $n=8$) from those in $Y_2+/+$ mucosa ($-27.8\pm10.3\,\mu A\ cm^{-2}$, $n=4$) indicating the predominance of Y_1-mediated responses to the full-length peptide. Subsequent addition of the α_2-adrenoceptor agonist, UK14,304 also elicited similar-sized reductions in I_{sc} in wild-type and knockout groups (Figure 1a for representative trace, pooled data not shown). In $Y_2+/+$ mucosa, the competitive Y_2 receptor antagonist BIIE0246 (1 μM) raised I_{sc} (by $2.5\pm0.4\,\mu A\,cm^{-2}$, $n=26$ and $2.1\pm0.3\,\mu A\,cm^{-2}$, $n=21$ in male and female tissue respectively, compared with vehicle controls containing BIBP3435 (1 μM) of $0.3\pm0.2\,\mu A\ cm^{-2}$, $n=6$ in male wild-type tissues) and this effect was lost from $Y_2-/-$ tissue ($0.6\pm0.6\,\mu A\ cm^{-2}$, $n=6$ and $0.3\,\mu A\ cm^{-2}$, $n=2$, male and female tissues respectively). Thus, the inhibitory Y_2 tone revealed by BIIE0246, as well as Y_2-stimulated agonist responses were predictably absent from $Y_2-/-$ male and female colon mucosae, with no changes in non-Y receptor-stimulated effects. The combination of Y_1 and Y_2 antagonists to $Y_2+/+$ mucosa resulted in a significant sustained elevation in I_{sc}, indicating a combined Y_1 and Y_2 receptor-mediated inhibitory tone in wild-type colon (Figure 2a, b).

Smooth muscle responses to the Y_2 preferred agonist PYY(3-36) and NPY± antagonists, BIBO3304 and BIIE0246

Longitudinal muscle contractile responses to PYY(3-36) (100 nM) were characterised (in $Y_2+/+$ ascending colon) by an initial increase in basal tone and a concomitant increase in frequency and amplitude of spontaneous activity (Figure 1c). These effects were present for 3–5 min in $Y_2+/+$, but were absent from $Y_2-/-$ colon (Figure 1c, d). PYY(3-36)-stimulated increases in $Y_2+/+$ basal tone (0.12 ± 0.02 g, $n=5$) were abolished by BIIE0246 (1 μM, 0.0 ± 0.0 g, $n=3$, $P<0.01$) the antagonist doing nothing *per se*. NPY (100 nM) also stimulated $Y_2+/+$ basal tone (Figure 1d) with associated increases in spontaneous activity (data not shown) and both components were unaffected by prior treatment with either the inactive enantiomer of Y_1 antagonist BIBP3226, BIBP3435 (300 nM), or the more selective Y_1 antagonist BIBO3304 (300 nM). NPY-stimulated $Y_2+/+$ basal tone was abolished by BIIE0246 (1 μM added in combination with BIBO3304, 300 nM, Figure 1d), indicating that Y_2 receptors predominantly mediate NPY-induced contraction in wild-type tissue. In $Y_2-/-$ colon, however, residual NPY-induced increases in basal tone (in the presence of BIBP3435) were abolished by BIBO3304 (Figure 1d), indicating that a Y_1 receptor-mediated component is present in Y_2 knockout longitudinal smooth muscle. CCh-stimulated contractions were not altered by any of the treatments above and there were no observable differences between muscarinic responses in male and female tissue (Figure 3a–d).

Mucosal responses to Y_4-preferring human pancreatic polypeptide (hPP) and Y_1-preferred $Pro^{34}PYY$± Y antagonists

At concentrations between 1 and 100 nM, human pancreatic polypeptide (hPP) was antisecretory with an EC_{50} of 3.7 nM in

