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The regulation of veratridine-stimulated electrogenic ion transport in mouse colon by neuropeptide Y (NPY), Y_1 and Y_2 receptors

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1 Neuropeptide Y (NPY) is a prominent enteric neuropeptide with prolonged antisecretory effects in mammalian intestine. Veratridine depolarises neurons consequently causing epithelial anion secretion across mouse colon mucosa. Our aim was to characterise functionally, veratridine-stimulated mucosal responses and to determine the roles for NPY, Y_1, and Y_2 receptors in modulating these neurogenic effects.

2 Colon mucosae (with intact submucous innervation) from wild-type mice (+/+) and knockouts lacking either NPY (NPY−/−), Y_1−/− or Y_2−/− were placed in Ussing chambers and voltage clamped at 0 mV. Veratridine-stimulated short-circuit current (I_{sc}) responses in +/+, Y_1 or Y_2 antagonist pretreated +/+ colon, Y_1−/− and NPY−/− colon were insensitive to cholinergic blockade by atropine (At; 1 μM) and hexamethonium (Hex; 10 μM). Tetrodotoxin (TTX, 100 nM) abolished veratridine responses, but had no effect upon carbachol (CCh) or vasoactive intestinal polypeptide (VIP)-induced secretory responses.

3 To establish the functional roles for Y_1 and Y_2 receptors, +/+ tissues were pretreated with either the Y_1 or Y_2 receptor antagonist (BIBO3304 (300 nM) or BIIE0246 (1 μM), respectively) and veratridine responses were compared with those from Y_1−/− or Y_2−/− colon. Neither BIBO3304 nor Y_1−/− altered veratridine-induced secretion, but Y_1 agonist responses were abolished in both preparations. In contrast, the Y_2 antagonist BIIE0246 significantly amplified veratridine responses in +/+ mucosa. Unexpectedly, NPY−/− colon exhibited significantly attenuated veratridine responses (between 1 and 5 min).

4 We demonstrate that electrogenic veratridine responses in mouse colon are noncholinergic and that NPY can act directly upon epithelia, a Y_1 receptor effect. The enhanced veratridine response observed in +/+ tissue following BIIE0246, indicates that Y_2 receptors are located on submucosal neurons and that their activation by NPY will inhibit enteric noncholinergic secretory neurotransmission.

5 We also demonstrate Y_1 and Y_2 receptor-mediated antisecretory tone in +/+ colon and show selective loss of each in Y_1 and Y_2 null colon respectively. In NPY−/− tissue, only Y_1-mediated tone was present, this presumably being mediated by endogenous endocrine peptide YY. Y_2 tone was absent from NPY−/− (and Y_2−/−) colon and we conclude that NPY activation of neuronal Y_2 receptors attenuates secretory neurotransmission thereby providing an absorptive electrolyte tone in isolated colon.
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Abbreviations: At, atropine; BIBO3304, ((*R*)-*N*-[[4-(aminocarbonylaminomethyl)phenyl)methyl]-N^2-(diphenylacetyl)-arginina-mide-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; DMSO, dimethylsulphoxide; Hex, Hexamethonium; KH, Krebs–Henseleit; NOS, nitric oxide synthase; NPY, neuropeptide Y; NPY−/−, NPY knockout mice; PG97–269, [Acetyl-His[1], D-Phe[2], Lys[15], Arg[16], Leu[17]]VIP(3–7)/GRF(8–27); PP, pancreatic polypeptide; Pro[34]PYY, human [Leu[31], Pro[34]]PYY; PYY, peptide YY; PYY(3–36), peptide YY(3–36); TTX, tetrodotoxin; VIP, vasoactive intestinal polypeptide; Y_1−/−, Y_1 receptor knockout; Y_2−/−, Y_2 receptor knockout; +/+, all wild type including NPY +/+, Y_1 +/+ and Y_2 +/+

Introduction

Neuropeptide Y (NPY) is a prominent neuropeptide located within myenteric and submucosal plexus neurons throughout the mouse colon, the latter providing a dense network of immunoreactive fibres within the lamina propria (Sang & Young, 1996). NPY and peptide YY (PYY), an intestinal hormone released from endocrine cells that are abundant in the descending colon (Ekblad & Sundler, 2002), preferentially stimulate Y_1 and Y_2 receptors. PYY(3–36), hydrolysed from PYY (Medeiros & Turner, 1994) and a Y_2 receptor preferring

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fragment (Michel *et al*., 1998) is the predominant form of postprandial circulating PYY (Grandt *et al*., 1994), and has been associated with decreased food intake in mice and humans (Batterham *et al*., 2002).

By virtue of their primary G_i-protein coupling in intestinal epithelia, NPY and PYY are both antisecretory peptides and cause an inhibition of ongoing adenylate cyclase activity (Servin *et al*., 1989). Studies of mouse isolated colon mucosa demonstrate an antisecretory role for NPY, PYY and the Y_1 receptor preferring analogue of PYY, Pro^{34}PYY, in reducing vasoactive intestinal polypeptide (VIP)-stimulated short circuit current (I_{sc}; Holliday *et al*., 2000). As expected Pro^{34}PYY responses were sensitive to pretreatment with the Y_1 receptor antagonist ((*R*)-*N*-[[4-(aminocarbonylaminomethyl)phenyl) methyl]-N^2-(diphenylacetyl)-argininamide-trifluoroacetate (BIBO3304) (Wieland *et al*., 1998), confirming an antisecretory role for this type of Y receptor in the mouse colon (Holliday *et al*., 2000). However, PYY responses were only partially attenuated by the Y_1 antagonist, demonstrating the ability of PYY to activate Y_1 and Y_2 receptors in mouse colon mucosa (Holliday *et al*., 2000). A role for the Y_2 receptor was subsequently confirmed by Cox *et al*. (2001) using the selective Y_2 receptor antagonist, BIIE0246 (Doods *et al*., 1999) and the combination of BIBO3304 and BIIE0246 was shown to abolish the antisecretory effects of PYY (Cox *et al*., 2001 and NPY (Cox *et al*., unpublished).

Pancreatic polypeptide (PP) is also a member of the NPY peptide family and causes a decrease in I_{sc} in murine and human colon mucosae (though not rat small intestine or colon mucosae) by preferentially stimulating Y_4 receptors (Tough & Cox, 1996; Cox *et al*., 2001; Cox & Tough, 2002). However, the antisecretory effects of PP in mouse colon are significantly smaller than those of either Pro^{34}PYY or PYY (Cox *et al*., 2001) suggesting that the former peptide and its receptor have a less significant role in regulating ion secretion, than the Y_1 receptor. The Y_5 receptor agonist Ala^{31}, Aib^{32}hNPY was only active at μM concentrations in mouse colon mucosa (Cox *et al*., 2001) while y_6 receptors do not appear to be present in adult mouse (Gregor *et al*., 1996). Taken together these data suggest that Y_1 and Y_2 receptors are the predominant Y receptor types mediating the antisecretory actions of NPY and PYY in mouse colon, a finding analogous to the human colon (Cox & Tough, 2002).

Immunohistochemical evidence for both epithelial and neuronal Y_1 receptors has been described in rat (Jackerott & Larsson, 1997) and human (Peaire *et al*., 1997; Mannon *et al*., 1999) intestine. In rat small intestine, Y_1 receptor immunoreactivity was colocalised with NPY on submucosal cell bodies and on both submucosal and myenteric nerve terminals (Jackerott & Larsson, 1997). Colocalisation was also demonstrated between a fraction of Y_1-positive nerve cell bodies and VIP and a separate Y_1-expressing population plus nitric oxide synthase (NOS) in submucosal cell bodies. A similar pattern of colocalisation between Y_1 and NOS was reported in human myenteric neurons, while NPY and Y_1 receptor colocalisation was observed in Henle's plexus neurons (Peaire *et al*., 1997). Y_1 receptor immunoreactivity exhibited a basolateral distribution in both crypt and surface epithelia of human colon (Mannon *et al*., 1999) and this matched the sidedness of Y_1 receptor-mediated responses observed in isolated human colon mucosa (Cox & Tough, 2002).

