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Constitutive neuropeptide Y Y_4 receptor expression in human colonic adenocarcinoma cell lines

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1 Three human adenocarcinoma cell lines, Colony-24 (Col-24), Col-6 and Col-1 have been studied as confluent epithelial layers able to transport ions vectorially in response to basolateral vasoactive intestinal polypeptide (VIP) and pancreatic polypeptides (PP).

2 Different species PP stimulated responses in Col-24 with Y_4-like pharmacology. Bovine (b)PP, human (h)PP and porcine (p)PP were equipotent (EC_{50} values 3.0–5.0 nM) while rat (r)PP, avian (a)PP and [Leu^{31}, Pro^{34}]PYY (Pro^{34}PYY) were significantly less potent. PYY was inactive. The PP pharmacology in Col-1 was comparable with Col-24. However, Col-6 cells were different; pPP had an EC_{50} intermediate (22.0 nM) between that of bPP (3.0 nM) and hPP (173.2 nM), with aPP and rPP being at least a further fold less potent.

3 Deamidation of Tyr^{36} in bPP (by *O*-methylation or hydroxylation) or removal of the residue resulted in significant loss of activity in Col-24.

4 GR231118 (1 μM) had no PP-like effects. In Col-24 and Col-1, GR231118 significantly attenuated bPP (30 nM) or hPP (100 nM) responses, but it did not alter bPP responses in Col-6. BIBP3226 and GR231118 both inhibited Y_1-mediated responses which were only present in Col-6.

5 RT–PCR analysis confirmed the presence of hY_4 receptor mRNA in Col-24 and Col-1 epithelia but a barely visible hY_4 product was observed in Col-6 and we suggest that an atypical Y_4 receptor is expressed in this cell line.

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Abbreviations: a, avian; b, bovine; BIBP3226, (R-N^2-(diphenylacetyl)-N-[(4-hydroxyphenyl)methyl]-D-argininamide); CHO, Chinese hamster ovary cells; Col, Colony (cell line); GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; GR231118, homodimeric Ile-Glu-Pro-Dpr-Tyr-Arg-Leu-Arg-Tyr-$CONH_2$; h, human; HEK293, human embryonic kidney 293 cells; HCA-7, human colonic adenocarcinoma cell line; HT-29, human adenocarcinoma cell line; I_{sc}, short circuit current; MuLV, murine leukaemia virus; PP, pancreatic polypeptide; PYY, peptide YY; p, porcine; Pro^{34}PYY, [Leu^{31}, Pro^{34}]PYY; r, rat; RT–PCR, reverse transcriptase-polymerase chain reaction; SRIF, somatotrophin release-inhibiting factor; VIP, vasoactive intestinal polypeptide

Introduction

Of the three endogenous, full-length pancreatic polypeptides (neuropeptide Y, NPY; peptide YY, PYY and pancreatic polypeptide, PP) the latter remains the least well understood. The first descriptions of a distinct PP-preference in tissues were from functional and binding studies with rat vas deferens and PC12 cells respectively (Schwartz *et al*., 1987; Jørgensen *et al*., 1990). These authors proposed the existence of a PP-specific receptor which was subsequently cloned and initially named the PP1 receptor (Lundell *et al*., 1995) though it is now designated the Y_4 receptor (Michel *et al*., 1998). Mammalian PPs exhibit high affinity for both Y_4 and Y_5 receptors and low affinities for the remaining known Y receptor types (Y_1, Y_2 and y_6). Both human (h) and bovine (b) PP exhibit a high affinity (<100 pM) for the human, rat and murine Y_4 receptor while rat (r) commonly exhibits (though not in all cases) an affinity 50–100 fold lower than hPP at the hY_4 receptor (for overview see Michel *et al*., 1998). A major difference between Y_4 and Y_5 receptors is the nonselective character of the latter. In addition to their stimulation by PP, Y_5 receptors are also activated by PYY, NPY, their C-terminal fragments as well as Pro^{34}-substituted analogues.

Given the lack of hPP activity in early studies with rodent intestinal preparations and the subsequent characterization of mucosal Y_1 or Y_2 receptor-mediated NPY/PYY antisecretory effects (Cox *et al*., 1988; Cox & Cuthbert, 1990; Cox & Krstenansky, 1991) it is not surprising that relatively little attention has been paid to PP in these models. However, more recently we and others have found certain mammalian gastrointestinal preparations with significant sensitivity to hPP (e.g. rabbit colon, Ballantyne *et al*., 1993; human adenocarcinoma cell lines, Cox & Tough, 1995; Tough & Cox, 1996; Holliday *et al*., 1997). In these systems hPP was inhibitory (like NPY or PYY) attenuating anion secretion that had been prestimulated by vasoactive intestinal polypeptide (VIP). In addition, rPP and hPP have now also been shown to inhibit spontaneous contractile activity in the rabbit isolated ileum (Félétou *et al*., 1999). Both species PP (and PYY) stimulated contraction of rat colon segments, at least

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in part by activating Y_4 receptors located on enteric neurones (Pheng *et al*., 1999). In fact all of the cloned Y receptor types have now been shown by reverse transcriptase-polymerase chain reaction (RT–PCR) analysis to be differentially expressed in rat small and large bowel, with Y_4 receptors being found in epithelial and non-epithelial layers in both regions (Goumain *et al*., 1998). Thus there is renewed interest in PP, the Y_4 receptor and their role(s) in the control of gastrointestinal function.

The present investigation arose from the initial observation that a confluent epithelial cell line (Colony-24, Col-24) derived from a human colonic adenocarcinoma cell line, HCA-7 (Marsh *et al*., 1993) exhibited two unexpected features. First, a lack of sensitivity to PYY was observed (unlike a related cell line, Col-6 which expresses Y_1 receptors, Tough & Cox, 1996) and secondly, inhibitory responses to hPP were obtained at low nM concentrations. Coincident with these findings we also discovered prolonged inhibitory hPP effects in *in vitro* preparations of human descending colon mucosae (Cox & Tough, submitted). We therefore set out to determine the detailed pharmacology of PP-mediated inhibitory responses in Col-24 cell layers and compared it with those exhibited by two related lines, namely Col-1 and Col-6.