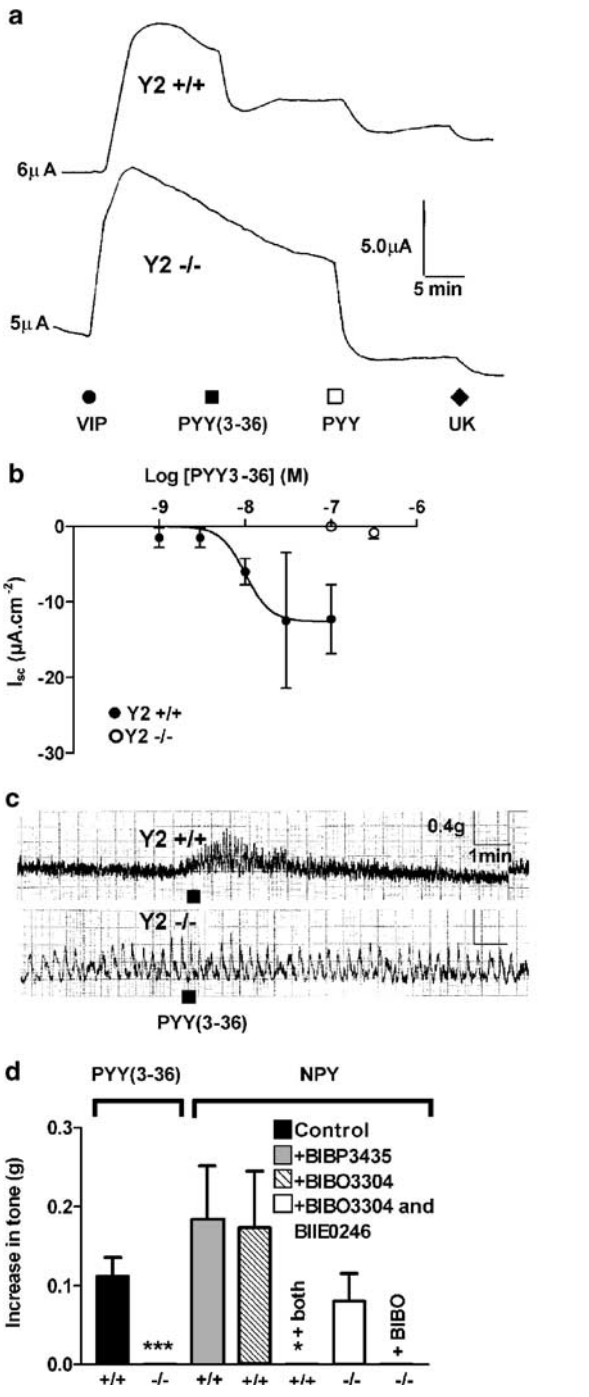

Figure 1 The presence or absence of PYY(3-36) responses in $Y_2+/+$ and $Y_2-/-$ mucosae (a, b) and longitudinal smooth muscle (c, d). (a) Representative responses to VIP (30 nM), PYY(3-36) (100 nM), PYY (10 nM) and UK14,304 (α2 adrenoceptor agonist) (1 μM) in $Y_2+/+$ (upper trace) and $Y_2-/-$ (lower trace). Values to the left of each trace in (a) are the basal I_{sc} prior to VIP addition. (b) Pooled PYY(3-36) data from male tissue in $Y_2+/+$ and $Y_2-/-$ colon. Values are the mean ± 1 s.e.m., $n=3$ throughout (where n represents the number of preparations). (c) Contractile effects of PYY(3-36) (100 nM) on smooth muscle in $Y_2+/+$ (upper trace) and $Y_2-/-$ (lower trace). (d) Pooled data showing PYY(3-36) or NPY (both at 100 nM) induced increases in tone in $Y_2+/+$ and $Y_2-/-$ colon, respectively. Each bar is the mean + 1 s.e.m. for between five and seven observations. Significant differences between NPY responses in the presence of both antagonists and vehicle control (BIBP3435, $*P\leqslant0.05$) and PYY(3-36) responses in $Y_2-/-$ compared with $Y_2+/+$ tissue, $***P<0.001$.