The lack of a commercially available Y_2 receptor antibody has precluded a detailed localisation of this receptor type. Nevertheless Y_2 receptor mRNA was found to be highly expressed in rat proximal and distal colon (Feletou *et al*., 1998), in the muscle of the ileum and ascending colon, as well as in the mucosal layers of the human ileum and descending colon (Ferrier *et al*., 2002). Taken together these studies suggest that Y_2 and Y_1 receptor activation can result in direct actions upon epithelial function as well as indirect neuronal modulatory roles in particular species and tissues. In the present study, we used the plant-derived alkaloid, veratridine, to depolarise intrinsic neurons (as a result of increased voltage-sensitive Na^+ permeability, Catterall, 1980), in order to elucidate functionally the pre- *versus* postjunctional modulatory roles afforded by Y_1 and Y_2 receptors in mouse descending colon.

Veratridine-stimulated responses had previously been characterised in mouse jejunum mucosal preparations and produced an increase in I_{sc}, associated with an increase in net Cl^- secretion (Sheldon *et al*., 1990). Both veratridine and veratrine were shown to stimulate the release of enteric neurotransmitters, for example, substance P (SP), VIP (Belai & Burnstock, 1988) and acetylcholine (ACh; Yau *et al*., 1986) from guinea pig myenteric neurons, and also VIP release from myenteric synaptosomes (Allescher *et al*., 1996). However, a role for NPY in contributing to, or modulating, veratridine-stimulated ion secretion has yet to be characterised. Using selective Y_1 and Y_2 receptor antagonists (BIBO3304 and BIIE0246, respectively) and mice lacking either the peptide (NPY−/−) or one receptor type (Y_1−/− and Y_2−/−) we now describe discrete functional roles for NPY and these two Y receptors in the mouse colon.

Methods

Tissue preparation

Descending colon was collected from both adult (>10 weeks old) Y_1+/+, Y_2+/+, NPY+/+, Y_1−/−, Y_2−/− and NPY−/− mice (all on the same mixed background, 129Sv/C57Bl6) which were asphyxiated with CO_2, followed by cervical dislocation. Tissues were immediately placed 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 routinely provided six mucosal preparations (with intact submucosal plexus innervation) termed 'mucosal preparations' throughout. These preparations were obtained by removing both overlying smooth muscle layers with the accompanying myenteric plexi, by dissection under a microscope. Mucosae were then placed in modified Ussing chambers (exposed area of 0.14 cm^2) bathing both sides with 5 ml oxygenated (95% O_2/5% CO_2) KH, maintained at 37°C.

Mucosae were voltage clamped at 0 mV using an automatic voltage clamp (DVC 1000, World Precision Instruments, Stevenage, U.K.) as previously described (Cox *et al*., 2001). All agents were added subsequently to the basolateral reservoir and changes in I_{sc} were recorded continuously. At least three animals contributed to each different data group. Tissues were not paired, and experiments with wild-type (+/+) and

knockout (−/−) mucosae were usually carried out on different days and not in parallel. Male and female mice were used in this study and data were pooled, as no significant differences between genders were found.

Investigation of neurogenic I_{sc} responses in mouse colon: TTX-sensitivity and antagonism of cholinoceptor responses

To investigate the tetrodotoxin (TTX, 100 nM) sensitivity of carbachol (CCh,10 μM) and VIP (30 nM) responses, TTX was added to tissues 15 min prior to either agonist, and forskolin (10 μM) was an internal control added at the end of each experiment. To investigate antagonism of muscarinic (atropine (At), 10 μM) and nicotinic (hexamethonium (Hex), 1 μM) receptors, these antagonists were added to untreated tissues 15 min prior to either CCh (10 μM) or nicotine (10 μM).

Chemical stimulation of enteric neurons with veratridine and modulation by BIBO3304 and BIIE0246

To stimulate all intrinsic neurons expressing voltage-sensitive Na^+ channels, veratridine (30 μM) was added to colonic preparations. Veratridine time-course experiments were recorded for 60 min, before TTX was added. To determine the contribution of ACh to veratridine-induced responses in wild-type and knockout tissues, a combination of At (10 μM) and Hex (1 μM) was added to untreated tissues 15 min before veratridine. To examine the modulation of veratridine-induced responses, the Y_1 antagonist, BIBO3304 (300 nM) or Y_2 antagonist, BIIE0246 (1 μM) were added 15 min prior to veratridine. Previous studies in our laboratory have demonstrated maximum inhibition of Y_1 or Y_2 agonist responses, respectively, by 300 nM BIBO3304 or 1 μM BIIE0246 (Cox *et al.*, 2001; Cox *et al.,* unpublished). In fact, these competitive antagonists both increased I_{sc} *per se*, revealing different degrees of Y_1 and Y_2 receptor-mediated inhibitory tone. The inactive enantiomer of Y_1 antagonist BIBP3226, 1 μM BIBP3435, was also used as an internal control, previous studies with +/+ mouse colon mucosa having shown it to have no effect upon Y_1 agonist responses, in contrast with BIBP3226 (1 μM) which abolished them (Cox *et al.,* unpublished). Each antagonist effect was analysed separately from their subsequent modulatory effect upon veratridine responses. The latter were recorded for 15 min, within which time the peak I_{sc} had been achieved and was beginning to wane. Respective agonists were then added to confirm specific antagonism, followed finally by TTX (100 nM).

Statistical analysis

Electrogenic I_{sc} responses were converted to $\mu A\,cm^{-2}$ and are quoted throughout as the mean ± 1 s.e.m. Student's unpaired *t*-test was used to compare either individual time points for veratridine responses, or agonist responses ± antagonist and a *P*-value of less than 0.05 was considered statistically significant. The responses of male and female Y_1+/+, Y_2+/+ and NPY+/+ animals (collectively abbreviated, +/+) to veratridine, were pooled as their genetic backgrounds were a similar mixture of 129Sv and C57Bl6 and importantly, that no significant differences were observed between these groups of animals (data not shown).

Materials

Pro^{34}PYY, PYY(3–36) and VIP were purchased from Bachem Ltd (Merseyside, U.K.) and aliquots were frozen and stored at −20°C, undergoing a single freeze-thaw cycle only. BIBO3304 (N^2-(diphenylacetyl)-*N*-[(4-hydroxy-phenyl)methyl]-D-arginine amide), BIBP3226 (N^2-(diphenylacetyl)-*N*-[(4-hydroxy-phenyl)methyl]-D-arginine amide), BIBP3435 (S)-N^2-diphenylacetyl)-*N*-[(4-hydroxyphenyl)methyl]-argininamide, and BIIE0246 ((S)-N^2-[[1-[2-[4-(R,S)-5,11-Dihydro-6(6H)-oxidobenz[b,e]azepin-11-yl]-1-piperazinyl]-2-oxoethyl]cyclopentyl]acetyl]-*N*-[2-[2-[1,2-dihydro-3,5(4H)-dioxo-1,2-diphenyl-3H-1,2,4-triazol-4-yl]ethyl]-argininamide) were obtained from Boehringer Ingelheim Pharma KG (Biberach an der Riss, Germany). The proposed $VPAC_1$ antagonist [Acetyl-His^1, D-Phe^2, Lys^{15}, Arg^{16}, Leu^{17}]VIP(3–7)/GRF(8–27) (PG97–269) (referred to as PG 97–269) was a gift from Patrick Robberecht (Universite Libre de Bruxelles, Belgium). Veratridine, CCh, nicotine, At, Hex and TTX were all purchased from Sigma (Poole, U.K.).

Results

Characterisation of veratridine-stimulated mucosal responses

Veratridine was added at time 0 min to the basolateral reservoir and resulted in an immediate increase in I_{sc}, which peaked 5–7 min later and then gradually returned towards basal I_{sc} levels over a period of 60 min (Figure 1a). Addition of TTX 15 min after veratridine reduced the intrinsic neuronal-elevated I_{sc} by >90% (Figure 1b). Pretreatment of tissues with a combination of both muscarinic and nicotinic antagonists (At and Hex) completely blocked subsequent CCh and nicotine I_{sc} responses, respectively (data not shown), but did not significantly alter veratridine-induced changes in I_{sc} in wild-type colon mucosa (Figure 1c). These data indicate that submucosal plexi cholinergic neurotransmission is not a significant component of the colonic mucosal response to veratridine.