Methods

Cell culture and short circuit current (I_{sc}) measurement

All cell lines were maintained in culture as described previously (Cox & Tough, 1995). Prior to measurement of electrogenic responses (I_{sc}) each line was grown on collagen-coated permeable supports and once confluent, cell layers on individual filters were placed between two halves of an Ussing chamber, voltage-clamped at 0 mV and the resultant I_{sc} measured continuously (Cox & Tough, 1995). A steady basal I_{sc} was achieved within 20 min and unless otherwise stated, confluent epithelial layers were pretreated with basolateral vasoactive intestinal polypeptide (VIP) for 20 min prior to addition of a single concentration of either PP or one of its analogues. The effects of BIBP3226 or GR231118 were monitored by adding either or both Y_1 antagonists to epithelial layers 10 min after VIP and 10 min prior to PP. Subsequent to PP additions, somatostatin 14–28 (SRIF, somatotrophin release-inhibiting factor, 100 nM) was added as an internal inhibitory control, and unless otherwise stated all peptide additions were made to the basolateral reservoir.

Statistical analysis of the mean changes in I_{sc} ($\pm$1 s.e.mean) to particular agonists from different data groups were performed using unpaired Student's *t*-test and a P value <0.05 was considered statistically significant. Concentration-response curves were constructed from single agonist additions per epithelial layer and pooled data was analysed using Graphpad Prism (v. 2.01, Graphpad Software Inc., California, U.S.A.). EC_{50} values (with 95% confidence limits) were calculated and for PP peptides the pEC_{50} values were compared (in Table 2) with those published previously by Walker *et al*. (1997).

Extraction of RNA

Epithelial cells (Col-24, Col-6, Col-1, a clonal line of Col-1 stably transfected with the hY_1 receptor, hY_1-4; HT-29, plus a stably transfected hY_4 HT-29 clone, hY_4-27) were grown to confluence in individual flasks ($1.4-1.7\times10^7$ cells ml^{-1}) trypsinized (0.5% w v^{-1} in versene) and centrifuged (1000 r.p.m. for 5 min) before being snap-frozen and stored at -30°C. RNA extraction and precipitation were performed following cell lysis in TRIzol reagent, according to the manufacturer's protocol (Life Technologies Ltd, Paisley, U.K.). Cell pellets were homogenized in 1 ml of TRIzol reagent and insoluble material removed by centrifugation at $12{,}000\times g$ for 10 min at 4°C. The supernatant was then incubated for 5 min at room temperature, extracted with 0.2 ml of phenol/chloroform and centrifuged at $12{,}000\times g$ for 15 min at 4°C. The aqueous phase ($\sim$0.6 ml) was then mixed with 0.5 ml isopropyl alcohol and incubated at room temperature for 15 min. RNA was precipitated by centrifugation at $12{,}000\times g$ for 10 min at 4°C and the resultant pellet rinsed with 1 ml of 75% ethanol and centrifuged at $7500\times g$ for 5 min at 4°C. The final pellet was air-dried and resuspended in 40 μl distilled H_2O containing 0.1% di-ethyl pyrocarbonate. Final RNA concentrations were determined by spectrophotometry (absorbance at 260 nm).

RT–PCR reactions

Six μg of total RNA were incubated in a total volume of 30 μl with 1 μl (10 U) of RNAse free-DNAse for 2 h at 37°C (DNAse was then heat-inactivated at 95°C for 10 min). Two μg of RNA was reverse transcribed in a total volume of 20 μl using 50 U murine leukaemia virus (MuLV) reverse transcriptase (RT) and oligo$(dT)_{15}$ at 42°C for 15 min, followed by heat inactivation at 95°C for 5 min, then cooling to 4°C. For each cell line a negative control lacking RT was included. Aliquots (2.5 μl) of the resultant cDNA mixtures, including negative controls, were used as templates for polymerase chain reaction (PCR) using specific primers for either the hY_4 receptor (see Table 1) or for the housekeeping gene, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH; Imperial Cancer Research Fund, Clare Hall, U.K.).

PCR was performed in Promega PCR buffer A containing 1.5 mM $MgCl_2$ with primers selective for the hY_4 receptor (see Table 1 for primer sequences, at 10 nmol throughout)

Table 1

Protein	*Primer position*	*Sequence* (5′ to 3′)		*PCR product* (bp)
hY_4	204/961	sense	CCT CTG CCT GAT GTG TGT	757
		antisense	TGG CAA GCA AGT GGC ACA	
hGAPDH	12/994	sense	TGA AGG TCG GAG TCA ACG GAT TTG GT	983
		antisense	CAT GTG GGC CAT GAG GTC ACA CAC	

The start primer positions are given for hY_4 cDNA (from GenBank U35232) and for hGAPDH mRNA (GenBank J02642).

following the protocol; 5 cycles at an annealing temperature of 60°C (denaturing at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min), then 5 cycles at an annealing temperature of 55°C, 25 cycles at an annealing temperature of 50°C and a final extension for 10 min at 72°C. All PCR products were analysed by electrophoresis on 1% agarose gels and were photographed under UV illumination.

Materials

All peptides were purchased from Peninsula Laboratories Inc. (Merseyside, U.K.) with the exception of bPP and all bPP analogues, which were gifts from Eli Lilly and Co. (Indianapolis, U.S.A.). GR231118 was a gift from Glaxo Wellcome Inc. (Research Triangle Park, NC, U.S.A.) while BIBP3226 was from Boehringer Ingelheim Pharma KG (Biberach an der Riss, Germany). Aqueous solutions of stock peptides and BIBP3226 were stored at −20°C and underwent a single freeze-thaw cycle only. Trypsin was purchased from Worthington, Lorne Laboratories, Reading, U.K. All other compounds were made up as aqueous solutions. Krebs–Henseleit solution constituents (in mM) were: 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. The sources of reagents used in RT–PCR were as follows; RNAse-free DNAse (Roche, Lewes, U.K.), MuLV Reverse Transcriptase kit (Perkin Elmer, Cheshire, U.K.), 10× reaction PCR buffer A and 25 mM $MgCl_2$ (Promega, Southampton, U.K.).