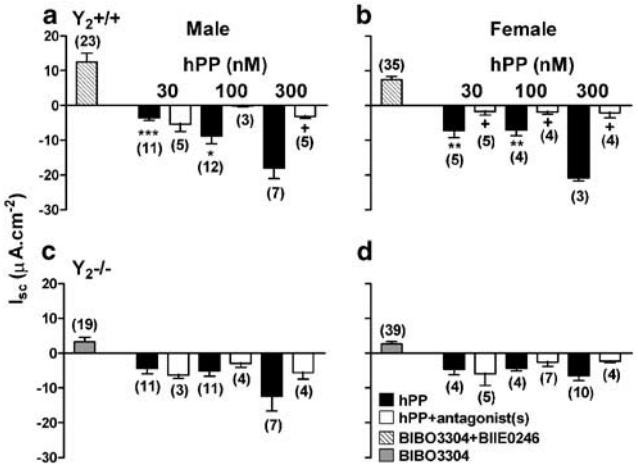

Figure 2 The effects of BIBO3304 (grey bars) or a combination of BIBO3304 and BIIE0246 (hatched bars) upon inhibitory hPP responses (open bars, in the presence of antagonist(s)) compared with control responses (black bars, in the absence of antagonist(s)) in $Y_2+/+$, male (a) and female (b), and $Y_2-/-$, male (c) and female (d) colon. All values are the mean ± 1 s.e.m. with n numbers in parentheses. Each 300 nM hPP response, in male and female $Y_2+/+$ tissue, was significantly larger (*$P\leqslant0.05$, **$P\leqslant0.01$, ***$P\leqslant0.001$; one-way ANOVA with Bonferonni's post-test) than the responses to 30 and 100 nM hPP in those groups. Unpaired Student's t-test was used to compare control hPP responses in the presence or absence of antagonist(s), where + $P\leqslant0.05$.

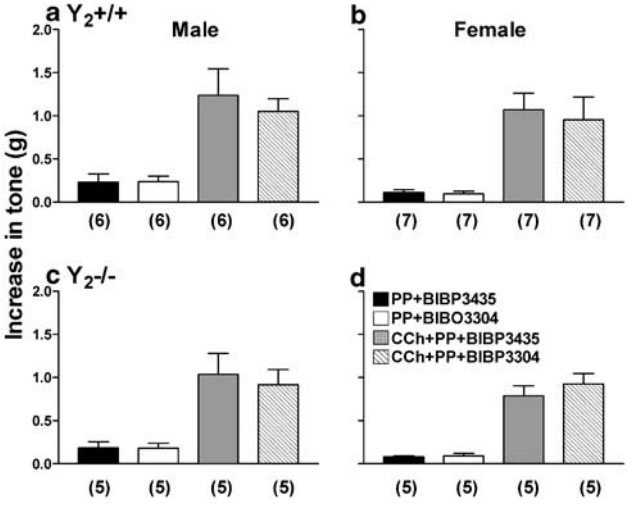

Figure 3 The effect of pPP (30 nM) upon basal tone of ascending colon preparations obtained from male (a) $Y_2+/+$ and female (b) $Y_2+/+$ mice and $Y_2-/-$ tissue from male (c) and female (d) mice. Responses in the presence of either BIBP3435 (300 nM, an inactive enantiomer of the Y_1 receptor antagonist, BIBP3226), or BIBO3304 (300 nM, a selective Y_1 receptor antagonist) are shown. Each bar is the mean + 1 s.e.m. with n numbers as shown in parenthesis. There are no significant differences between BIBP3435- and BIBO3304-pretreated pPP responses or between responses from male and female tissue.

male and 9.9 nM in female $Y_2+/+$ colon (Table 1). Within this concentration range, the sensitivity to exogenous hPP was approximately halved in female $Y_2-/-$ colon (where notably plasma PP levels doubled, Sainsbury *et al*., 2002) and in male $Y_2-/-$ mucosa the response curve was shifted an order of magnitude to the right compared with male $Y_2+/+$ tissue (Table 1, and the former exhibited three-fold elevated PP levels compared with male $Y_2+/+$ controls). In Figure 2, we show the effects of competitive antagonists (in combination, hatched bars or BIBO3304 alone in grey bars) followed by inhibitory responses to three hPP concentrations only (30, 100 and 300 nM) either in the absence or presence of Y_1 and Y_2 antagonists. In $Y_2+/+$ colon treated with BIBO3304 (300 nM) and BIIE0246 (1 μM), Y_4 receptors remain unaffected and can be stimulated by a Y_4-preferring agonist hPP. Thus, the antagonist combination raised I_{sc} levels (Figure 2a, b) indicating endogenous Y_1 and Y_2 receptor-mediated inhibitory tone in wild-type colon mucosae. Secondly, in male and female $Y_2+/+$ tissues, control 300 nM hPP responses were significantly larger than those at 100 nM (and were excluded from EC_{50} calculations). Antagonist treatment significantly reduced these high concentration responses to the levels observed with 30 or 100 nM hPP (Figure 2a, b) showing that at 300 nM hPP is able to stimulate Y_1 (and probably Y_2) as well as Y_4 receptors in this tissue.

