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The functional investigation of a human adenocarcinoma cell line, stably transfected with the neuropeptide Y Y_1 receptor

[1]Nicholas D. Holliday & [1,2]Helen M. Cox

Epithelial Research Unit, The Royal College of Surgeons of England, 35-43 Lincoln's Inn Fields, London, WC2A 3PN

1 The human adenocarcinoma cell line, HT-29, has been stably transfected with the cDNA sequence for the rat neuropeptide Y (NPY) Y_1 receptor, and three Y1 clones (Y1-4, Y1-7 and Y1-16) have been isolated which express high levels of specific [^{125}I]-PYY binding. We have studied the functional responses or lack of responses to peptide YY (PYY) and its analogues in the three transfected clones and HT-29 wild type (wt) cells.

2 Vasoactive intestinal polypeptide (VIP) produced long-lasting increases in short-circuit current (SCC) in both HT-29 wt cells and the Y1 clones. VIP EC_{50} values were 8.4-11.7 nM in all four cases. The elevation in SCC after a maximal concentration of VIP (30 nM) was significantly greater in Y1-7 cells than in either HT-29 wt epithelia or the other Y1 cell lines.

3 PYY (100 nM) and human pancreatic polypeptide (hPP; 1 μM) were ineffective in HT-29 wt cells under either basal or stimulated conditions. In contrast, basolateral additions of PYY reduced both basal and VIP-stimulated SCC in all three Y1 clones. After VIP, the PYY EC_{50} values (in nM) were 18.6 in Y1-4, 8.0 in Y1-7 and 52.5 in Y1-16. hPP (1 μM) produced only small and transient responses in each transfected cell type.

4 The Y_1 receptor agonist, [Leu^{31}, Pro^{34}] NPY (1 μM) was also effective in the three Y1 cell lines. In the Y1-7 clone the EC_{50} value for the effect of this peptide was 149 nM, 18.6 fold less potent than PYY.

5 PYY and the Y_1-selective non-peptide antagonist, BIBP 3226 displaced [^{125}I]-PYY binding from Y1-7 cell membranes with K_i values of 2.0 and 3.1 nM respectively. In the Y1-7 clone, BIBP 3226 fully inhibited the reductions in VIP-stimulated SCC induced by 30 nM PYY, with an IC_{50} of 27.2 nM and 30 nM BIBP 3226 caused a parallel rightward shift on the PYY concentration-response curve, with an approximate pK_B of 8.0.

6 HT-29 clones stably expressing the Y_1 receptor therefore show responses to PYY and its analogues that are characteristic of that subtype, and the Y1-7 clone in particular will be useful in the assessment of novel Y_1-specific drugs. This approach will also allow the functional study of NPY Y_1 receptors with selected mutations.

Keywords: Neuropeptide Y; pancreatic polypeptides; Y_1 receptors; vasoactive intestinal polypeptide; epithelial chloride secretion; HT-29 cell line

Introduction

Neuropeptide Y (NPY) and its endocrine analogue, peptide YY (PYY), are potent modulators of gastrointestinal function (Cox, 1993). In addition to their inhibitory actions on blood flow and motility (Sheikh, 1991), both peptides attenuate basal and agonist-stimulated epithelial chloride secretion, for example in mucosal preparations from the rat jejunum (Cox & Cuthbert, 1988; Cox *et al.*, 1988) and distal colon (NPY only; Strabel & Diener, 1995), and inhibit fluid secretion in the human small intestine *in vivo* (Playford *et al.*, 1990). These effects in rat intestine are mediated via a direct action on epithelial receptors located on the basolateral cell surface (Cox *et al.*, 1988). Two receptor subtypes which recognise both NPY and PYY have been identified according to their relative affinities for the analogue [Leu^{31}, Pro^{34}] NPY (a specific agonist for Y_1 receptors; Fuhlendorff *et al.*, 1990) and C-terminal fragments of NPY (which preferentially activate the Y_2 subclass; Wahlestedt *et al.*, 1986). The development of Y_1-selective non-peptide antagonists, most notably BIBP 3226 (Rudolf *et al.*, 1994) has further aided the full characterization of this receptor subtype. There is so far no evidence to suggest that Y_1 and Y_2 receptors can be distinguished by their ability to activate different second messenger pathways; both act through G proteins of the $G_{i/o}$ subclass to inhibit adenylyl cyclase or to elevate intracellular calcium, dependent on the cell type in which they are expressed (Wahlestedt & Reis, 1993).

