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Selective inhibition of neuropeptide Y Y_1 receptors by BIBP3226 in rat and human epithelial preparations

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Abstract

BIBP3226 (N^2-(diphenylacetyl)-*N*-[(4-hydroxyphenyl)methyl]-D-arginine amide) has been used to examine the presence of neuropeptide Y Y_1 receptors in 3 gastrointestinal epithelial preparations, namely the rat jejunum and descending colon mucosae and a human colonic adenocarcinoma cell line. The selective Y_1 receptor antagonist (1 μM BIBP3226) had no significant effect upon either peptide YY (PYY) responses or on electric field stimulated changes in electrogenic ion transport in rat jejunum mucosa. Partial inhibition of PYY responses was observed following BIBP3226 pretreatment of rat colon mucosal preparations in the presence and absence of tetrodotoxin. Responses to the Y_1 selective agonist [Leu^{31},Pro^{34}]neuropeptide Y ([Leu^{31},Pro^{34}]NPY) in descending colon preparations were significantly attenuated by BIBP3226 (1 μM). The same concentration of antagonist abolished responses to PYY and [Leu^{31},Pro^{34}]NPY but had no effect upon human pancreatic polypeptide (hPP) in monolayer cultures of the human adenocarcinoma cell line, Colony-6. Schild analysis of BIBP3226 antagonism of PYY responses in Colony-6 cells provided a pA_2 value of 7.9 with a Hill slope of 1.03, indicating competitive antagonism at these epithelial Y_1 receptors.

Keywords: Neuropeptide Y; BIBP3226; Y_1 receptor antagonist; Gastrointestinal epithelium

1. Introduction

Neuropeptide Y (NPY), peptide YY (PYY) and other members of the pancreatic polypeptide (PP) family are now believed to activate at least 5 different receptor subtypes, 3 of which (Y_1, Y_2 and Y_4) have been cloned. Together NPY, PYY and PP exert multiple effects upon gastrointestinal processes (Cox, 1993). NPY, which is extensively expressed in myenteric and submucous neurones throughout the intestinal tract and PYY (located in endocrine cells, particularly in the lower bowel) have similar actions upon many intestinal preparations. Both peptides will inhibit gastrointestinal motility (Holzer et al., 1987; Krantis et al., 1988) and attenuate epithelial ion secretion (Hubel and Renquist, 1986; Cox et al., 1988) as well as stimulate feeding (Leibowitz, 1989) and modulate emesis (Harding and McDonald, 1989). In the rat jejunum previous functional and binding studies with a series of agonists (Servin et al., 1989; Cox and Cuthbert, 1990; Cox and Krstenansky, 1991) have shown that jejunal epithelial cells express NPY receptors of the Y_2-type. Human colon has been found to express high levels of Y_1 receptor mRNA (Wharton et al., 1993) and Mannon et al. (1994) have been able to activate the expression of Y_1-like receptors following butyrate treatment of a cell line derived from a human colonic tumour, HT-29. We have recently identified a Y receptor in a human colonic adenocarcinoma cell line with an apparently unusual agonist potency order, although it most resembles the Y_1 receptor in character (Cox and Tough, 1995).

The recent discovery of selective antagonists for the Y_1 receptor (Rudolf et al., 1994; Leban et al., 1995; Serradeil-Le Gal et al., 1995) now provides us with the opportunity to definitively assess this receptor type's involvement in responses to PYY and NPY. Here we have tested the nonpeptide antagonist BIBP3226 (Rudolf et al., 1994) in 3 epithelial preparations, namely the rat jejunum mucosa (a Y_2-like system), the rat descending colon (a mixed receptor

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population, Cox unpublished observations) and the human adenocarcinoma cell line, Colony-6 (Y_1-like but with some unusual features; Cox and Tough, 1995).