Sensitivity of CCh and VIP responses to TTX pretreatment

TTX was added to mucosal sheets once a stable basal I_{sc} was achieved and it caused a small sustained decrease in I_{sc} *per se* (Figure 2a and b). Previously we reported that the Y_1 receptor preferred agonist, Pro^{34}PYY stimulated prolonged reductions in I_{sc} that were insensitive to TTX pretreatment while in contrast, Y_2-preferred agonist PYY(3–36) responses were significantly reduced (>80%) by the neurotoxin (Cox *et al.*, 2003; Hyland *et al.*, 2003). Neither CCh (Figure 2a) nor VIP-induced peak I_{sc} responses (Figure 2b) were altered by TTX. These data suggests the responses to CCh and VIP are predominantly, if not exclusively, epithelial in origin in mouse descending colon mucosa. We also tested the $VPAC_1$ antagonist, PG97–269 in the hope that it would block VIP-induced anion secretion, however, this analogue had no

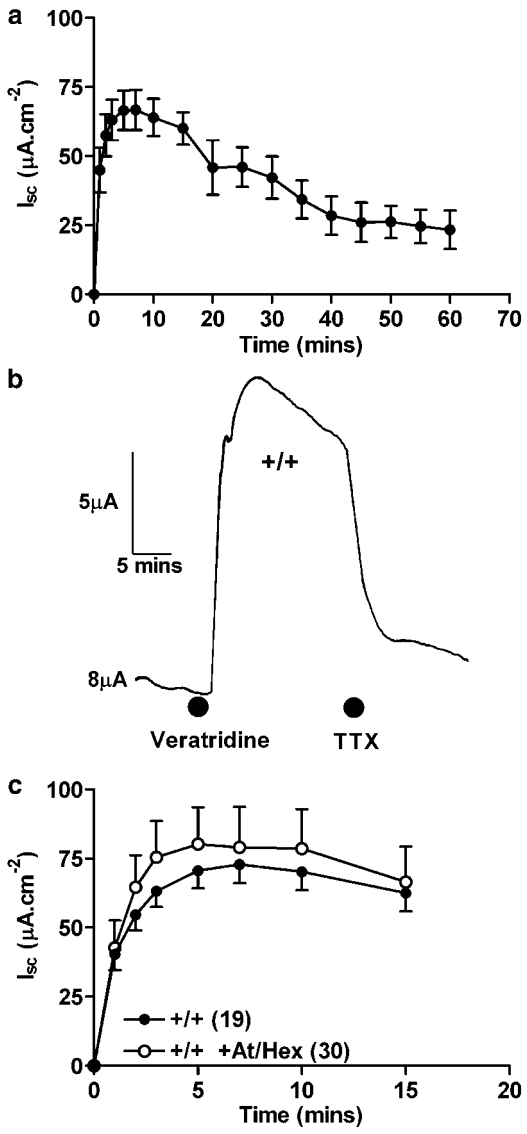

Figure 1 The pooled veratridine (30 μM) I_{sc} responses over a period of 60 min (a) $n=5$–19, and the sensitivity of a representative veratridine response to TTX (100 nM at 15 min, in b). In (c), a comparison of veratridine responses $\pm$ cholinergic blockade with atropine (At, 1 μM) and hexamethonium (Hex, 10 μM) is shown. Numbers of observations are otherwise given in parenthesis and data (in a and c) is the mean $\pm$ 1 s.e.m. A Student's unpaired *t*-test was used to compare veratridine responses $\pm$ At/Hex and $P>0.05$ for all time points shown.

significant effect (up to a concentration of 3 μM) upon subsequent VIP (30 nM) responses (data not shown).

Lack of regulation of veratridine-induced increases in I_{sc} by the Y_1 receptor

Neither pretreatment of +/+ tissue with the Y_1 receptor antagonist, BIBO3304 (300 nM, Figure 3a) nor knockout of the Y_1 receptor (Figure 3b) significantly altered the time-course of veratridine-induced ion transport. Cholinergic blockade did not alter veratridine-stimulated I_{sc} in either Y_1 antagonist pretreated or $Y_1-/-$ colon mucosae (Figure 3c and d) but did abolish subsequent responses to CCh and nicotine (data not shown).

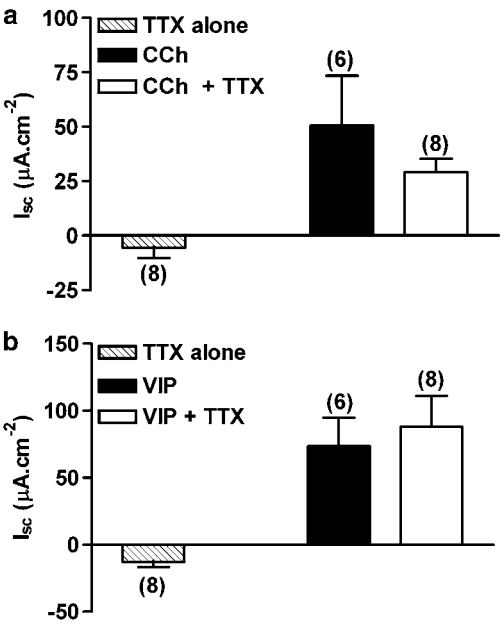

Figure 2 The effects of TTX pretreatment alone (100 nM, hatched bars) and upon subsequent CCh (10 μM, a) and VIP (30 nM, b) responses. Each bar is the mean $\pm$ 1 s.e.m. with *n* numbers shown in parenthesis. Student's unpaired *t*-test was used to compare control and TTX-treated data groups for either agonist and no significant differences were observed.

Regulation of veratridine-induced increases in I_{sc} by the Y_2 receptor

In contrast, pretreatment of +/+ tissue with the Y_2 antagonist BIIE0246 significantly increased veratridine responses (between 2 and 15 min, Figure 4a). A small but insignificant increase in veratridine-induced secretion was observed in $Y_2-/-$ tissue compared with +/+ colon (Figure 4b). As seen with $Y_1-/-$ colon, and +/+ tissues pretreated with a Y_1 antagonist, there was no effect of cholinergic blockade upon +/+ neurogenic responses in the presence of Y_2 anatgonist, BIIE0246 (Figure 4c). However, pretreatment of $Y_2-/-$ tissue with At and Hex markedly increased veratridine-induced responses indicating a potential role for ACh in modulating submucosal neurotransmission in $Y_2-/-$ colon. CCh responses were significantly decreased in $Y_2-/-$ colon; $56.8\pm18.8\,\mu A\,cm^{-2}$ (+/+, $n=4$) compared with $18.8\pm5.8\,\mu A\,cm^{-2}$ ($Y_2-/-$, $n=9$, $P<0.05$) in $Y_2-/-$ colon epithelia.

Antagonism of Y_1 and Y_2 receptors in $Y_2-/-$ and $Y_1-/-$ colon, respectively

Addition of BIBO3304 alone to $Y_2-/-$ tissue reduced veratridine-induced responses (Figure 5a) significantly so, 1–3 min after veratridine addition. This is in contrast to +/+ tissue where the Y_1 antagonist had no significant effect upon veratridine-stimulated I_{sc} (Figure 3a). Pretreatment of $Y_1-/-$ colon mucosa with Y_2 antagonist, BIIE0246 increased neurogenic changes in I_{sc} (Figure 5b), but to a lesser extent than observed with +/+ colon (Figure 4a).