Results

I_{sc} responses to VIP, hPP and SRIF but not PYY in Col-24 cells

Confluent layers of voltage-clamped Col-24 epithelia were sensitive to increasing concentrations of VIP and analysis of pooled data (from single additions of VIP, $n=3-4$ throughout) resulted in an EC_{50} value of 2.9 nM (2.0–4.1, 6 d.f.). This was comparable with the VIP EC_{50} values obtained previously from Col-1 (6.6 nM; 2.8–15.2 nM, 3 d.f.; Holliday *et al*., 1997) and Col-6 cells (1.6 nM; 1.52–1.64, 6 d.f.; Cox & Tough, 1995). Following VIP pretreatment (10 nM, which stimulated a prolonged elevation of I_{sc}, Figure 1) subsequent basolateral addition of hPP (100 nM) or bPP (30 nM) but not PYY (100 nM, not shown) produced reductions in VIP-elevated I_{sc}. The $Y_1/Y_4/Y_5$ agonist, [Leu^{31}, Pro^{34}]PYY (Pro^{34}PYY, 100 nM) also attenuated VIP-elevated I_{sc} but with an apparent half maximal concentration of 1 μM (see pooled data in Figure 2A). A threshold concentration of 100 nM was obtained for rPP (data not shown) while PYY was without effect at 100 nM (0.0 ± 0.0 μA cm^{-2}, $n=4$, Figure 2A). SRIF (100 nM) consistently abolished any remaining elevated I_{sc} (see Figure 1).

Structure-activity studies with different species PP in Col-24 compared with Col-1 and Col-6

Following VIP-pretreatment inhibitory electrogenic responses to hPP exhibited an EC_{50} value of 4.9 nM (0.7–28.0 nM, 3 d.f.), with a maximal effect of -5.1 ± 0.9 μA cm^{-2} ($n=18$) at 100 nM hPP (Figure 2A). Figure 2A shows the pooled data and relative potencies obtained with different species PP (except bPP and aPP), Pro^{34}PYY and porcine (p) PYY. Human PP, pPP and bPP exhibited similar nM potencies and efficacies (Figure 2A,B; Table 2) while rPP (which is 78% homologous with hPP) and Pro^{34}PYY were at least two orders of magnitude less potent than hPP. A threshold concentration of 300 nM was obtained for aPP, which exhibits only 40% sequence homology with hPP (-0.5 ± 0.0 μA cm^{-2}, $n=3$).

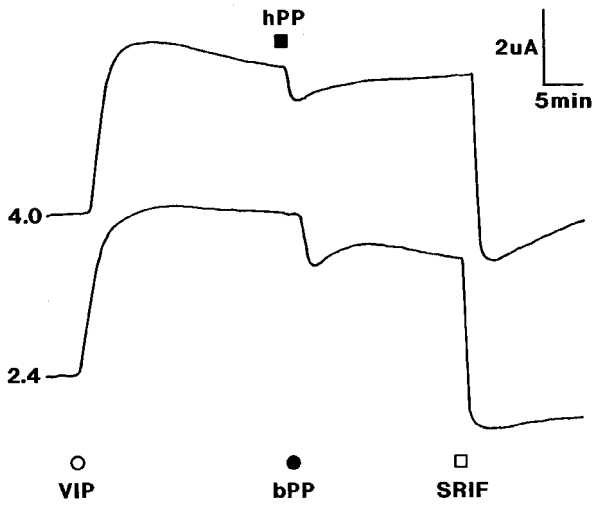

Figure 1 Representative traces showing responses to hPP (100 nM) or bPP (30 nM) in Col-24 cells pretreated with VIP (10 nM). Each filter received a final addition of somatostatin (SRIF, 100 nM), which completely inhibited the remaining VIP-elevated I_{sc}. Basal I_{sc} values are shown in μA to the left of each trace (note the exposed area was 0.2 cm^2).

Previous functional studies have shown that Col-6 epithelia were sensitive to hPP, albeit at high nM concentrations (Cox & Tough, 1995). In addition these cells express Y_1 receptors (Pro^{34}NPY and PYY responses being abolished by pretreatment with BIBP3226, 1 μM) but hPP responses were resistant to the Y_1 antagonist (Tough & Cox, 1996). In the present study each species PP produced maximal reductions in I_{sc} that were similar, with the exception of the concentration-response curve for pPP which was significantly greater than bPP ($P<0.01$) and hPP ($P<0.02$) but no different to the maximal Pro^{34}PYY response (data not shown). Pooled data resulted in the pEC_{50} values listed in Table 2 (with asterisks for the two agonists exhibiting higher maximal responses).

In order to complete the comparison, the same species PPs were tested in Col-1 layers and analysis of this pooled functional data is presented in Table 2. These epithelia were equally sensitive to hPP, bPP and pPP (although the three peptides were a fold less potent in Col-1 compared with Col-24 layers) and no responses were observed with either rPP (100 or 300 nM, $n=2-4$) or aPP (300 nM, $n=2$). Pro^{34}PYY was also without effect at 100 nM and 1 μM ($n=4$) in Col-1 cells.

Structure-activity studies with different species PP and bPP analogues in Col-24

A series of bPP analogues were included in structure-activity studies and deamidation of the C-terminal Tyr^{36}