2. Materials and methods

2.1. Materials

BIBP3226 (N^2-(diphenylacetyl)-*N*-[(4-hydroxyphenyl)methyl]-D-arginine amide) was a kind gift from Dr. H.N. Doods and Dr. K. Rudolf (Dr. Karl Thomae GmbH, Biberach, Germany). PYY and all related peptides were purchased from Peninsula Laboratories Inc. (Merseyside, UK) and aliquots were frozen and stored at −20°C. Piretanide was a gift from Hoechst Pharmaceuticals (Milton Keynes, UK) and trypsin was purchased from Worthington Biochemicals Corp. (Freehold, New Jersey, USA). Tetrodotoxin was obtained from Sigma (Poole, UK). The constituents of Krebs-Henseleit (KH) were (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 11.1. Dulbecco's modified Eagle medium (DMEM) was purchased from Gibco (Paisley, UK) and the supplements, foetal calf serum, kanamycin and amphotericin were from ICN Biomedicals Ltd (Thame, UK).

2.2. In vitro preparations and cell culture

Male Sprague-Dawley rats (200–300 g) were killed and a 6–8 cm length of jejunum and/or the whole of the descending colon were removed and placed in oxygenated KH solution. The serosa and muscularis propria of both gastrointestinal areas were removed from the underlying mucosae by blunt dissection under a microscope (as described previously; Cox et al., 1988). The remaining jejunum and colonic mucosae had intact submucosal innervation and the descending colon preparations were assumed also to have an intact mucosal plexus innervation, as described originally by Andres et al. (1985).

Human colonic adenocarcinoma Colony-6 cells (Col-6) were grown in DMEM supplemented with foetal calf serum (10%), amphotericin (1.2 μg/ml) and kanamycin (100 μg/ml). Once these epithelial cells had reached confluence in culture flasks (area 25 cm^2) they were trypsinized (0.5% trypsin in versene, w/v) and seeded onto collagen-coated Millipore filters as described previously (Cox and Tough, 1994).

2.3. Short-circuit current measurement

Mucosal preparations (exposed area, 0.6 cm^2) or confluent layers of Col-6 epithelia (0.2 cm^2) on filters were placed between two halves of Perspex Ussing chambers and bathed in oxygenated (95% O_2/5% CO_2) 12 or 15 ml KH solution each side at 37°C, pH 7.4. All epithelial preparations were voltage-clamped at zero potential (WP Instruments, Sarasota, FL, USA) and allowed to stabilise for at least 30 min. The short-circuit current was monitored continuously on pen recorders during both stabilisation and experimentation periods. All peptides, tetrodotoxin and BIBP3226 were added to the basolateral surface and resultant peak changes in SCC were converted to μA/cm^2.

Electric field stimulation (EFS) studies were performed with rat jejunum preparations where a pair of silver sheet electrodes was placed one on either side of the tissue in between the Ussing chamber halves. Tissues were bathed in KH as described above and once a stable basal short-circuit current had been achieved, the electrodes were attached to a Pulsemaster A300 stimulator (WP Instruments) and trains of 5 stimuli (0.6 ms duration and 5 Hz frequency) were delivered at 7 min intervals. Four trains were given before and after BIBP3226 application (1 μM) and 4 further stimuli were given following basolateral atropine (1 μM).

2.4. Analyses

Concentration–response profiles were obtained cumulatively, increasing peptide concentrations being added without intermediate washing. Each peptide response curve was analysed using the iterative curve-fitting programme, Graphpad Prism (version 2.0, Graphpad Software Inc, San Diego, USA). EC_{50} values were calculated for each preparation and values were then pooled (mean ± 1 S.E.M.) within particular groups. Peak heights of short-circuit current responses following EFS were pooled from each train of 4 stimuli and the means ± 1 S.E.M. are quoted as μA/cm^2. Statistical analysis of absolute changes in short-circuit current (μA/cm^2) or EC_{50} values was performed using Student's unpaired *t*-test and a *P*-value < 0.05 was considered statistically significant. One-way ANOVA was used to assess significance levels for PYY and [Leu^{31},Pro^{34}]NPY response curves following various pretreatments of colonic preparations.