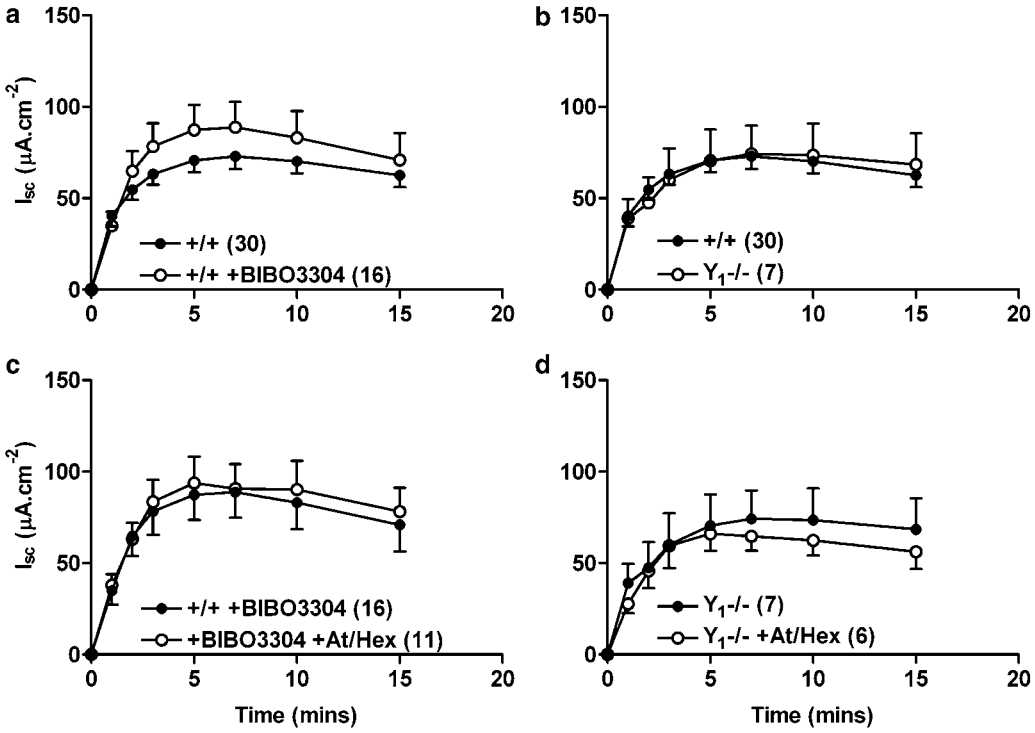

Figure 3 The effects of either Y_1 receptor antagonism (BIBO3304, 300 nM, in a) or Y_1 receptor knockout ($Y_1-/-$, in b) upon veratridine-induced (30 μM, added at time 0 min) I_{sc} responses. Cholinergic blockade with atropine (At, 1 μM) and hexamethonium (Hex, 10 μM, c and d) did not significantly alter veratridine responses in either BIBO3304-pretreated tissue (c) or in $Y_1-/-$ tissue (d). Each pooled mucosal veratridine response was compared wild-type ($+/+$) responses. Data are the mean $\pm$ 1 s.e.m. and n numbers are given in parenthesis.

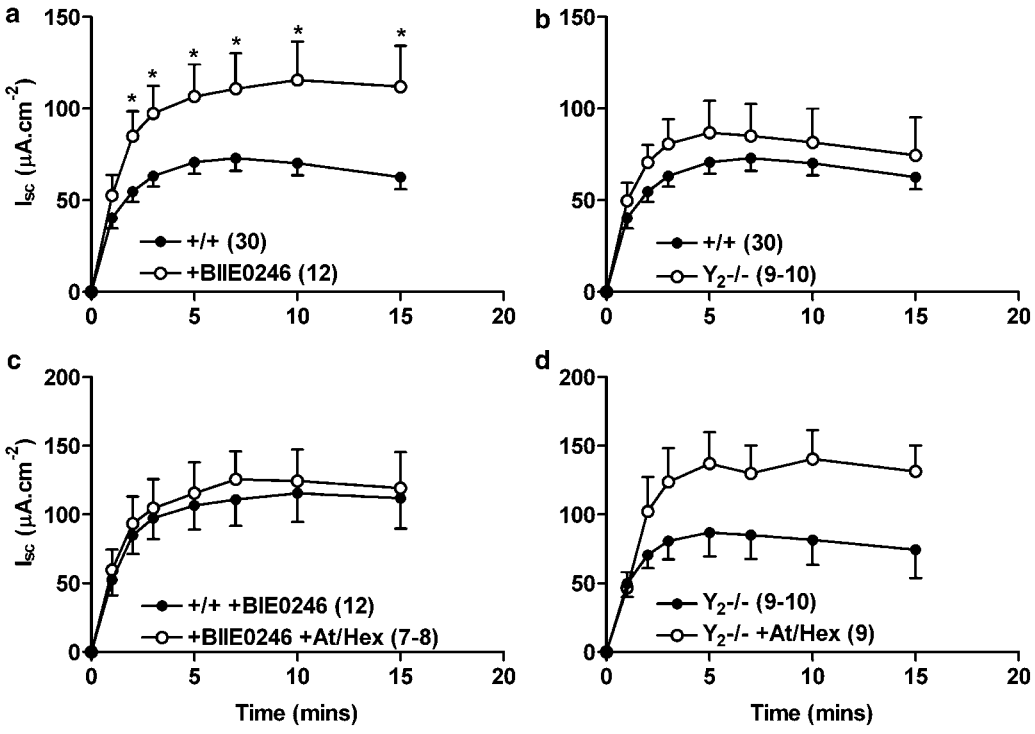

Figure 4 The effects of either Y_2 receptor antagonism (BIIE0246, 1 μM, a), or Y_2 receptor knockout ($Y_2-/-$, b) upon veratridine-induced I_{sc} responses compared with wild-type ($+/+$) controls. The effects of cholinergic blockade (atropine, At, 1 μM and hexamethonium, Hex, 10 μM) upon BIIE0246-pretreated $+/+$ tissue (in c), or $Y_2-/-$ tissue (in d) are shown for comparison. Veratridine (30 μM) was added at time 0 min. Each point is the mean $\pm$ 1 s.e.m and n numbers are given in parenthesis. A Student's unpaired t-test was used to compare individual time points and significant differences are shown; $^*P<0.05$.

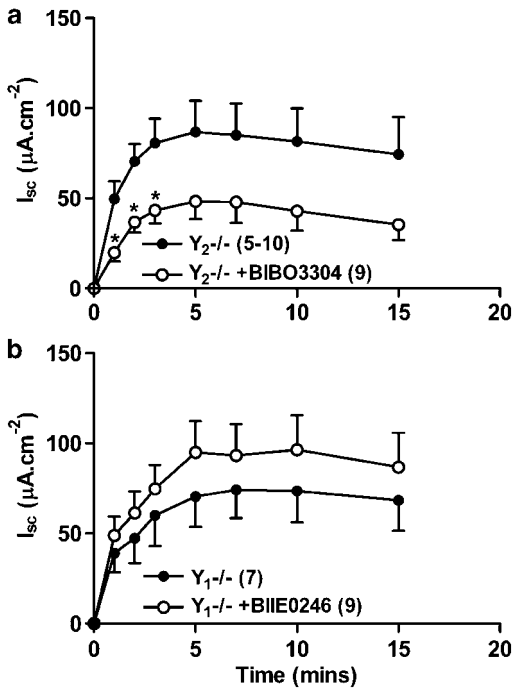

Figure 5 The effect of either Y_1 receptor antagonist pretreatment (BIBO3304, 300 nM) of $Y_2-/-$ colon (in a) or Y_2 antagonism (BIIE0246, 1 μM) of $Y_1-/-$ mucosa (in b). Veratridine (30 μM) was added at time 0 min and data are the mean $\pm$ 1 s.e.m., with n numbers shown in parenthesis. A Student's unpaired t-test was used for comparison between individual pairs of time points in a; $^*P<0.05$.

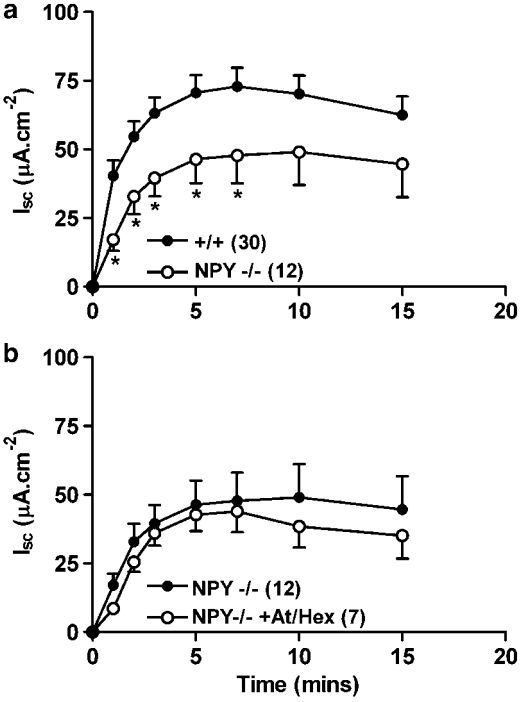

Figure 6 A comparison of NPY$-/-$ and wild-type ($+/+$) colon mucosal veratridine responses in (a); and the effects of cholinergic blockade upon the same in NPY$-/-$ colon in (b). Veratridine (30 μM) was added at time 0 min, and each data point is the mean $\pm$ 1 s.e.m. with n numbers in parenthesis. A Student's unpaired t-test was used to compare individual pairs of time points and $^*P<0.05$.