In the rat jejunum and the rat distal colon, the antisecretory activity of NPY and PYY appears to be mediated by epithelial Y_2 receptors, since NPY (13-36) is an effective agonist (Cox & Cuthbert, 1990; Strabel & Diener, 1995). However, in the human colon high concentrations of Y_1 receptor mRNA are expressed in epithelial crypt cells (Wharton *et al.*, 1993). In this study we have therefore stably transfected the human adenocarcinoma cell line HT-29, which does not show [^{125}I]-PYY binding (Mannon *et al.*, 1994), with the cDNA sequence coding the rat Y_1 receptor (Eva *et al.*, 1990). The resultant clones provide an opportunity to investigate the functional consequences of the activation of a clearly defined NPY receptor on epithelial ion transport, in the absence of other mucosal cell types. Some of this work has previously been published in abstract form (Holliday & Cox, 1995).

Methods

Cell culture and transfection

All cell lines were maintained in 25 cm^2 flasks (Falcon, Marathon Labs Inc., U.S.A.) containing Dulbecco's Modified Eagle's medium (DMEM; Gibco, Paisley U.K.; 5 ml) supplemented with glucose (25 mmol l^{-1}), foetal calf serum (10%; ICN Biomedicals Inc., California, U.S.A.), kanamycin

[1]Present address: Dept. of Pharmacology, UMDS, St. Thomas' Hospital, Lambeth Palace Road, London SE1 7EH.
[2]Author for correspondence.

(100 $\mu g\ ml^{-1}$; ICN) and amphotericin B (1.2 $\mu g\ ml^{-1}$; ICN). They were incubated at 37°C in a water-saturated atmosphere of 95% O_2/5% CO_2 and passaged when confluent by trypsinization (0.5% w/v in versene; Worthington Biochemical Corporation).

The wild type (wt) HT-29 human adenocarcinoma cell line was obtained from the Imperial Cancer Research Fund, London, U.K. The pTEJ8-FC5 expression vector (Johansen *et al.*, 1990), containing the rat Y_1 receptor cDNA sequence under the control of the human ubiquitin promoter, was kindly provided by Prof. T. Schwartz (Laboratory for Molecular Endocrinology, Copenhagen, Denmark). The construct was transfected into HT-29 wt cells by co-precipitation with calcium phosphate followed by glycerol shock (15%) for 4 min. Stably transfected clones were identified by their resistance to geneticin (1.0 mg ml^{-1}) conferred by the neomycin phosphotransferase gene contained within the vector. Three clones, Y1-4, Y1-7 and Y1-16, showed high levels of Y_1 receptor expression and were selected for further studies.

Radioligand binding

Radioligand binding studies using membrane preparations from the HT-29 wt or the Y1-7 cell lines were carried out as follows. Cell (2 x 175 cm^2 confluent flasks) were gently scraped off the plastic and homogenized in ice-cold membrane buffer (triethanolamine 10 mM; phenylmethylsulphonyl fluoride (PMSF) 0.1 mM; pH 7.6) and centrifuged at 20,000 *g* for 10 min at 4°C. After a second homogenization in membrane buffer and a further centrifugation at 20,000 *g*, the isolated membranes were resuspended in ice-cold 10 mM N-Tris-(hydroxymethyl)-methyl-2-aminoethanesulphonic acid (TES) containing 0.1 mM PMSF (pH 7.6; 2-3 ml). Protein content was determined by the method of Bradford (1976); membrane preparations typically contained 3-5 μg protein μl^{-1}. Membrane suspensions (30 μl aliquots) were immediately incubated for 2 h at 22°C in incubation buffer (composition, mM: HEPES 10, KCl 5, $NaHCO_3$ 25, $MgSO_4$ 1.2, K_3PO_4 1.2, $CaCl_2$ 2.5 and bovine serum albumin 0.5% w/v; pH 7.4) containing bacitracin (0.1 mg ml^{-1}) and [^{125}I]-PYY (16-21 pM), in the absence or presence of increasing concentrations of unlabelled PYY or BIBP 3226 (final volume 500 μl). Membranes were separated from free ligand by rapid filtration through Whatman GF/B filters presoaked in 0.3% polyethylenimine, each filter washed with 10 ml ice-cold incubation buffer (pH 7.4) and counted in a gamma counter.

Measurement of short-circuit current

Confluent HT-29 wt, Y1-4, Y1-7 or Y1-16 epithelial layers (passages 3-15) in 25 cm^2 flasks were trypsinized and seeded onto collagen-coated Millipore filters (0.2 cm^2 aperture) as described by Cuthbert *et al.* (1987). After 7-14 days, confluent epithelia on filters were mounted between two halves of perspex Ussing chambers and bathed in oxygenated (95% O_2/5% CO_2) Krebs-Henseleit solution (KHS; pH 7.4) at 37°C (12 or 15 ml in each reservoir). They were voltage-clamped at zero potential (WP Instruments, Florida, U.S.A.) and the resulting short-circuit current (SCC) was continuously recorded on pen recorders. Peptides and other agents were added to the basolateral side of the epithelial layer (unless otherwise stated in the text), and the peak changes in SCC were measured and calculated as $\mu A\ cm^{-2}$. The morphology of cells growing on filters was established by light microscopy, using 4 μm thick paraffin embedded sections of bisected filters stained with haematoxylin and eosin.