3. Results

BIBP3226 (1 μM) had no significant effect upon basal short-circuit current in preparations of rat jejunum mucosa (38.7 ± 12.5 μA/cm^2, $n = 6$, before BIBP3226 and 39.8 ± 13.0 μA/cm^2, $n = 6$, after drug) nor did the antagonist significantly alter the responses either to basolateral PYY (100 nM: −18.2 ± 3.5 μA/cm^2, $n = 4$, compared with controls of −21.4 ± 3.8 μA/cm^2, $n = 5$) or to EFS where control responses were 3.1 ± 0.8 μA/cm^2 ($n = 3$, and these responses were not significantly different from controls recorded in earlier studies using the same stimulation parameters, Cox and Cuthbert, 1990). In the presence of BIBP3226 (1 μM) EFS responses were 3.0 ± 0.6 μA/cm^2 ($n = 3$) and following atropine (1 μM) 2.4 ± 0.1 μA/cm^2

($n = 3$). Subsequent application of PYY (100 nM) to these preparations abolished the residual responses to EFS as seen in earlier studies (Cox and Cuthbert, 1990). PYY and NPY responses in this preparation are not sensitive to tetrodotoxin (100 nM; Cox et al., 1988).

Rat descending colon mucosal preparations were sensitive to tetrodotoxin (100 nM). The neurotoxin produced a sustained reduction in basal short-circuit current from 87.9 $\pm$ 4.7 $\mu A/cm^2$ ($n = 12$) to 39.5 $\pm$ 2.9 $\mu A/cm^2$ ($n = 12$). The effects of tetrodotoxin upon concentration–response curves to PYY, NPY and two selective agonists, [Leu^{31},Pro^{34}]NPY (Y_1-receptor-selective) and PYY(3–36) (Y_2-receptor-preferring) are shown in Fig. 1. In the absence of tetrodotoxin the EC_{50} values (in nM) were: 10.3 $\pm$ 4.0 ($n = 9$), 24.6 $\pm$ 4.5 ($n = 8$), 39.8 $\pm$ 12.5 ($n = 6$) and 2.8 $\pm$ 1.6 ($n = 9$) for PYY, NPY, [Leu^{31},Pro^{34}]NPY and PYY(3–36) respectively. In untreated preparations the Y_2-preferring, C-terminal fragments, NPY(2–36) reduced short-circuit current with an EC_{50} of 4.0 $\pm$ 1.6 nM ($n = 4$) and NPY(13–36) was also inhibitory with an EC_{50} of 23.0 $\pm$ 7.4 nM ($n = 3$). Human PP (hPP) exhibited a threshold concentration of 100 nM and reduced basal short-circuit current by -10.7 ± 0.7 $\mu A/cm^2$ ($n = 3$) at 1 μM. Tetrodotoxin did not significantly alter the EC_{50} values of these agonists, but the maxima of peptide response curves were significantly reduced (as shown in Fig. 1) compared with respective controls: e.g., for [Leu^{31},Pro^{34}]NPY (3 nM – 1 μM) and for PYY(3–36) (at 30 and 100 nM). EC_{50} values in the presence of tetrodotoxin were 7.3 $\pm$ 1.9 ($n = 4$), 31.1 $\pm$ 12.3 ($n = 4$), 72.3 $\pm$ 25.5 ($n = 3$) and 4.2 $\pm$ 2.5 ($n = 5$) for PYY, NPY, [Leu^{31},Pro^{34}]NPY and PYY(3–36), respectively.