The effects of NPY knockout on veratridine-stimulated secretion in mouse descending colon

Somewhat surprisingly, veratridine responses were significantly blunted (between 1 and 7 min, Figure 6a) in NPY$-/-$ tissue compared with $+/+$ controls. When NPY$-/-$ mucosae were pretreated with At and Hex (Figure 6b) there was no significant alteration in the profile of the neurogenic responses, as seen previously with both $+/+$ (Figure 1c) and $Y_1-/-$ (Figure 3d) tissues. The sensitivity of NPY$-/-$ colon to exogenous Y_1 agonist, $Pro^{34}PYY$ (EC_{50} 16.3 nM (14.1–20.0) in $+/+$ colon, compared with 16.8 nM (10.3–25.8) in NPY$-/-$) or the Y_2 receptor-preferred fragment, PYY(3–36) (EC_{50} 12.4 nM (5.4–28.7) in $+/+$ tissues, compared with 29.4 nM (17.9–48.4) in NPY$-/-$ colon) was not significantly different, either in terms of potency or efficacy.

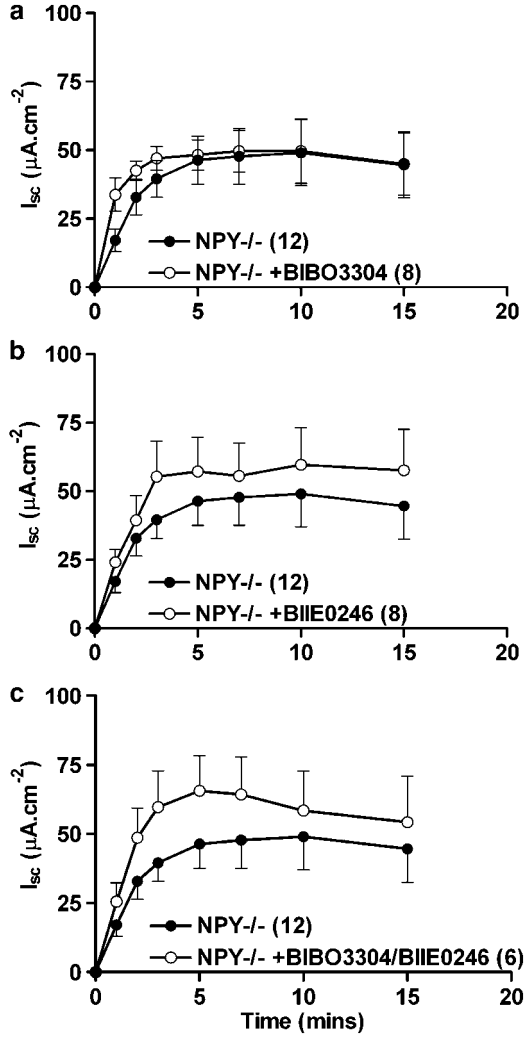

Figure 7 The effects of either single, Y_1 receptor antagonism (in a, BIBO3304, 300 nM) or in (b); Y_2 receptor blockade (BIIE0246, 1 μM) or in (c); combined Y_1 and Y_2 antagonism in NPY$-/-$ colon throughout. Veratridine (30 μM) was added at time 0 min. Data are the mean $\pm$ 1 s.e.m. with n numbers given in parenthesis. Student's unpaired t-test comparisons revealed there were no significant differences between any time points.

The effects of individual and combined Y_1 and Y_2 receptor antagonism upon NPY−/− colon

In order to investigate a role for enteroendocrine cell-derived PYY and PYY(3–36) upon submucosal neuromodulation, we measured veratridine-stimulated changes in I_{sc} in the presence of BIBO3304 and/or BIIE0246 in NPY−/− colon. BIBO3304 pretreatment of NPY−/− tissue caused a more rapid rate of I_{sc} increase after veratridine (1–3 min) but this was not significant (Figure 7a). BIIE0246 pretreatment of NPY−/− colon mucosae resulted in a nonsignificant amplification of the neurogenic response (the maximum difference at 3 min was, $39.5 \pm 6.7\,\mu A\,cm^{-2}$, $n=12$; and following BIIE0246 pretreatment, $55.3 \pm 13.0\,\mu A\,cm^{-2}$, $n=8$; Figure 7b). The combination of BIBO3304 and BIIE0246 (Figure 7c) appeared additive but again, this change in the veratridine response was not statistically significant.

The effects of either BIBO3304 or BIIE0246 alone upon naive and VIP-stimulated mucosae

The competitive Y_1 receptor antagonist, BIBO3304 when added to untreated +/+ colon (Figure 8a) or to VIP-stimulated (30 nM for 15 min) tissue (Figure 8b) resulted in a significant elevation of I_{sc} *per se*, compared to vehicle controls (DMSO) and both responses were significantly larger than controls ($P<0.01$). Like BIBO3304, the Y_1 antagonist, BIBP3226 (1 μM) also raised basal I_{sc} ($9.6 \pm 2.0\,\mu A\,cm^{-2}$, $n=6$) while its inactive enantiomer, BIBP3435 (1 μM) had no significant effect ($0.6 \pm 0.4\,\mu A\,cm^{-2}$, $n=6$). Y_1-mediated $Pro^{34}NPY$ responses (30 nM) were abolished by BIBP3226 (controls; $-47.3 \pm 7.8\,\mu A\,cm^{-2}$, $n=6$, and plus BIBP3226; $-0.6 \pm 0.6\,\mu A\,cm^{-2}$, $n=6$) while BIBP3435 had no significant effect upon agonist responses ($-39.6 \pm 13.1\,\mu A\,cm^{-2}$, $n=6$).

The effect of Y_1 antagonist, BIBO3304 was predictably absent from Y_1−/− colon both in naive tissues (Figure 8a) and following VIP pretreatment (Figure 8b). In untreated +/+ colon mucosa, BIBO3304's effects were insensitive to At and Hex (in controls $6.9 \pm 1.4\,\mu A\,cm^{-2}$ ($n=24$) compared with $6.3 \pm 1.3\,\mu A\,cm^{-2}$ ($n=12$) in experimentals) which suggests that submucous plexus-derived ACh does not contribute to Y_1 tone. Interestingly, Y_1 inhibitory tone was significant in basal and VIP-pretreated Y_2−/− and NPY−/− tissue (Figure 8a and b) and comparable with that observed in +/+ tissues.

The Y_2 receptor antagonist, BIIE0246 when added to untreated +/+ tissue did not significantly elevate basal I_{sc} above vehicle control (DMSO; Figure 8c), however, following VIP, it did significantly increase I_{sc} in both +/+ and Y_1−/− tissue (Figure 8d). Furthermore, the effects of the competitive Y_2 antagonist were predictably absent from Y_2−/− tissue and also notably from NPY−/− colonic tissues ± VIP (Figure 8c and d). The inhibitory Y_2 tone revealed by BIIE0246 following VIP pretreatment of +/+ and Y_1−/− tissues was therefore absent from both Y_2−/− and NPY−/− mucosae.

Discussion

Characterisation of veratridine-stimulated mucosal responses

Different types of enteric neuron have been classified in guinea pig colon; these include cholinergic secretomotor and non-cholinergic secretomotor neurons, interneurons and sensory