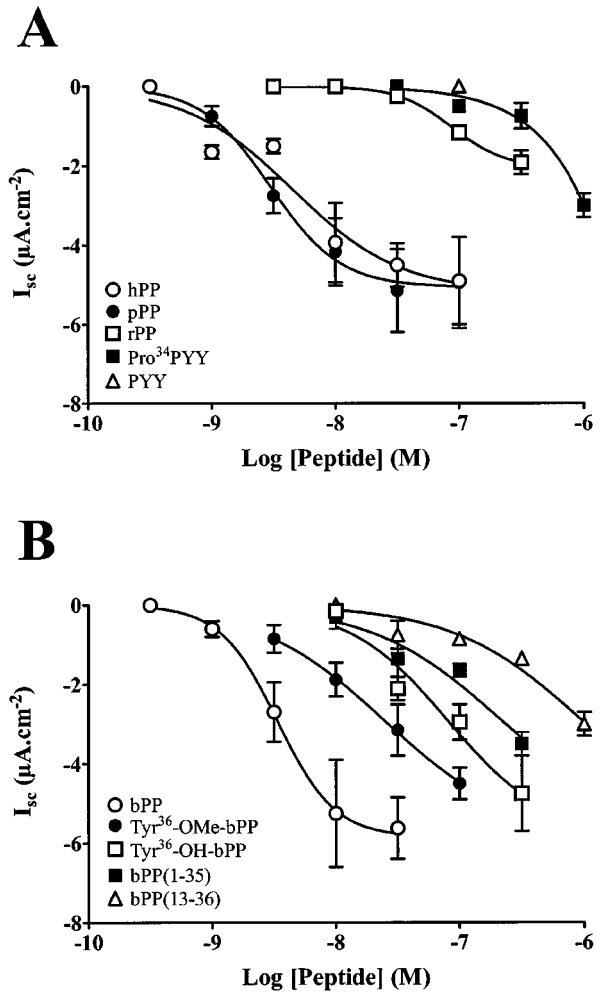

Figure 2 (A) Concentration-response curves for pooled data from Col-24 layers pretreated with VIP (10 nM) and then single additions of either different species PP, Pro^{34}PYY or PYY. All values are the mean$\pm$1 s.e.mean from between 3–18 observations. These curves were used to calculate the pEC_{50} values listed in Table 2. (B) Structure-activity studies of bPP and four bPP analogues. All values are the mean$\pm$1 s.e.mean from between 3–18 observations. Each curve was used to calculate the pEC_{50} values shown in Table 2.

residue by either hydroxylation (Tyr^{36}-OH bPP) or *O*-methylation (Tyr^{36}-OMe bPP) reduced the potency by 23.8 and 7.4 fold respectively (compared with native bPP). There did not appear to be any effect upon agonist efficacy (Figure 2B). C-terminal truncation resulted in further loss of biological activity, bPP(1–35) being approximately 80-times less potent than full length bPP. Increasing N-terminal truncations also resulted in significant loss of potency; bPP(4–36) and bPP(5–36) being approximately 80-times and bPP(13–36) 300-times less potent than native bPP. The order of potency observed with these bPP analogues is summarized in Table 2 and compared with previously published radioligand binding assays utilising Chinese hamster ovary (CHO) cell membranes transfected with hY_4 receptors (Gehlert *et al*., 1996) and functional data for hY_4-mediated inhibition of forskolin-stimulated cyclic AMP levels in stably transfected L-M(TK$^-$) cells (Walker *et al*., 1997).

Sidedness and desensitization of PP responses in VIP-pretreated layers of Col-24

Basolateral addition of a maximally effective concentration of bPP (30 nM) significantly attenuated the electrogenic effects of a second application of bPP (30 nM) 20 min later (Figure 3A). However, if the first bPP addition was made to the apical compartment then a significantly smaller reduction in I_{sc} was observed ($P=0.002$). Subsequent basolateral addition of bPP (30 nM) 20 min after apical pretreatment produced reductions in I_{sc} that were not significantly different from controls ($P=0.8$).

Following pretreatment of Col-24 with 10 nM VIP, significant desensitization of subsequent responses to bPP (30 nM, after 30 nM bPP, $P<0.001$) and hPP responses (100 nM, $P<0.05$) was observed (Figure 3B). Porcine PP responses were also significantly attenuated (30 nM, $P<0.01$) following bPP addition, although rPP responses were not (300 nM, $P=0.12$, Figure 3B). In all cases the remaining VIP-elevated I_{sc} was abolished by subsequent addition of SRIF (100 nM, data not shown).

The inhibitory effects of BIBP3226 (1 μM) and GR231118 (1 μM) upon bPP responses in Col-24, Col-6 and Col-1 cells

Neither the nonpeptide Y_1 antagonist, BIBP3226 nor the bridged antiparallel dipeptide, GR231118 (both at 1 μM) had any significant effect upon I_{sc} levels (each changed the I_{sc} by 0.7 ± 0.1 μA cm^{-2}, $n=26$; and 0.7 ± 0.1 μA cm^{-2}, $n=8$ respectively and together by, 0.6 ± 0.1 μA cm^{-2}, $n=12$) in Col-24. Following pretreatment with BIBP3226 alone there was no significant difference in the size of bPP (30 nM) responses ($P=0.7$, Figure 4A). In contrast GR231118 alone significantly attenuated bPP responses ($P<0.01$) and there was no further inhibition following a combination of BIBP3226 and GR231118 ($P<0.01$, Figure 4A). GR231118 also attenuated hPP (100 nM) responses in Col-24, but this was not statistically significant (controls were; -6.0 ± 1.0 μA cm^{-2}, $n=5$ and plus GR231118; -3.0 ± 0.5 μA cm^{-2}, $n=4$, $P=0.07$). Neither BIBP3226 nor GR231118 alone or together had any significant effect upon the inhibitory responses to basolateral SRIF (100 nM, added after bPP, Figure 4B).

Col-1 basal I_{sc} levels were similarly unaffected by additions of either BIBP3226 (1 μM) or GR231118 (1 μM) however a significant inhibition of hPP responses (100 nM) was observed consequent to GR231118 pretreatment. Control hPP effects were; -2.2 ± 0.2 μA cm^{-2} ($n=5$); following BIBP3226 hPP responses were unchanged (-2.5 ± 0.3 μA cm^{-2}, $n=3$, $P=0.3$) but after GR231118 they were attenuated by 64% (-0.8 ± 0.1 μA cm^{-2}, $n=4$, $P<0.001$).