BIBP3226 (1 μM) was tested against PYY and [Leu^{31},Pro^{34}]NPY responses in colonic preparations, in the absence and presence of tetrodotoxin (Fig. 2A,B). EC_{50} calculations for PYY were not significantly altered, being 8.8 $\pm$ 2.2 nM ($n = 9$) and 6.6 $\pm$ 2.2 nM ($n = 10$; for controls – tetrodotoxin and + tetrodotoxin, respectively); 6.9 $\pm$ 1.8 nM (+ BIBP3226 alone, 1 μM, $n = 10$) and 12.7 $\pm$ 6.0 nM ($n = 9$) in the presence of both tetrodotoxin and BIBP3226. Again there was a significant reduction in the size of PYY responses in the presence of tetrodotoxin (compared with controls) and BIBP3226 (1 μM) further reduced responses in the presence of the neurotoxin ($P < 0.05$ at PYY concentrations of 30 and 100 nM). [Leu^{31},Pro^{34}]NPY concentration–response curves, as expected, exhibited significant sensitivity to BIBP3226 and the combination of tetrodotoxin and BIBP3226 virtually abolished responses to the Y_1-selective analogue (Fig. 2B). The control EC_{50} was 49.8 $\pm$ 14.1 nM ($n = 4$) and after tetrodotoxin, 16.7 $\pm$ 4.5 nM ($n = 4$, $P = 0.053$ compared with [Leu^{31},Pro^{34}]NPY + tetrodotoxin; data in Fig. 1)

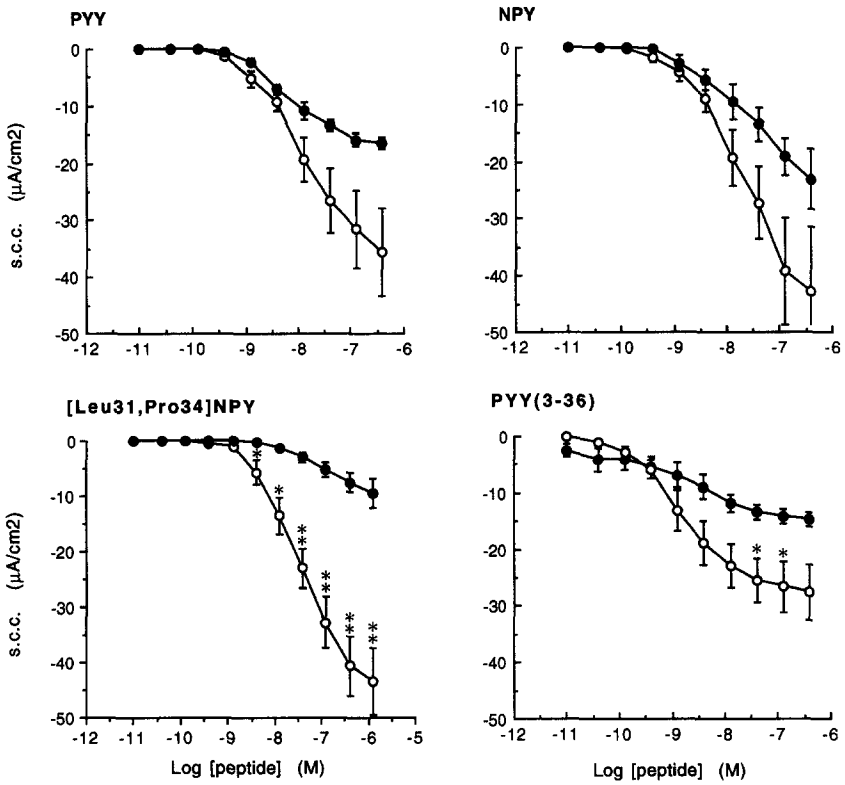

Fig. 1. Concentration–response curves for PYY, NPY, [Leu^{31},Pro^{34}]NPY and PYY(3–36) in mucosal-submucosal preparations of rat descending colon in the presence (●) or absence (○) of tetrodotoxin (100 nM). Each point is the mean $\pm$ 1 S.E.M. from 4–9 observations. * $P < 0.05$, ** $P < 0.01$.