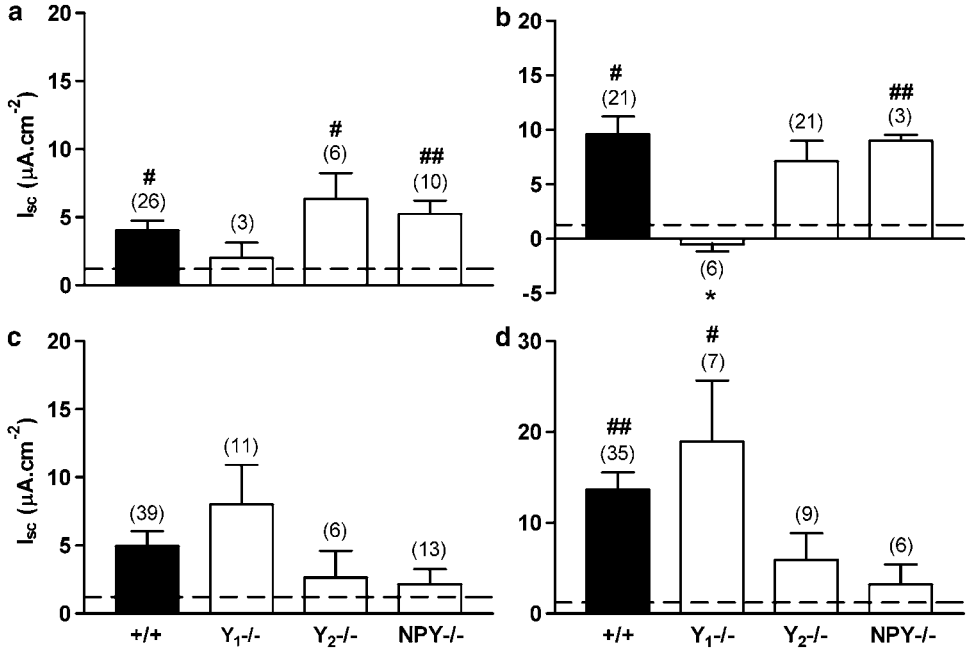

Figure 8 Blockade of endogenous Y_1-inhibitory tone (by BIBO3304, 300 nM) in (a): untreated controls, or (b): following VIP (30 nM) pretreatment of different genotype mouse colon mucosae. Inhibition of Y_2-inhibitory tone (with BIIE0246, 1 μM) in naive (c) or VIP-pretreated (d) wild-type (+/+), Y_1−/−, Y_2−/− or NPY−/− colon mucosae. The dashed line in all histograms represents the vehicle control (DMSO, 0.01%). Data are the mean ± 1 s.e.m. with n numbers shown in parenthesis. A one-way ANOVA using Dunnett's post-test was used to compare +/+ responses with those in −/− tissues, and $^{*}P \leqslant 0.05$ was considered significant. A Student's unpaired t-test was used to compare BIBO3304 and BIIE0246 effects with vehicle controls and these significant differences are denoted by hatches ($^{\#}P \leqslant 0.05$, $^{\#\#}P \leqslant 0.01$).

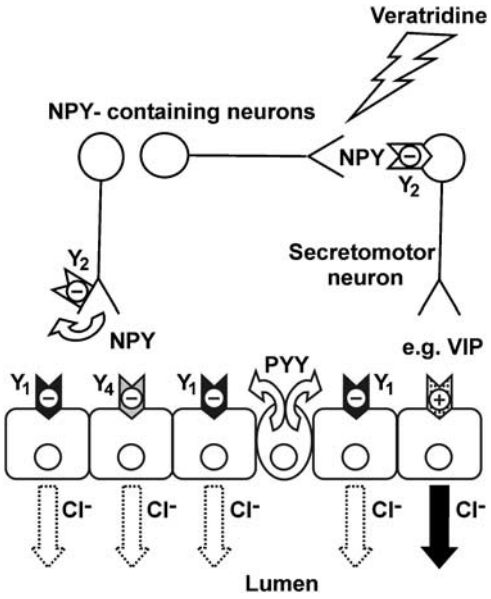

Figure 9 Schematic diagram depicting potential sites of action for neuronal NPY and endocrine PYY upon different targets in wild-type mouse descending colon mucosa. Veratridine nonselectively stimulates all intrinsic submucous neurons. Endocrine PYY may coactivate neuronal Y_2 receptors as well as epithelial Y_1 receptors, the latter predominating and resulting in a sustained inhibition of epithelial Cl^- secretion. NPY released from submucosal secretomotor neurons can feedback to inhibit further NPY release (a Y_2 receptor-mediated effect) and also, when released from interneurons, can inhibit (again *via* Y_2 receptors) other NANC secretomotor neurons. The NANC neurotransmitter in this final secretomotor neuron cannot as yet be positively identified, but is most likely to be VIP. Stimulation of epithelial VIP receptors results in activation of epithelial Cl^- secretion, while epithelial Y_1 (or Y_4) receptor activation will inhibit mucosal anion secretion.

neurons (Furness, 2000; Furness *et al*., 2004). In this study, we have used the *veratrum* alkaloid, veratridine to nonselectively stimulate submucosal neurons. The mixture of neurotransmitters released from stripped mouse jejunum (Sheldon *et al*., 1990) and rabbit colon (Plass *et al*., 1994) results ultimately in a sustained mucosal response, recorded as a prolonged increase in I_{sc}. Following stimulation of intrinsic submucosal neurons in mouse descending colon, the balance of neurotransmitter release also appears to be prosecretory and resulted in a prolonged increase in I_{sc} (Figure 1a and b).

Although approximately 20% of submucosal neurons in the mouse colon are cholinergic (Sang & Young, 1998), veratridine-induced responses were not significantly altered by the combination of muscarinic and nicotinic antagonists, At and Hex (Figure 1c). This was also the case in stripped mouse jejunum (Sheldon *et al*., 1990). These data would suggest that veratridine-stimulated responses in mouse colon and jejunum are predominantly noncholinergic, and implicate a major role for noncholinergic submucosal neurotransmission in contributing to the observed increase in I_{sc}.

NOS and VIP have been colocalised in submucosal cell bodies and fibres in mouse colon (Sang & Young, 1996) however, our preliminary data suggested we would be unable to investigate a role for either neurotransmitter in regulating veratridine-stimulated secretion in this study. In our hands the proposed $VPAC_1$ receptor antagonist, PG 97–269 (Gourlet *et al*., 1997), did not significantly alter VIP-induced secretory responses in mouse colon. This suggests either that the analogue has low affinity for murine $VPAC_1$ receptors, or the involvement of other VIP receptor types, possibly $VPAC_2$ in mediating the effects of the neuropeptide in this tissue. In addition, the VIP fragment VIP(10–28), which displayed noncompetitive antagonism of VIP responses in rat intestine (Cox & Cuthbert, 1989) did not significantly alter VIP responses in mouse colon (data not shown). Thus, the lack of a selective VPAC receptor antagonist precluded further investigation of this most likely of secretomotor transmitters and its contribution to veratridine-induced mucosal responses.

We also encountered problems when attempting to investigate a role for NO in veratridine-stimulated I_{sc} responses in mouse colon. At similar concentrations used by Rao *et al*. (1994) in mouse ileum, we found that the NOS inhibitor, N_ω-nitro-L-arginine did not alter I_{sc} in colon mucosa. At a low concentration (137 μM) and at higher concentrations (1 and 2 mM) the NO substrate, L-Arg did not alter I_{sc}, however at 3 mM a sustained increase in I_{sc} was observed. It is unlikely that the latter was a specific effect however, as this response was mimicked by D-Arg (3 mM, data not shown).

Y_1 receptor-mediated mechanisms

Neither Y_1 receptor blockade nor Y_1 receptor knockout had significant effects upon veratridine-induced changes in I_{sc} in $+/+$ mouse colon (Figure 3a). Nevertheless the small increase in veratridine-induced secretion in the presence of BIBO3304 may indicate a minor prejunctional neuronal role for Y_1 receptors in wild-type colon. Y_1 receptors were colocalised with NOS in human submucosal colonic neurons (Peaire *et al*., 1997), and in rat small intestinal submucosal neurons (Jackerott & Larsson, 1997). In the hypothalamus NPY actions mediated *via* Y_1 (and Y_2) receptors can directly stimulate NOS-containing neurons (Fetissov *et al*., 2003). Our study has not demonstrated whether NO results in a secretory or antisecretory response in mouse colon. However, if NO resulted in the former, then blockade of prejunctional Y_1 receptors could potentially cause release of NO and subsequently enhance secretory responses following veratridine stimulation. This mechanism may also explain the significant attenuation of veratridine responses observed in $Y_2-/-$ colon (Figure 5a) following BIBO3304 pretreatment.

Y_1 receptors have also been colocalised with VIP in nerve cell bodies throughout the rat intestine in submucosal neurons (Jackerott & Larsson, 1997). If a similar pattern of colocalisation is present in mouse colon submucous innervation, then blockade of Y_1 receptors with BIBO3304 could potentially increase VIP release, thereby enhancing veratridine-stimulated secretion. This mechanism might offer an explanation for the slight enhancement observed with BIBO3304 upon veratridine-stimulated ion secretion in $+/+$ mouse colon (Figure 3a). However, we know that Y_1 receptors are located predominantly on epithelia in mouse colon and we suggest that their activation by endogenous PYY provides the majority of the inhibitory Y_1 tone we observe in $+/+$ and NPY$-/-$ (Figure 7a and c) mouse colon mucosa.