In male and female $Y_2-/-$ colon, BIBO3304 alone caused a small but significant elevation in basal I_{sc} (Figure 2c, d) indicating a similar degree of Y_1 receptor-mediated endogenous tone as described for male 129Sv colon mucosa (Cox *et al*., 2001). In $Y_2-/-$ mucosae from either gender, BIBO3304 pretreatment did not significantly inhibit hPP effects (Figure 2c, d). Notably in male $Y_2-/-$ mucosa, the hPP concentration–response curve was shifted to the right (Table 1).

The potency of exogenous Y_1-preferred agonist, $Pro^{34}PYY$ (0.3–300 nM) was not significantly reduced when comparing $Y_2-/-$ responses with those in respective wild types (Table 1). The $Pro^{34}PYY$-response curve maxima (which were eight to 10 times greater than those for either hPP or PYY(3-36)) were unchanged in the four groups and BIBO3304 (300 nM) abolished $Pro^{34}PYY$ antisecretory effects (data not shown). Additionally, the inactive enantiomer of BIBP3226, BIBP3435 (1 μM) had no significant effect upon either $Pro^{34}PYY$ responses or other Y agonist effects in colon mucosal sheets (data not shown).

Smooth muscle responses to pPP in $Y_2+/+$ and $Y_2-/-$ ± either BIBO3304 or BIBP3435

In smooth muscle preparations pPP (30 nM) increased basal tone in both $Y_2+/+$ and $Y_2-/-$ tissue and these responses were not significantly altered by pretreatment with BIBO3304 or BIBP3435 (Figure 3). Neither compound altered basal tone *per se* (data not shown). Responses to pPP after either BIBO3304 or BIBP3435 were no different from those recorded in untreated male tissues (0.26 ± 0.09 g, $n=5$ in $Y_2+/+$ colon; 0.08 ± 0.03 g, $n=3$, $Y_2-/-$ tissue). Subsequent CCh responses were unaffected either by BIBO3304 or BIBP3435 treatment, and were no different between male, female, wild-type or knockout groups (Figure 3).

Effects of the Y_2 antagonist BIIE0246 and TTX upon Y_2-preferred PYY(3-36) responses in murine and human colon mucosae

The competitive Y_2 antagonist, BIIE0246 (1 μM) increased I_{sc} in both murine and human colon mucosae and these effects were significantly attenuated by TTX (100 nM, Figure 4a, b)

indicating that Y_2 receptors are present on inhibitory submucous neurones and that they are tonically active in both tissues. While TTX alone did not reduce mouse colon basal I_{sc} (from $26.5 \pm 6.9\,\mu$A cm^{-2}, $n=9$ to $24.7 \pm 6.7\,\mu$A cm^{-2}, $n=9$), it did significantly inhibit subsequent antisecretory PYY(3-36) responses (by 87.2%, $P<0.001$, Figure 4a). This agonist activation of Y_2 receptors was predominantly neurogenic although a residual $\sim$10% of the PYY(3-36) response (30 nM) was not TTX sensitive, indicating a minor postjunctional component, probably epithelial Y_2 receptor expression in this tissue. The identity of the final effector inhibiting I_{sc} across the wild-type mouse colon epithelium could be one of a number of inhibitory enteric neuropeptides, such as somatostatin (sst). A selective sst_2 receptor antagonist, CYN-154806 (1 μM, Feniuk *et al*., 2000; Tough & Cox, 2002) was tested. However, it had no effect upon PYY(3-36) responses (controls $-5.7 \pm 1.1\,\mu$A cm^{-2}, $n=7$; plus CYN-154806, $-6.6 \pm 0.9\,\mu$A cm^{-2}, $n=7$), but it significantly reduced sst (30 nM) responses (from $-50.0 \pm 8.5\,\mu$A cm^{-2}, $n=7$ to $-10.2 \pm 3.4\,\mu$A cm^{-2}, $n=7$, $P<0.001$).