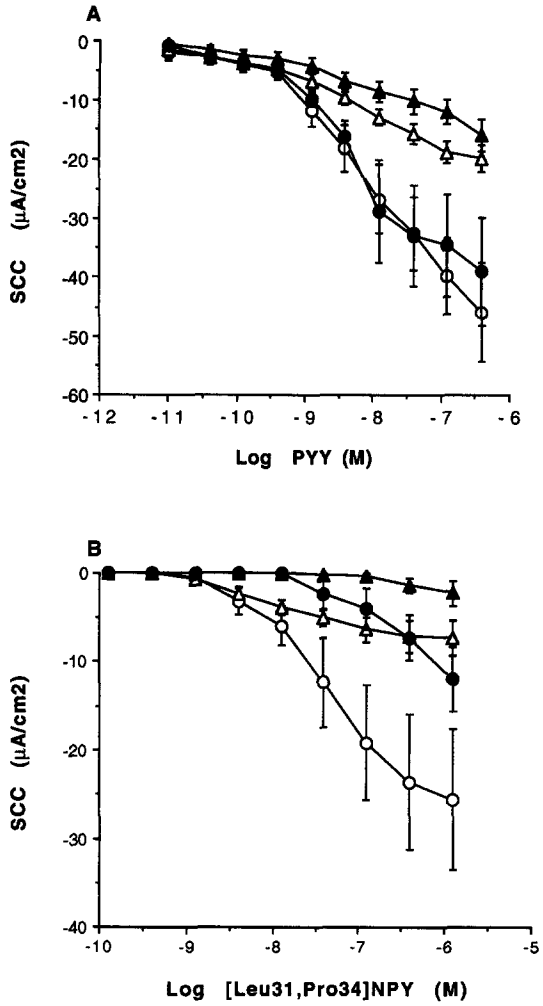

Fig. 2. The effects of increasing concentrations of (A) PYY and (B) [Leu^{31},Pro^{34}]NPY either alone (O) or in the presence of either TTX (100 nM, Δ) or BIBP3226 (1 μM, ●) or a combination of TTX and BIBP3226 (▲) in rat descending colon. Each point is the mean ± 1 S.E.M. from 4–10 observations throughout. One-way analysis of variance found no significant difference ($P = 0.07$) between PYY response curves, but [Leu^{31},Pro^{34}]NPY responses were significantly different ($P = 0.01$).

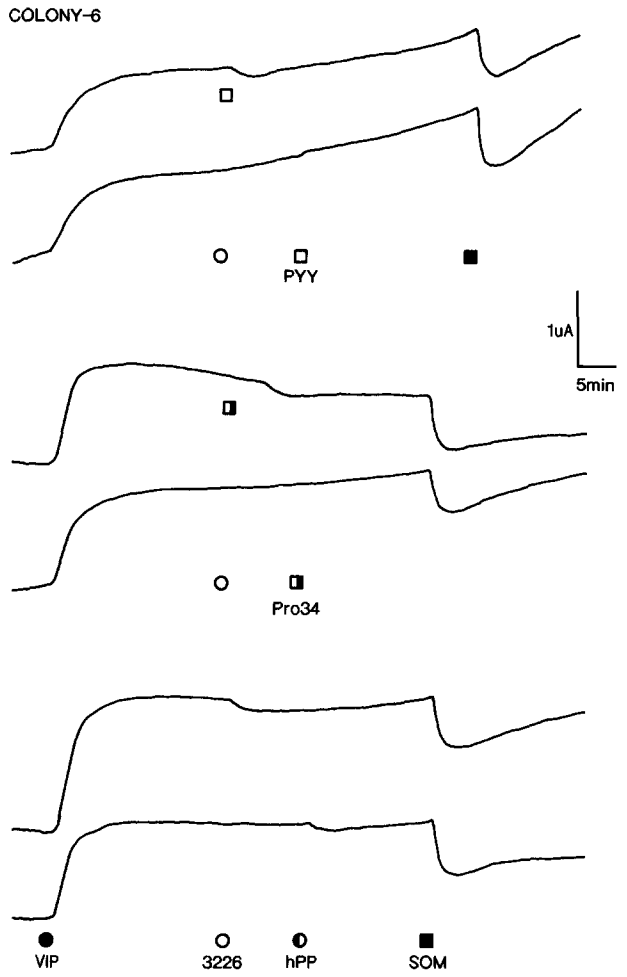

Fig. 3. Representative traces of SCC recordings from Col-6 epithelial layers. All preparations were pretreated with 10 nM VIP (●) for at least 20 min before addition of either PYY (10 nM, □) [Leu^{31},Pro^{34}]NPY (100 nM, ◨) or hPP (300 nM, ◑) and finally to somatostatin 14–28 (100 nM, ■). BIBP3226 1 μM (○) was added between the VIP and NPY analogue additions in only the lower of each pair of traces.