Previous studies with Col-6 epithelia have already shown that BIBP3226 abolished both PYY (10 nM) and Pro^{34} NPY (100 nM) responses but that the Y_1 antagonist had no effect upon hPP (100 nM) responses (Tough & Cox, 1996). Maximal Pro^{34} PYY responses (100 nM, -2.5 ± 0.5 μA cm^{-2}, $n=4$) in Col-6 cells were significantly greater than either bPP (100 nM, -0.9 ± 0.2 μA cm^{-2}, $n=5$, $P=0.01$) or PYY-induced maximal reductions in I_{sc} (100 nM, -1.3 ± 0.2 μA cm^{-2}, $n=6$, $P<0.05$) indicating that the former peptide is able to stimulate both the Y_1 and a PP-sensitive response at

Table 2 A comparison of the pEC_{50} and pK_i values with calculated relative potencies (RP, set to unity for bPP) for different species PP and analogues of bPP in epithelial preparations and CHO cells transfected with hY_4 receptors

	Col-24 epithelia		*Col-6 epithelia*		*Col-1 epithelia*		*hY_4 cyclic AMP assay* (Walker *et al*., 1997)		*hY_4 in CHO* (Gehlert *et al*., 1996)	
Peptide	*pEC_{50}* (nM)	*RP*	*pEC_{50}* (nM)	*RP*	*pEC_{50}* (nM)	*RP*	*pEC_{50}*	*RP*	K_i (nM)	*RP*
bPP	8.47±0.03	1.0	8.57±0.16	1.0	7.51±0.14	1.0	9.80±0.08	1.0	0.04	1.0
hPP	8.31±0.44	1.4	6.76±0.07	57.7	7.59±0.20	0.8	10.06±0.06	0.7	0.02	0.6
pPP	8.52±0.05	0.9	7.65±0.09*	n.a.	7.32±0.08	1.5	–	–	–	–
rPP	>6.5	>80.0	~6.0	~300	>6.5	–	8.69±0.09	12.9	0.05	1.5
aPP	>>6.0	>>300	~6.0	~300	>6.5	–	7.11±0.16	~500	0.96	27.5
Tyr^{36}-OMe bPP	7.60±0.01	7.4	–	–	–	–	–		0.07	2.0
Tyr^{36}OH bPP	7.09±0.11	23.8	–	–	–	–	–		0.89	25.4
bPP(1–35)	~6.5	~80.0	–	–	–	–	–		2.80	80.0
bPP(4–36)	~6.5	~80.0	–	–	–	–	–		0.31	8.9
bPP(5–36)	~6.5	~80.0	–	–	–	–	–		1.80	51.4
bPP(13–36)	>6.0	>300	–	–	–	–	6.44±0.16	>2000	0.46	13.1
Pro^{34}PYY	~6.0	~300	7.80±0.32*	n.a.	>6.0	–	9.26±0.16	3.5	0.08	2.3

Functional data for Col-24, Col-6 and Col-1 cells are compared with that published by Walker *et al*. (1997) who stably transfected L-M(TK$^-$) cells with hY_4 cDNA and then measured Y-agonist inhibition of forskolin-stimulated cyclic AMP levels. Also included is binding data from Gehlert *et al*. (1996); who used [^{125}I]-PYY as the radioligand in displacement assays. *Denotes a significantly greater efficacy compared with PP analogues in Col-6, and n.a. means not applicable.

higher nM concentrations. BIBP3226 (1 μM, which *per se* increased I_{sc} after VIP by 0.7 ± 0.1 μ A cm^{-2}, $n=19$) significantly inhibited but did not abolish Pro^{34} PYY (100 nM) responses (controls, -2.4 ± 0.4 μA cm^{-2}, plus BIBP3226 -0.6 ± 0.2 μA cm^{-2}, both $n=6$, $P<0.01$). The Y_1 antagonist however, had no significant effect upon either bPP (30 nM) or SRIF (100 nM) responses (Figure 5A,C) but it did significantly attenuate PYY responses (30 nM, Figure 5B, $P<0.05$). Similarly GR231118 alone significantly inhibited PYY ($P<0.05$) but had no effect upon either bPP or SRIF responses. After both GR231118 and BIBP3226 addition (which increased I_{sc} by 1.7 ± 0.4 μA cm^{-2}, $n=5$) PYY responses were again significantly inhibited ($P<0.05$), while bPP and SRIF were unchanged (Figure 5). Thus BIBP3226 had no effect in Col-24 or Col-1 epithelia, but it did inhibit (as expected) Y_1-mediated responses that are only found in Col-6 cells. GR231118 in contrast, had no significant effect upon bPP responses but did inhibit PYY effects in Col-6, while in Col-24 cells bPP responses were significantly attenuated. Similar levels of bPP inhibition were obtained with GR231118±BIBP3226 in Col-24 while there was no effect in Col-6 cells.

RT–PCR detection of hY_4 receptor in Col-24 and related cell lines

Following RT amplification, PCR products of the predicted size (757 bp) were observed from RNA extracted from Col-24 cells and positive controls, i.e. HT-29 epithelia stably transfected with hY_4 receptor (hY_4-27) but not from negative control, wild-type HT-29 epithelia (Figure 6). A faint band of similar size was evident for Col-6 cells plus RT under UV illumination (though too faint to be reproduced in Figure 6). Other positive controls were included for comparison and were as follows; Col-1 (which exhibit sensitivity to hPP, Holliday *et al*., 1997) and its derivative, stably transfected hY_1 clonal line (hY1–4). All negative controls lacking RT were devoid of product.

Discussion

The functional data presented above shows that Col-24 epithelial cells derived from a human adenocarcinoma cell line express Y_4 receptors and that when stimulated by the preferred agonists, namely hPP, bPP or pPP, a reduction in VIP-stimulated I_{sc} was obtained. These three species PP were equipotent in Col-24 layers with rPP, Pro^{34}PYY and aPP being significantly less effective and PYY essentially inactive. This potency order resembles that of other Y_4-expressing tissues, including gastrointestinal epithelial cells (Gilbert *et al*., 1988; Gingerich *et al*., 1991) and the absence of PYY effects excludes the involvement of Y_5 receptors in Col-24 cells. The potency order for the same species PP was however, different in Col-6 epithelia (Table 2). In particular, the potency of hPP was much lower (EC_{50} of 173 nM compared with 3.0 nM for bPP) than expected for the hY_4 receptor, while pPP was seven times less potent than bPP. These three mammalian PPs are 95% homologous with nonconservative substitutions at position 6 (where negatively charged Glu^6 in bPP is replaced for a nonpolar Val^6 residue in hPP and pPP) and position 11 (uncharged Asn^{11} in both bPP and hPP versus acidic Asp^{11} in pPP) together with a single conservative replacement of Glu^{23} in bPP and pPP for an Asp^{23} in hPP. Whilst none of these substitutions altered peptide potency or efficacy at the hY_4 receptor expressed in Col-24 cells, the loss of a negative charge within the N-terminal polyproline helix of bPP appeared to significantly reduce the peptide potency (of hPP) in Col-6 cells. Subtle changes in the tertiary structure and polarity of PP will alter peptide-receptor interactions differentially and to date no similar wide spread in potencies for bPP, pPP and hPP has been observed in any other tissue. We therefore conclude that this unusual PP pharmacology in Col-6 epithelia is due to the presence of a novel Y_4-like receptor in these cells, and it is notable that RT–PCR analysis of Col-6 RNA yielded barely visible hY_4 product.