Data evaluation

Cells on individual filters that did not respond to secretory agents were not included in the data analysis. Concentration-response curves were constructed from single additions of the desired peptide to the basolateral compartment (unless otherwise stated), and EC_{50} values were calculated (including 95% confidence limits) from the pooled data using the iterative curve-fitting programme, Graphpad Inplot (version 3.01, Graphpad Software Inc, San Diego, U.S.A.). Statistical analysis of data groups was carried out with Student's unpaired *t* test, with a significance level of $P<0.05$. The pK_B value for BIBP 3226 was calculated from the Gaddum equation:

$$pK_B = \log [A'/A-1] - \log [B]$$

where A and A' are the agonist EC_{50} values in the absence and presence of antagonist concentration [B].

Binding studies were performed using quadruplicate assay samples for each concentration of unlabelled ligand. Specifically bound counts where observed comprised 40-60% of the total binding. Mean specific counts from quadruplicates were expressed as fmol [^{125}I]-PYY bound mg^{-1} membrane protein, and single displacement curves for each experiment were calculated using Graphpad Inplot. The IC_{50} values obtained were converted to binding affinities (K_i) using the Cheng-Prussof equation.

Chemicals and solutions

KHS had 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 and glucose 11.1. All non-labelled peptides were obtained from Peninsula Labs Inc. (Merseyside, U.K.), dissolved in distilled water and stored as aliquots at − 20°C until required. [^{125}I]-Peptide YY was purchased from NEN Dupont (Stevenage, U.K.). Forskolin, IBMX (3-isobutyl-1-methylxanthine), carbachol and clonidine were obtained from the Sigma Chemical Company (Poole, U.K.). BIBP 3226 ((**R**)-N^2-(diphenylacetyl)-*N*-[(4-hydroxyphenyl)methyl]-argininamide) was kindly provided by Dr H. Doods (Dr. Karl Thomae GmbH, Biberach, Germany). Piretanide was a gift from Hoechst Pharmaceuticals. Forskolin and IBMX were dissolved in 95% ethanol; other compounds were made up as aqueous stock solutions. The following abbreviations have been used in the text: porcine vasoactive intestinal polypeptide (VIP), rat/porcine peptide YY (PYY), rat/porcine peptide YY(13-36) (PYY (13-36)), porcine [Leu^{31}, Pro^{34}] neuropeptide Y ([Leu^{31}, Pro^{34}] NPY), human pancreatic polypeptide (hPP), somatostatin 14-28 (Som), forskolin (Forsk), carbachol (CCh) and clonidine (Clon).

Results

Basal parameters and responses to VIP, somatostatin 14-28 and clonidine

HT-29 wt epithelia and the three clones stably transfected with the Y_1 receptor (Y1-4, Y1-7 and Y1-16) formed multiple layers of cells when grown on collagen-coated filters. In each case, morphological indicators of polarization were rare, although occasional groups of cells adjacent to the filter exhibited a characteristic alignment of nuclei in the basolateral domain. Cysts (Marsh *et al.*, 1993) were observed in epithelial layers from all cell types, but were most pronounced in the Y1-7 clone.

The basal transepithelial resistances and levels of SCC for HT-29 wt cells are shown in Table 1. While basal resistances and SCC for Y1-4 and Y1-16 epithelia were equivalent to those measured in HT-29 wt cells, they were significantly greater in the Y1-7 clone ($P<0.001$ in both cases compared to wt). In response to VIP, SCC was rapidly stimulated in all four cell lines, reaching peak levels at 8-10 min and remaining elevated above baseline for at least 25 min after application (Figure 1). A maximal concentration of VIP (30 nM) produced a peak increase in SCC of $+17.2 \pm 2.3\ \mu A\ cm^{-2}$ ($n=28$) in HT-29 wt epithelia, which was sensitive to the addition of the loop diuretic, piretanide (200 μM). Equivalent values were ob-

Table 1 Basal resistance, basal SCC and responses to VIP in HT-29 wt and Y1 clones