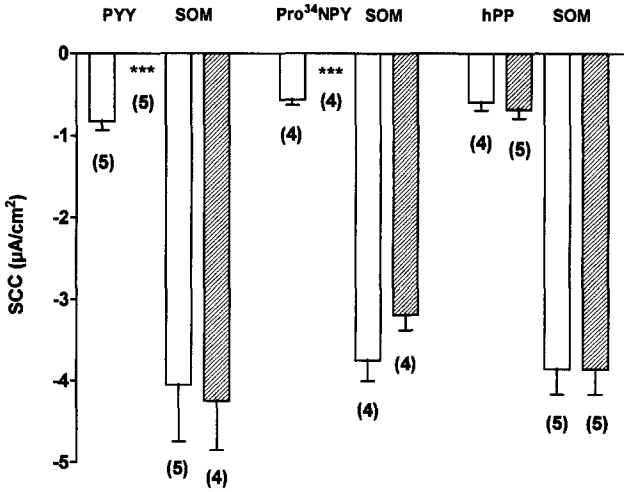

Fig. 4. Histogram of the peak responses to PYY, [Leu^{31},Pro^{34}]NPY, hPP and somatostatin 14–28 (SOM) from control (open columns) and BIBP3226 (1 μM, hatched columns) pretreated Col-6 epithelial layers. The number of observations in each group is shown in parentheses. *** $P < 0.001$.

while the presence of BIBP3226 (1 μM) shifted the control [Leu^{31},Pro^{34}]NPY curve to the right with a projected $EC_{50} > 1$ μM.

Antisecretory responses to PYY in layers of human Col-6 epithelia were also sensitive to the Y_1 antagonist. Following addition of VIP (10 nM as described previously; Cox and Tough, 1995) which elevated short-circuit current (Fig. 3) subsequent addition of BIBP3226 (1 μM) had no effect upon this new current, but it abolished responses to PYY (10 nM) and [Leu^{31},Pro^{34}]NPY (100 nM). There was however, no significant effect upon the size or shape of hPP (300 nM) responses after VIP and BIBP3226 (Figs. 3 and 4). Somatostatin 14–28 (SOM) induced prolonged reductions in short-circuit current (as expected from previous studies; Ferrar et al., 1990; Cox and Tough, 1995; and Fig. 3) and these were unchanged by pretreatment with BIBP3226 (Fig. 4). Increasing concentrations of the antag-

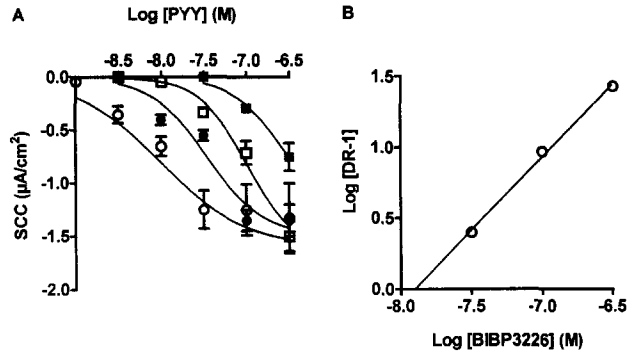

Fig. 5. Competitive inhibition of PYY responses by BIBP3226 in Col-6 epithelial layers. *Panel A:* Rightward shift of PYY response curves, in controls (○) and following addition of 30 nM (●) 100 nM (□) and 300 nM (■) BIBP3226. Each point is the mean ± 1 S.E.M. from 3 observations. *Panel B:* Schild plot of data shown in A, giving a pA_2 value of 7.9 and slope of 1.03.

onist (i.e., 30, 100 and 300 nM) resulted in parallel rightward shifts of the PYY response curve (Fig. 5A) and Schild analysis gave a pA_2 value of 7.9 with a slope of 1.03 (Fig. 5B).