In human colon mucosa, TTX significantly reduced basal I_{sc} (from $66.2 \pm 18.5\,\mu$A cm^{-2}, $n=6$ to $17.8 \pm 8.9\,\mu$A cm^{-2}, $n=5$; $P<0.05$, Figure 4b). Subsequent PYY(3-36) (100 nM) responses were abolished by BIIE0246 (as shown previously, Cox & Tough, 2002) and by TTX ($P<0.01$, Figure 4b). Thus, Y_2 receptors in human colon mucosa are also predominantly prejunctional and reduce electrogenic epithelial ion transport, a mechanism similar to that observed in murine colon mucosa (Figure 4a).

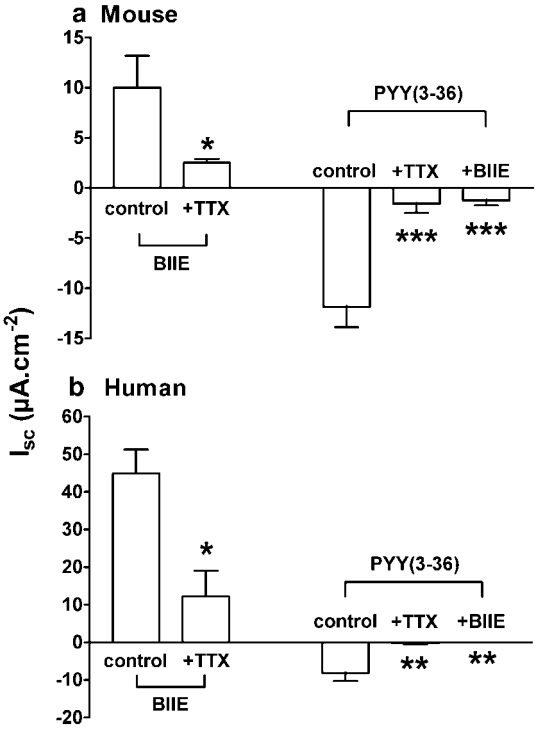

Figure 4 The effects of BIIE0246 (1 μM) and TTX (100 nM) *per se* and upon subsequent PYY(3-36) (30 nM in (a) and 100 nM in (b)) responses in (a); wild-type mouse descending colon mucosa and in (b), human colon mucosae. Each bar is the mean $\pm$ 1 s.e.m. from between three and six observations and $*P \leqslant 0.05$, $**P \leqslant 0.01$, $***P \leqslant 0.001$.

Effects of TTX upon PYY(3-36)-induced contractile responses in mouse colon smooth muscle

In wild-type ascending colon, TTX (200 nM) caused an increase in basal tone (0.34 ± 0.08 g, $n=7$) and spontaneous activity (data not shown), neither of which were significantly altered in $Y_2-/-$ tissue (basal tone; 0.21 ± 0.03 g, $n=7$). PYY(3-36) effects following TTX were no different from those in naïve tissues (0.13 ± 0.04 g, $n=6$; compared with controls of 0.12 ± 0.02 g, $n=5$) and are therefore direct excitatory effects upon longitudinal smooth muscle.