The cloning and characterization of the Y_4 receptor from human (Lundell *et al*., 1995; Bard *et al*., 1995) rat (Gerald *et*

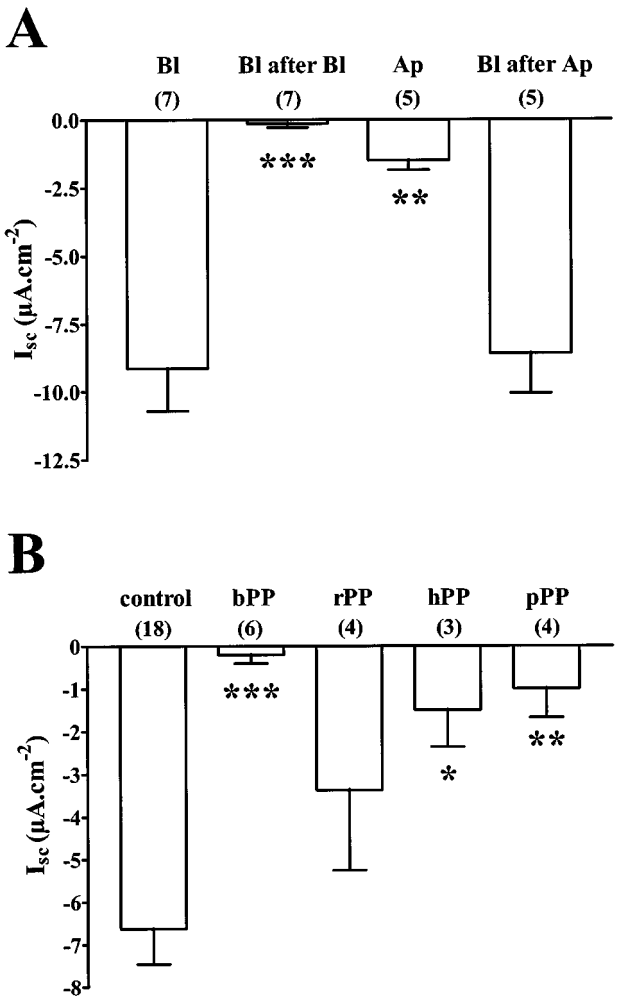

Figure 3 (A) A comparison of response sizes after either basolateral (Bl) or apical (Ap) addition of bPP (30 nM, following 10 nM VIP). Each bar is the mean−1 s.e.mean and the number of observations in each group are shown in parentheses. The size of 30 nM bPP responses following an earlier basolateral bPP addition were significantly attenuated compared with Bl controls (**$P<0.01$) as were Ap responses in naive cell layers (***$P<0.001$). (B) Cross-desensitization between responses to bPP (30 nM, control) and different species PP following pretreatment with VIP (10 nM). Initial additions of either 30 nM bPP, 300 nM rPP, 100 nM hPP or 30 nM pPP were made, 20 min later 30 nM bPP was added and subsequent reductions in I_{sc} recorded. Each bar is the mean−1 s.e.mean of the latter responses (with the number of observations in parentheses) and significant reductions in bPP responses were as shown; *$P<0.05$; **$P<0.01$ and, ***$P<0.001$.

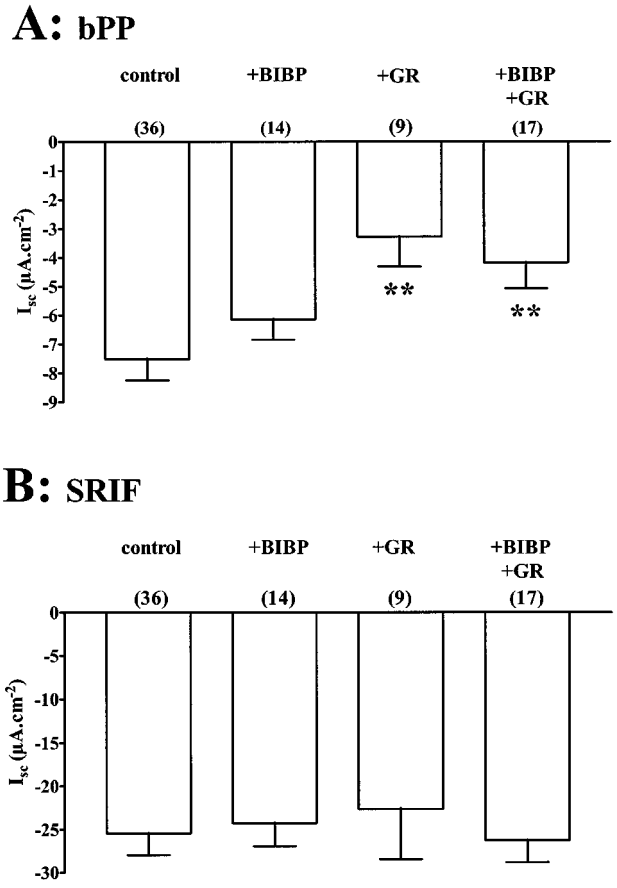

Figure 4 The effects of BIBP3226 (BIBP, 1 μM) or GR231118 (GR, 1 μM) or a combination of the two in Col-24 cells, upon (A) bPP responses (30 nM) and (B) SRIF responses (100 nM) following pretreatment with VIP (10 nM). All bars are the mean−1 s.e.mean with the number of observations shown in parentheses. Significant differences between control bPP responses and those following GR231118 ± BIBP3226 (both at 1 μM) were; **$P\leqslant0.01$.