4. Discussion

We have shown that BIBP3226 selectively inhibits PYY and [Leu^{31},Pro^{34}]NPY responses in Col-6 epithelia and partially blocks responses to PYY and particularly [Leu^{31},Pro^{34}]NPY in the rat colon, but it does not (at the same concentration of 1 μM) have any effect upon responses to either PYY or EFS secretory responses in the rat jejunum. This confirms the original receptor characterisation as non-Y_1 ([Leu^{31},Pro^{34}]NPY and its analogues are inactive; Cox and Krstenansky, 1991), and since C-terminal fragments are effective (Cox and Cuthbert, 1990), the jejunal epithelial NPY receptor is classified as Y_2-like. Responses to C-terminal fragments in rat descending colon preparations (see data above) indicate that Y_2 receptors are also present in this gastrointestinal area. Many potential antagonists have been tested on the Y_2-like system of the rat jejunum mucosa in recent years and all have either failed to inhibit NPY and PYY responses (e.g., PYX_1 or PYX_2; Tatemoto et al., 1992) or at best been nonselective inhibitors (Cox, unpublished data). A selective Y_2 receptor antagonist is eagerly awaited to assist confirmation of these classifications.

The potency BIBP3226 exhibited in Col-6 epithelia is very similar to that observed in other human Y_1 receptor systems (Rudolf et al., 1994; Abounader et al., 1995; Wieland et al., 1995) where pA_2 values between 7.5 and 8.5 have been reported. Y_1 receptors in rat tissues appear to be only slightly less sensitive to BIBP3226 (Doods et al., 1995), but all quoted pK_b values are, so far, greater than 7.5. In rat descending colon the antisecretory effects of PYY and NPY were partially (though not significantly) inhibited by tetrodotoxin (as also observed by Strabel and Diener, 1995, for NPY). Responses to the Y_1-selective agonist [Leu^{31},Pro^{34}]NPY were significantly attenuated, indicating a preponderance of Y_1 receptors on prejunctional membranes. tetrodotoxin-resistant responses to both PYY and [Leu^{31},Pro^{34}]NPY were further inhibited by pretreatment with BIBP3226 and the antagonist virtually abolished responses to the Y_1 agonist, indicating that this receptor subtype also exists in colonic epithelial surfaces in contrast with the rat jejunum. PYY(3–36) responses were also sensitive to tetrodotoxin but less so than [Leu^{31},Pro^{34}]NPY responses, indicating that receptors of the Y_2-type are located within both pre- and postjunctional membranes in the rat colon. Again, the availability of a Y_2-selective antagonist would help clarify the pharmacology of this system and thereby provide information concerning the potential therapeutic benefits of a Y_2-selective agonist as an antidiarrhoeal. In fact, infusions of PYY (Playford et al., 1990) or NPY (Holzer-Petsche et al., 1991) will attenuate acute hypersecretion in humans and the relative clinical benefits of a nonselective versus a selective Y receptor agonist have yet to be determined.

In conclusion, the rat descending colon expresses a combination of Y_1- and Y_2-like receptors within both prejunctional nerve terminals and basolateral epithelial membranes, with a predominance of Y_1 receptors at prejunctional (submucosal or mucosal nerve terminals) and Y_2 receptors on postjunctional epithelial surfaces. No evidence was found for the existence of Y_1 receptors in rat jejunum, BIBP3226 having no significant effect upon PYY responses in this preparation. The human colonic adenocarcinoma cell line (Col-6), in contrast, expresses Y_1 receptors together with another, as yet unidentified, BIBP3226-insensitive Y receptor that can be stimulated by hPP (albeit at higher nM concentrations). Future studies will attempt to discover the nature of this low-affinity PP receptor.

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