Conclusion

General features

The reduced body weight observed in male and female $Y_2-/-$ mice is consistent with the lean phenotype observed previously in these germline knockout mice, where the long-term loss of hypothalamic Y_2 receptors has been associated with elevations in orexigenic NPY and Agouti-related peptide (AgRP) and with coincident decreases in anorexic POMC and cocaine-and amphetamine-regulated transcript (CART) mRNA in the arcuate nucleus (Sainsbury *et al*., 2002). Y_2 receptors in this nucleus are thought to mediate the inhibitory food intake response observed in humans and mice, that occurs for up to 12 h following elevated postprandial PYY(3-36) (Batterham *et al*., 2002). Male $Y_2-/-$ mice, in addition to being lean, also exhibit significantly increased circulating PP levels (Sainsbury *et al*., 2002) compared with $Y_2+/+$ and a transgenic PP overexpressing mouse with elevated circulating levels of PP also exhibits a lean phenotype (Ueno *et al*., 1999). Conversely, human obesity syndromes and the genetically obese *ob*/*ob* mouse demonstrate reduced PP plasma levels. How raised PP occurs as a consequence of germ line Y_2 receptor knockout remains unclear, although sexual dimorphism in the functioning of the hypothalamo-pituitary-adrenal axis is evidently partially responsible (Sainsbury *et al*., 2002). Such changes in circulating PP will not only alter hypothalamic mechanisms, but also peripheral tissue sensitivities to the hormone and potentially to other Y agonists with overlapping pharmacology (for example, hPP can activate murine Y_1 as well as Y_4 receptors).

Predicted losses in sensitivity to the Y_2-preferred agonist, PYY(3-36) in $Y_2-/-$ compared with $Y_2+/+$ colon mucosa and smooth muscle

Y_2 receptors predominantly mediate PYY(3-36) responses (up to 100 nM) in colon mucosa and smooth muscle. This agonist's concentration–response curve in $Y_2+/+$ mucosa was comparable with that from 129Sv mouse colon mucosa (Cox *et al*., 2001). No sensitivity to this fragment was observed in either female or male $Y_2-/-$ mucosae, up to concentrations of 100 nM. The small decreases in I_{sc} seen to 300 nM PYY(3-36) in $Y_2-/-$ were abolished by BIBO3304 pretreatment, showing that the fragment can stimulate Y_1 as well as Y_2 receptors, albeit at high nM concentrations.

Endogenous PP is predicted to preferentially stimulate Y_4 receptors, although costimulation of Y_1 receptors may also occur (Cox *et al*., 2001). The consequence of either or both of these events would attenuate electrogenic anion secretion across mucosal

preparations thereby lowering basal I_{sc} levels. Such a pattern was observed with $Y_2-/-$ mucosae (Table 1) and correlates with the robust elevations in circulating PP levels established in $Y_2-/-$ mice of both genders compared to $Y_2+/+$ mice (Sainsbury *et al*., 2002). The absence of differences in VIP-stimulated I_{sc} responses and basal mucosal resistances, between the four tissues, argues against nonspecific mucosal changes in knockout tissues.

In $Y_2+/+$ ascending colon longitudinal smooth muscle, Y_2 receptors exclusively mediate PYY(3-36) and NPY contractile effects (Figure 1d). The effects of Y agonists that we observe in mouse ascending colon are similar to the tonic contractions recorded in rat ascending colon longitudinal smooth muscle by Ferrier *et al*. (2000) and Pheng *et al*. (1999). In the latter, NPY, PYY and PP responses were abolished by TTX and partially inhibited by atropine, indicating that activation of Y receptors (probably Y_2 and Y_4) resulted in ACh and NANC transmitter release to cause contraction indirectly. RT–PCR analysis of rat colon showed expression of Y_1 and Y_4 receptor mRNA (Ferrier *et al*., 2000) and there were no Y_2-mediated NPY(13-36) effects. Y_2 mRNA was also lacking from 'nonepithelial' preparations of rat colon, where PCR-based detection identified Y_1, Y_4 and Y_5 receptor mRNA (Goumain *et al*., 1998). It is important also to note that the Y_6 receptor is not expressed in adult mouse tissue (Gregor *et al*., 1996). Thus, our data clearly demonstrate a functional role for Y_2 receptors in mouse colon and indicate disparities between species, not only in terms of the Y receptor type(s) involved, but also the mechanism by which Y agonist-mediated excitation occurs. The TTX insensitivity of PYY(3-36) and also PP contractile responses (the latter were also atropine- and neurokinin-insensitive, Singh *et al*., 2002; Tough *et al*., 2002) indicate that both peptides act predominantly directly upon mouse longitudinal smooth muscle to cause contraction. In $Y_2-/-$ tissue, we also observed a Y_1 receptor-mediated excitatory component, which was abolished with BIBO3304. Whether this represents a compensatory upregulation of the Y_1 receptor type specifically in smooth muscle remains to be determined.