al., 1996; Lundell *et al.*, 1996) and murine tissue (Gregor *et al.*, 1996), has provided comparative information concerning this receptor's pharmacology and distribution (see review, Michel *et al.*, 1998). Human Y_4 mRNA is predominantly expressed in the colon, small intestine, pancreas and prostate (Lundell *et al.*, 1995) and recently we have observed prolonged inhibitory effects with nM concentrations of hPP added to mucosal preparations of human descending colon. These responses were resistant to pretreatment with either Y_1 or Y_2 receptor selective antagonists (Cox & Tough, submitted). The latter observation was unexpected because the majority of mammalian gastrointestinal mucosae studied to date show little if any sensitivity to hPP or other species PP (for review see Cox, 1998). Certain human epithelial cell lines however do exhibit PP-sensitivity (Cox & Tough, 1995; Holliday *et al.*, 1997) with Col-6 cells exhibiting an additional complicating characteristic; constitutive co-expression of the Y_1 receptor (Tough & Cox, 1996) plus the abnormal Y_4-like responses discussed above. Expression of multiple Y receptor types in mucosal preparations has also been demonstrated by Goumain *et al.* (1998) using rat jejunum and colonic tissue; who identified differential patterns of Y_1, Y_2, Y_4 and Y_5 receptor expression using a RT–PCR based approach. This sensitive strategy found Y_4 receptor mRNA in both surface and crypt epithelia (plus non-epithelial, submucosal targets) together with Y_2 receptor mRNA in the jejunum, where only the latter receptors would be predicted from mucosal functional (Cox *et al.*, 1988; Cox & Krstenansky, 1991) and binding studies (Laburthe *et al.*, 1986). In the rat colon, Y_4 receptor expression was also observed in epithelia (with Y_2 mRNA) and in the underlying non-epithelial layers (with both Y_1 and Y_5 receptor mRNA). The functional relevance of albeit low levels of Y_4 and Y_5 receptor expression in these discrete gastrointestinal locations from rat remains to be determined.

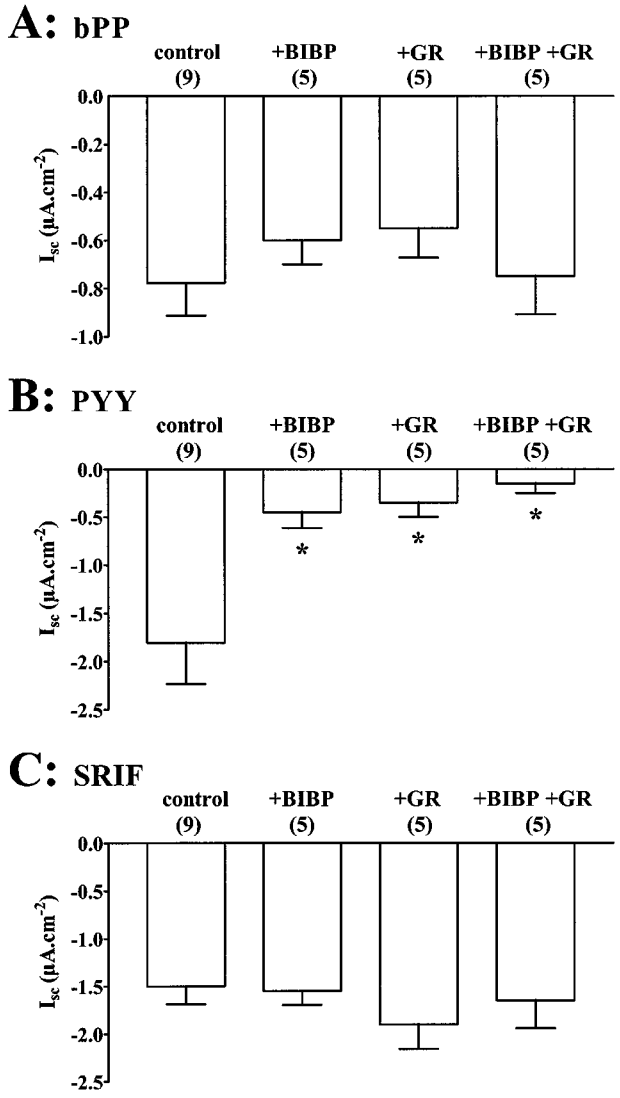

Figure 5 The effects of BIBP3226 (BIBP, 1 μM) or GR231118 (GR, 1 μM) either alone or together in Col-6 cells, upon responses to: (A) bPP (30 nM), (B) PYY (30 nM) and (C) SRIF (100 nM) following pretreatment with VIP (30 nM). All bars are the mean − 1 s.e.mean with the number of observations shown in parentheses. Significant differences between control PYY responses and those following GR231118 ± BIBP3226 (both at 1 μM) were as shown; $*P<0.05$.

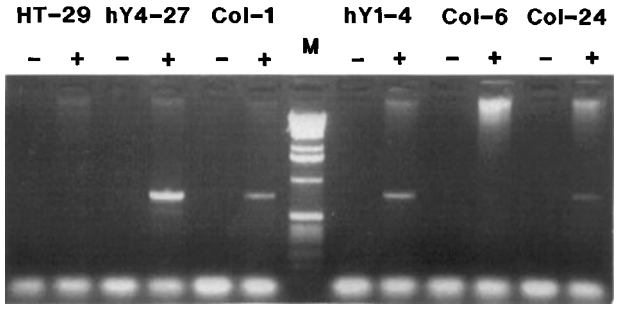

Figure 6 RT−PCR detection of mRNA for hY_4 receptor (757 bp) in different epithelial cell lines. Pairs of lanes are ± reverse transcriptase (RT) and were in order from left to right; HT-29, hY4-27 (an HT-29 clone stably transfected with hY_4 receptor cDNA), Col-1, marker lane, hY1-4 (a Col-1 clone stably transfected with the hY_1 receptor cDNA), Col-6, and finally Col-24.