Y_2 receptor-mediated mechanisms

In contrast to Y_1 receptor blockade, Y_2 receptor antagonism significantly increased veratridine-induced changes in I_{sc},

although the increase observed in $Y_2-/-$ colon was not significant (Figure 4a and b). This suggests that in $+/+$ colon, Y_2 receptors have a significant role in the modulation of enteric submucosal neurotransmission. Use of BIIE0246 has confirmed a prejunctional role for Y_2 receptor modulation of neurotransmitter release in a wide range of central and peripheral tissues such as the kidney and spleen (Malmström *et al*., 2002a, b), hippocampus (Weiser *et al*., 2000), hypothalamus (King *et al*., 2000), rat heart, and guinea pig trachea and vas deferens (Smith-White *et al*., 2001). The Y_2 receptor has an autoinhibitory role in the regulation of NPY release. Addition of BIIE0246 to rat hypothalamic slices after neuronal stimulation (King *et al*., 2000), and following electrical field stimulation of spleen and kidney sympathetic nerves (Malmström *et al*., 2002a) results in a significant increase in NPY release. Previous studies have demonstrated a role for Y_2 receptor-mediated blockade of inhibitory postsynaptic potentials in guinea pig submucosal neurons (Cunningham *et al*., 1994). Therefore, increased NPY release in the presence of BIIE0246 could subsequently inhibit the release of an antisecretory neurotransmitter (e.g. NPY) in mouse colon submucosal neurons, observed as an increase in veratridine-induced secretion in this study (Figure 4a). Consistent with a prejunctional location for Y_2 receptors, previous studies have shown that BIIE0246 effects *per se* were TTX sensitive (Cox *et al*., 2003) and here we found Y_2-inhibitory tone was absent from $NPY-/-$ colon (Figure 8c and d), suggesting that it is NPY dependent in mouse descending colon.

Y_2 receptor knockout increased veratridine-induced secretion but not significantly so and the unusual sensitivity to cholinergic blockade (Figure 4d), accompanied by changes in sensitivity to CCh (data not shown), might suggest a reorganisation of cholinergic neural circuits in the colonic submucous plexi of this null mouse. Further characterisation of veratridine effects in the presence of At and Hex is required in $Y_2-/-$ tissue, in order to determine which neurotransmitters are contributing to the enhanced cholinergic component observed in the colon from these knockout mice. Another unexpected finding with $Y_2-/-$ colon mucosa was that the Y_1 antagonist BIBO3304, significantly attenuated veratridine responses (between 1 and 3 min; Figure 5a) in contrast to a lack of effect in $+/+$ colon. Thus, absence of Y_2 receptors may either have uncovered a functional role for prejunctional Y_1 receptors or a compensatory ectopic expression of neuronal Y_1 receptors in this null mouse. The latter is unlikely because we have demonstrated that there is no change in the TTX insensitivity of Y_1-mediated, $Pro^{34}PYY$ (30 nM) responses in $Y_2-/-$ tissues (Cox *et al.,* unpublished). The mechanism by which Y_1 receptor blockade decreases veratridine-induced responses in $Y_2-/-$ colon could involve nitr-ergic or VIP-ergic pathways, as both NOS and VIP are colocalised with different populations of Y_1-positive submucous neuron cell bodies in rat intestine and human colon (Jackerott & Larsson 1997; Peaire *et al*., 1997). Blockade of Y_1 receptors that would usually attenuate antisecretory, rather than secretagogue neurotransmission (e.g. VIP) would result in the blunting of neurogenic responses, as we observed in $Y_2-/-$ colon mucosa (Figure 5a).

Absence of NPY reveals PYY-mediated inhibitory mechanisms in mouse colon

$NPY-/-$ mice provided a model that allowed conclusions to be drawn about the mechanism by which endocrine PYY and its product PYY(3–36) modulate submucosal neurotransmission. The decrease in veratridine-stimulated secretion that we observed was unexpected, as NPY added to rat (Cox & Cuthbert, 1988) and mouse (Holliday *et al*., 2000; Cox *et al*., 2001) colon mucosae is antisecretory and causes prolonged, concentration-dependent decreases in I_{sc}. In the guinea pig ENS however, Y_2 receptors have been implicated in the attenuation of inhibitory postsynaptic potentials in caecal submucosal neurons (Cunningham *et al*., 1994). This could offer a potential explanation for the significant decrease in veratridine-induced secretion in $NPY-/-$ mouse colon. NPY, depending upon the neurochemical coding of the target neuron, could potentially inhibit secretory, or disinhibit antisecretory neurotransmitter release in the vicinity of the epithelial lining, thereby decreasing veratridine-induced neurogenic increases in I_{sc} (Figure 9). In addition to which, knockout of the NPY gene identified a potential prejunctional neuromodulatory role for PYY and its product PYY(3–36) in mouse colon, as the Y_2 antagonist, BIIE0246 alone and in combination with the Y_1 antagonist, increased veratridine responses (Figure 7a–c) in the absence of NPY.

Therefore, in mouse descending colon the antisecretory effect of NPY and PYY is modulated by an indirect mechanism involving Y_2 receptors located on noncholinergic neurons (Figure 9). Further studies characterising the neurochemical coding of these noncholinergic submucosal Y_2 neurons, that may also express NPY, VIP and/or NOS (Sang & Young, 1996), would greatly enhance our understanding of the nature of neurotransmitter release regulated by this Y receptor type.

Y_1 and Y_2 receptor-mediated antisecretory tone in the colon

The use of competitive antagonists, selective for either the Y_1 or the Y_2 receptor has allowed us to establish different ongoing antisecretory tone, the latter being sensitive to TTX and therefore submucous neuron mediated (Cox *et al*., 2001; Hyland *et al*., 2003). Using different null mice we observed a predictable loss of Y_1 tone from $Y_1-/-$ colon (Figure 8a and b) and Y_2 tone from $Y_2-/-$ colon mucosa (Figure 8c and d). In $NPY-/-$ colon, where the endogenous agonist for both receptor types is PYY released from endocrine cells, Y_2 tone is not apparent (Figure 8d), while Y_1 tone is present and to the same degree as observed in $+/+$ colon (Figure 8a and b). We conclude therefore that endogenous PYY predominantly provides the epithelial Y_1 receptor-mediated antisecretory tone, while neuronal NPY activates Y_2 receptors on, as yet unidentified submucosal nerves to provide Y_2 receptor antisecretory tone in mouse colon.

This study has demonstrated the complexity of NPY and Y receptor regulation of epithelial Cl^- secretion in mouse colon. The functional end point, a change in I_{sc} is a result of a coordinated effect of neuronal and epithelial Y receptor stimulation. We have demonstrated differential roles for neuronal Y_2 receptors and epithelial Y_1 receptors. Therefore NPY (or PYY and its product, PYY(3–36)) activating prejunctional Y_2 receptors on NANC neurons, depending on the nature of the neuron (secretory or antisecretory), will indirectly attenuate epithelial transport mechanisms, measured as reductions in I_{sc}. Our data would suggest that these intrinsic neurons are secretory in nature. From a

therapeutic perspective *in vivo* human studies have demonstrated a profound antisecretory role for PYY in the small intestine of ileostomy patients (Playford *et al*., 1990), and i.v. infusion of NPY-inhibited PGE_2-stimulated fluid secretion in healthy human volunteers (Holzer-Petsche *et al*., 1991). Our data describes several pathways that may regulate the antisecretory effect of NPY and PYY in mouse colon. This is particularly relevant as studies from our laboratory using mouse (Holliday *et al*., 2000; Cox *et al*., 2001; Hyland *et al*., 2003) and human (Cox & Tough, 2002; Hyland *et al*., 2003) isolated colon have demonstrated significant similarities in Y receptor function. Specifically, Y_1 receptors are predominantly epithelial, while Y_2 receptor mechanisms are neuronal (Cox & Tough, 2002; Hyland *et al*., 2003) and significant Y_1 and Y_2 receptor-mediated antisecretory tone exists in both the mouse and the human-isolated colon.

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References

ALLESCHER, H.D., KURJAK, M., HUBER, A., TRUDRUNG, P. & SCHUSDZIARRA, V. (1996). Regulation of VIP release from rat enteric nerve terminals: evidence for a stimulatory effect of NO. *Am. J. Physiol.,* **271,** G568–G574.

BATTERHAM, R.L., COWLEY, M.A., SMALL, C.J., HERZOG, H., COHEN, M.A., DAKIN, C.L., WREN, A.M., BRYNES, A.E., LOW, M.J., GHATEI, M.A., CONE, R.D. & BLOOM, S.R. (2002). Gut hormone PYY(3–36) physiologically inhibits food intake. *Nature,* **418,** 650–654.

BELAI, A. & BURNSTOCK, G. (1988). Release of calcitonin gene-related peptide from rat enteric nerves is Ca^{2+}-dependent but is not induced by K^+ depolarization. *Regul. Pept.,* **23,** 227–235.