Wild-type colon longitudinal smooth muscle therefore contracts following stimulation of Y_2 and Y_4 receptors and there was no evidence of inhibitory tone in this preparation, in contrast with colonic mucosae from both human and mouse. It is clear, however, that in murine colon mucosa Y_2 receptors are located predominantly on submucosal neurones (Figure 4) and that a significant level of Y_2-mediated inhibitory tone is present in this tissue. Human colon mucosa also exhibits a marked Y_2 tone which was also TTX-sensitive. Subsequent PYY(3-36) responses were abolished by either TTX or the Y_2 antagonist, BIIE0246, indicating a solely prejunctional Y_2-mediated mechanism.

Unpredicted functional changes in $Y_2-/-$ compared with $Y_2+/+$ tissues

The increases in PP EC_{50} values in male $Y_2-/-$ mucosae could be a consequence of sustained Y_4 receptor stimulation (this reducing basal epithelial I_{sc} slightly in $Y_2-/-$ mucosa) by elevated endogenous PP (Sainsbury *et al*., 2002). At concentrations of 30 nM (and higher, Cox *et al*., 2001) hPP coactivates murine Y_1 as well as Y_4 receptors (Figure 2a, b, Tough *et al*., 2002). Thus, prolonged elevations of endogenous PP levels in male $Y_2-/-$ could also be manifested by reductions in Y_1-agonist potency, which our observations with $Pro^{34}PYY$ do suggest. The EC_{50} values for $Pro^{34}PYY$ were greatest in male $Y_2-/-$ tissue (from mice where PP levels of $\sim$15 nM have been recorded), next highest in male $Y_2+/+$ mucosa (where PP levels were $\sim$5 nM), followed by female $Y_2-/-$ and $Y_2+/+$ tissue (where PP levels were $\sim$2 and $\sim$1 nM respectively, Sainsbury *et al*., 2002). In female $Y_2-/-$ tissues, hPP potency was reduced by half compared with that in female $Y_2+/+$. The observation that both female groups exhibited reduced hPP sensitivity compared with those from males (Table 1) implicates additional gender-specific differences that have altered female tissue sensitivity to exogenous peptide.

In smooth muscle, PP responses (30 nM) were not sensitive to BIBO3304 in any group and there were no significant differences in the size of these peptides or CCh responses between $Y_2+/+$ and $Y_2-/-$ preparations (Figure 3). The cellular mechanisms underpinning PP-mediated contractile effects in intestinal smooth muscle do involve voltage-gated Ca^{2+} channels since responses are abolished by nifedipine (Singh *et al*., 2002). All the Y receptor types are coupled to $G_{i/o}$, pertussis toxin (PT)-sensitive inhibition of adenylate cyclase (for review, see Michel *et al*., 1998), but in neuroblastoma cells (Lynch *et al*., 1994) a PT-insensitive, direct coupling to receptor-operated Ca^{2+} channels provides for depolarisation and resultant contraction. We have yet to determine the exact cellular mechanism(s) involved in these Y receptor-mediated smooth muscle responses.

From these functional studies, we conclude that Y_2 receptors are differentially expressed, being present postjunctionally on murine smooth muscle (together with Y_4 receptors), while in mucosal preparations Y_2 receptors are predominantly prejunctional. Activation of the latter by the endogenous Y_2 agonist PYY(3-36) results in intrinsic inhibitory Y_2 tone and therefore attenuates epithelial ion transport indirectly in human and wild-type mouse colon. Germline knockout of Y_2 receptors predictably led to loss of sensitivity to the preferred Y_2 agonist, PYY(3-36) in isolated preparations, while the associated elevation in circulating PP levels in $Y_2-/-$ mice resulted in functional blunting, not just of exogenous PP (Y_4-mediated) responses, but also of $Pro^{34}PYY$ (Y_1-mediated) antisecretory effects.

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