In Col-24 epithelial layers, loss of the C-terminal Tyr^{36}-amide residue by either hydroxylation (Tyr^{36}-OH bPP) or by *O*-methylation (Tyr^{36}-OMe bPP) reduced peptide potency 24- and 7-times respectively. This was in keeping with the effects of deamidation observed upon displacement of [^{125}I]-PYY from either transfected hY_4 receptors in CHO membranes (Gehlert *et al*., 1996) or canine basolateral membranes (Gingerich *et al*., 1991). C-terminal truncations of bPP resulted in further loss of biological activity, in a manner similar to that observed in earlier studies with native and C-terminally truncated NPY and PYY peptides acting upon Y_2-expressing mucosae and epithelial membranes (Servin *et al*., 1989; Cox & Cuthbert, 1990; Cox & Krstenansky, 1991).

The selective targeting of Y_4 receptors to the basolateral domain of Col-24 layers also follows the same pattern of sidedness exhibited by other Y receptors in epithelial preparations (Cox *et al*., 1988; Cox & Tough, 1995; Holliday *et al*., 1997). The characterization of specific [^{125}I]-PP binding to basolateral membranes from dog small intestine (Gilbert *et al*., 1988; Gingerich *et al*., 1991) concurs with our findings. Homologous PP desensitization was also observed in Col-24 cells within a 20 min period, indicating that the different species PP (bPP, hPP and pPP) were activating the same population of Y_4 receptors. Cross-desensitization between rPP and bPP was not significant most probably because rPP (even at a concentration of 300 nM) was unable to stimulate the same proportion of receptors as 30 nM bPP or pPP, or 100 nM hPP. These findings contrast with those described for hY_4 receptors stably expressed in CHO cells, where no significant desensitization was observed following 24 h preincubations with 10 nM hPP (Voisin *et al*., 2000). Y_4 receptor desensitization is clearly occurring in the Col-24 line within 20 min, albeit at receptor levels that are significantly lower (no measurable specific [^{125}I]-hPP binding is observed in Col-24 membranes; Cox, unpublished observations) than those quoted by Voisin *et al*. (2000) B_{max} of 5.6 ± 0.4 pmol. 10^{-6} cells in CHO membranes.

The absence of measurable PP-like effects in epithelial layers with GR231118 (formerly known as 1229U91, Daniels *et al*. (1995) or GW1229, Bitran *et al*. (1997)) is contrary to previous studies where the dipeptide has been found to be both a Y_4 agonist and a Y_1-selective antagonist (Schober *et al*., 1998; Parker *et al*., 1998). We chose a concentration of GR231118 (1 μM) which is expected to be maximally effective at Y_4 receptors, given the consistent subnanomolar K_i values observed from binding studies (Gehlert *et al*., 1996; 1997; Parker *et al*., 1998; Dumont & Quirion, 2000). Also, the attenuation of forskolin-stimulated cyclic AMP levels afforded by GR231118 in hY_4-expressing cells, was half-maximal at 2.5 nM (Parker *et al*., 1998) and 7.0 nM (Schober *et al*., 1998) and although somewhat less potent than hPP the homodimeric peptide was a full agonist in both of these studies. We have already shown that GR231118 blocks Y_1 receptor mediated effects in different epithelial preparations, e.g. in the rat descending colon mucosa (where a pA_2 of 8.1 was obtained), in rY_1-transfected HT-29 epithelia (pIC_{50} of 7.7) and in Col-6 cells which constitutively express Y_1 receptors (pIC_{50} of 7.5; Cox *et al*., 1997). Thus in the present investigation we chose BIBP3226 as the selective Y_1 antagonist (Doods *et al*., 1995) having previously shown that

the nonpeptide attenuates Y_1-mediated inhibitory responses in Col-6 epithelia (pA_2 of 7.9; Tough & Cox, 1996). Alone BIBP3226 had no significant effect upon bPP responses in Col-24 cells while GR231118 attenuated responses by 56% and the combination of BIBP3226 and GR231118 (both at 1 μM) were similarly inhibitory, leaving SRIF responses unaffected. Whilst we cannot exclude the possibility that GR231118 is an antagonist at epithelial Y_4 receptors we favour the alternative explanation that it is acting as a weak partial agonist in Col-24 and Col-1 cells. In Col-6 cells GR231118 and BIBP3226 are able to block Y_1-mediated PYY responses but not bPP, hPP or SRIF responses. Taken together this data is consistent with the hypothesis that Col-6 cells are expressing a novel PP receptor.

RT–PCR analyses confirmed the expression of hY_4 receptors in Col-24 (and Col-1 cells plus other chosen positive controls) but the presence of a very faint 757 bp product in Col-6 cells indicates negligible levels of native Y_4 receptor expression. Future studies will seek to establish whether a novel Y_4-like receptor is indeed expressed in the latter cell line. Heterogeneity of Y_4 receptors has already been suggested in the rat brain, following the identification of differential labelling patterns with [^{125}I]-bPP and [^{125}I]-[Leu^{31}, Pro^{34}]PYY in the interpeduncular nucleus (Gehlert *et al*., 1997). Dumont & Quirion (2000) have also utilised [^{125}I]-GR231118 to label a combination of Y_1 and Y_4 receptors in rat brain membrane homogenates (and human embryonic kidney (HEK) 293 cells transfected with either rat Y_1 or Y_4 receptors). Their data showed that in the presence of an optimal concentration of Y_1 antagonist (BIBO3304) [^{125}I]-GR231118 was capable of selectively labelling Y_4 receptors with high affinity and without interference from Y_5 receptors, a distinct advantage over [^{125}I]-PP radiolabelling. These authors also found mismatches in autoradiographic studies, BIBO3304-insensitive [^{125}I]-GR231118 labelling the area prostrema and discrete layers of the hippocampal formation with only partial sensitivity for hPP. The existence of further PP-preferring Y receptor types distinct from Y_4 or Y_5 (or y_6) receptors in brain and gastrointestinal tissue now awaits molecular confirmation.

In conclusion we have described the targeting of Y_4 receptors to the basolateral domains of a human adenocarcinoma cell line (Col-24) and shown that activation of these receptors by a range of PP analogues, results in an order of potency similar to that observed for other hY_4 receptor-expressing cells. However, the different structure-activity relationships and insensitivity to GR231118 observed in Col-6 epithelia, suggests that an atypical Y_4 receptor is constitutively expressed here. Col-24 and Col-1 cells will be especially useful as simple epithelial models comparable with the more complex mucosal preparations from human colon which express Y_4, together with Y_1 and Y_2 receptor types.

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