CATTERALL, W.A. (1980). Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. *Annu. Rev. Pharmacol. Toxicol.,* **20,** 15–43.

COX, H.M. & CUTHBERT, A.W. (1988). Neuropeptide Y antagonises secretagogue evoked chloride transport in rat jejunal epithelium. *Pflugers Arch.,* **413,** 38–42.

COX, H.M. & CUTHBERT, A.W. (1989). Secretory actions of vasoactive intestinal polypeptide, peptide histidine isoleucine and helodermin in rat small intestine: the effects of putative VIP antagonists upon VIP-induced ion secretion. *Regul. Pept.,* **26,** 127–135.

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

COX, H.M. & TOUGH, I.R. (2002). Neuropeptide Y, Y_1, Y_2 and Y_4 receptors mediate Y agonist responses in isolated human colon mucosa. *Br. J. Pharmacol.,* **135,** 1505–1512.

COX, H.M., TOUGH, I.R. & HYLAND, N.P. (2003). Neuropeptide Y Y_1 and Y_2 receptors mediate inhibitory tone in murine and human colon mucosae. *Neurogastroenterol. Motil.,* **15,** 195–237.

CUNNINGHAM, S.M., MIHARA, S. & LEES, G.M. (1994). Y_2-receptor-mediated selective inhibition of slow, inhibitory postsynaptic potential in submucous neurons of guinea-pig caecum. *Br. J. Pharmacol.,* **113,** 883–888.

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. & SUNDLER, F. (2002). Distribution of pancreatic polypeptide and peptide YY. *Peptides,* **23,** 251–261.

FELETOU, M., RODRIGUEZ, M., BEAUVERGER, P., GERMAIN, M., IMBERT, J., DROMAINT, S., MACIA, C., BOURRIENNE, A., HENLIN, J.M., NICOLAS, J.P., BOUTIN, J.A., GALIZZI, J.P., FAUCHERE, J.L., CANET, E. & DUHAULT, J. (1998). NPY receptor subtypes involved in the contraction of the proximal colon of the rat. *Regul. Pept.,* **75–76,** 221–229.

FERRIER, L., SEGAIN, J., BONNET, C., CHERBUT, C., LEHUR, P., JARRY, A., GALMICHE, J. & BLOTTIERE, H. (2002). Functional mapping of NPY/PYY receptors in rat and human gastrointestinal tract. *Peptides,* **23,** 1765.

FETISSOV, S.O., XU, Z.Q., BYRNE, L.C., HASSANI, H., ERNFORS, P. & HÖKFELT, T. (2003). Neuropeptide y targets in the hypothalamus: nitric oxide synthesizing neurons express Y_1 receptor. *J. Neuroendocrinol.,* **15,** 754–760.

FURNESS, J.B. (2000). Types of neurons in the enteric nervous system. *J.Auton. Nerv. Syst.,* **81,** 87–96.

FURNESS, J.B., JONES, C., NURGALI, K. & CLERC, N. (2004). Intrinsic primary afferent neurons and nerve circuits within the intestine. *Prog. Neurobiol.,* **72,** 143–164.

GOURLET, P., DE NEEF, P., CNUDDE, J., WAELBROECK, M. & ROBBERECHT, P. (1997). *In vitro* properties of a high affinity selective antagonist of the VIP1 receptor. *Peptides,* **18,** 1555–1560.

GRANDT, D., SCHIMICZEK, M., BEGLINGER, C., LAYER, P., GOEBELL, H., EYSSELEIN, V.E. & REEVE, J.R. (1994). Two molecular forms of peptide YY (PYY) are abundant in human blood: characterization of a radioimmunoassay recognizing PYY 1–36 and PYY 3–36. *Regul. Pept.,* **51,** 151–159.

GREGOR, P., FENG, Y., DECARR, L.B., CORNFIELD, L.J. & MCCALEB, M.L. (1996). Molecular characterization of a second mouse pancreatic polypeptide receptor and its inactivated human homologue. *J. Biol. Chem.,* **271,** 27776–27781.

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

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

HYLAND, N.P., SJÖBERG, F., TOUGH, I.R., HERZOG, H. & COX, H.M. (2003). Functional consequences of neuropeptide Y Y_2 receptor knockout and Y_2 antagonism in mouse and human colonic tissues. *Br. J. Pharmacol.,* **139,** 863–871.

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

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.

MALMSTRÖM, R.E., LUNDBERG, J.N. & WEITZBERG, E. (2002a). Effects of the neuropeptide Y Y_2 receptor antagonist BIIE0246 on sympathetic transmitter release in the pig *in vivo*. *Naunyn Schmiedebergs Arch. Pharmacol.,* **365,** 106–111.

MALMSTRÖM, R.E., LUNDBERG, J.O. & WEITZBERG, E. (2002b). Auto-inhibitory function of the sympathetic prejunctional neuropeptide Y Y_2 receptor evidenced by BIIE0246. *Eur. J. Pharmacol.,* **439,** 113–119.

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

MEDEIROS, M.D. & TURNER, A.J. (1994). Processing and metabolism of peptide-YY: pivotal roles of dipeptidylpeptidase-IV, aminopeptidase-P, and endopeptidase-24.11. *Endocrinology,* **134,** 2088–2094.

MICHEL, M.C., BECK-SICKINGER, A., COX, H., DOODS, H.N., HERZOG, H., LARHAMMAR, D., QUIRION, R., SCHWARTZ, T. & WESTFALL, T. (1998). XVI. International Union of Pharmacology 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 subtype Y_1 within human colon: evidence for NPY targeting a subpopulation of nitrergic neurons. *J. Auton. Nerv. Syst.,* **67,** 168–175.

PLASS, H., WACHTER, C. & TURNHEIM, K. (1994). Neurogenic chloride secretion induced by scorpion venom and veratrine in rabbit colon. *Naunyn. Schmiedebergs. Arch. Pharmacol.,* **350,** 403–409.

PLAYFORD, R.J., DOMIN, J., BEACHAM, J., PARMAR, K.B., TATEMOTO, K., BLOOM, S.R. & CALAM, J. (1990). Preliminary report: role of peptide YY in defence against diarrhoea. *Lancet,* **335,** 1555–1557.

RAO, R.K., RIVIERE, P.J., PASCAUD, X., JUNIEN, J.L. & PORRECA, F. (1994). Tonic regulation of mouse ileal ion transport by nitric oxide. *J. Pharmacol. Exp. Ther.,* **269,** 626–631.

SANG, Q. & YOUNG, H.M. (1996). Chemical coding of neurons in the myenteric plexus and external muscle of the small and large intestine of the mouse. *Cell Tissue Res.,* **284,** 39–53.

SANG, Q. & YOUNG, H.M. (1998). The identification and chemical coding of cholinergic neurons in the small and large intestine of the mouse. *Anat. Rec.,* **251,** 185–199.

SERVIN, A.L., ROUYER-FESSARD, C., BALASUBRAMANIAM, A., SAINT, P.S. & LABURTHE, M. (1989). Peptide-YY and neuropeptide-Y inhibit vasoactive intestinal peptide- stimulated adenosine 3′,5′-monophosphate production in rat small intestine: structural requirements of peptides for interacting with peptide-YY-preferring receptors. *Endocrinology,* **124,** 692–700.

SHELDON, R.J., MALARCHIK, M.E., BURKS, T.F. & PORRECA, F. (1990). Effects of nerve stimulation on ion transport in mouse jejunum: responses to Veratrum alkaloids. *J. Pharmacol. Exp. Ther.,* **252,** 636–642.

SMITH-WHITE, M.A., HARDY, T.A., BROCK, J.A. & POTTER, E.K. (2001). Effects of a selective neuropeptide Y Y_2 receptor antagonist, BIIE0246, on Y_2 receptors at peripheral neuroeffector junctions. *Br. J. Pharmacol.,* **132,** 861–868.

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

WEISER, T., WIELAND, H.A. & DOODS, H.N. (2000). Effects of the neuropeptide Y Y_2 receptor antagonist BIIE0246 on presynaptic inhibition by neuropeptide Y in rat hippocampal slices. *Eur. J. Pharmacol.,* **404,** 133–136.

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 BIBO 3304 and its effect on feeding in rodents. *Br. J. Pharmacol.,* **125,** 549–555.

YAU, W.M., DORSETT, J.A. & YOUTHER, M.L. (1986). Calcium-dependent stimulation of acetylcholine release by substance P and vasoactive intestinal polypeptide. *Eur. J. Pharmacol.,* **120,** 241–243.

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