Cell line	*Basal resistance* (Ω cm^2)	*Basal SCC* (μA cm^{-2})	*Peak response to 30 nM VIP* (μA cm^{-2})	*VIP EC_{50}* (nM)
HT-29 wt	23.4±1.5 (93)	4.2±0.8 (93)	+17.2±2.3 (28)	11.7 (6.2–22.4)
Y1-4	26.0±1.1 (123)	2.7±0.8 (123)	+21.4±1.6 (49)	10.7 (4.4–26.3)
Y1-7	34.3±1.0*** (288)	7.0±0.5*** (288)	+40.8±1.9*** (147)	8.4 (5.4–12.9)
Y1-16	29.4±1.3 (105)	4.3±0.7 (105)	+22.8±1.5 (50)	10.7 (3.0–38.5)

Single numbers in parentheses indicate n values. VIP EC_{50} values (with 95% confidence limits, 3 d.f.) were calculated from concentration-response relationships obtained from single basolateral additions of VIP (1–100 nM; $n=3-160$). ***$P<0.001$ compared to HT-29 wt.

tained for the Y1–4 and Y1–16 clones (Table 1); however, Y1–7 cells exhibited significantly greater secretory responses compared to wt (+40.8±1.9 μA cm^{-2}; $n=160$; $P<0.001$). Concentration-response curves constructed for VIP yielded EC_{50} values of 8.4–11.7 nM for all cell lines (Table 1).

Both somatostatin 14-28 (Som; 100 nM) and the α_2-adrenoceptor agonist clonidine (Clon; 10 μM) were effective antisecretory agents in both HT-29 wt epithelia and the three Y1 clones. In HT-29 wt cells prestimulated with VIP (100 nM), the peak response to Som was −4.6±0.9 μA cm^{-2} ($n=14$), while Clon decreased SCC by −3.1±1.8 μA cm^{-2} ($n=4$). Similar sized reductions were observed in Y1–4 and Y1–16 epithelial layers; they were larger in Y1–7 cells where decreases in SCC after Som and Clon were −21.2±5.1 μA cm^{-2} ($n=6$; $P<0.001$ compared to wt) and −6.3±1.6 μA cm^{-2} ($n=3$; $P=0.25$). EC_{50} values for the effect of Som on SCC elevated by 30 nM VIP were (in nM) 39.0 (17.9–85.0) in HT-29 wt, 23.9 (10.7–53.3) in Y1–4, 42.1 (28.2–62.7) in Y1–7 and 13.2 (9.9–17.5) in Y1–16 cells ($n=2-9$; 3 degrees of freedom (d.f.)).

Functional responses to PYY under basal and VIP-stimulated conditions

In HT-29 wt epithelia, PYY (100 nM) had no effect in unstimulated cells ($n=3$), nor did either PYY (100 nM) or hPP (1 μM) produce any response after SCC had been elevated by VIP (30 nM) ($n=6$ and $n=4$ respectively). In contrast all three Y_1-transfected cell lines responded to PYY (100 nM) with reductions in both the baseline and VIP-stimulated SCC (Figure 1). Decreases in basal levels of SCC were transient, giving peak responses of −1.3±0.4 μA cm^{-2} ($n=3$) in Y1–4, −3.6±0.9 μA cm^{-2} ($n=12$) in Y1–7 and −0.9±0.2 μA cm^{-2} ($n=7$) in Y1–16 epithelia. In Y1–7 cells the magnitude of this response was to some extent linearly correlated with the initial SCC ($r^2=0.79$). After 30 nM VIP, PYY (100 nM) decreased SCC by −2.3±0.4 μA cm^{-2} in Y1–4 ($n=8$), −5.5±0.9 μA cm^{-2} ($n=8$) in Y1–7 and −2.4±0.5 μA cm^{-2} ($n=4$) in Y1–16 cells. Concentration-response relationships obtained for PYY after stimulation with 30 nM VIP are shown in Figure 2. The calculated EC_{50} value in the Y1–16 clone (52.5 nM: 43.3–63.6, 4 d.f.) was significantly higher than those in either Y1–4 (18.6 nM; 8.3–41.7, 3 d.f.) or Y1–7 cells (8.0 nM; 6.2–10.3, 3 d.f.; $P<0.05$ for both). Higher concentrations of PYY were associated with an increase in the transient nature of the response (Figure 3). PYY at 3 nM and 10 nM produced sustained decreases in SCC which reached a maximum at 4–6 min after application of the peptide; responses to 30 nM and 100 nM PYY peaked earlier ($t_{peak}=2-3$ min) and decayed to between 30–40% of the peak reduction in SCC after 10 min.

The effect of [Leu^{31}, Pro^{34}] NPY, PYY (13-36) and hPP

The Y_1 selective agonist [Leu^{31}, Pro^{34}] NPY (1 μM) produced decreases in SCC after 30 nM VIP of −0.7±0.4 μA cm^{-2}

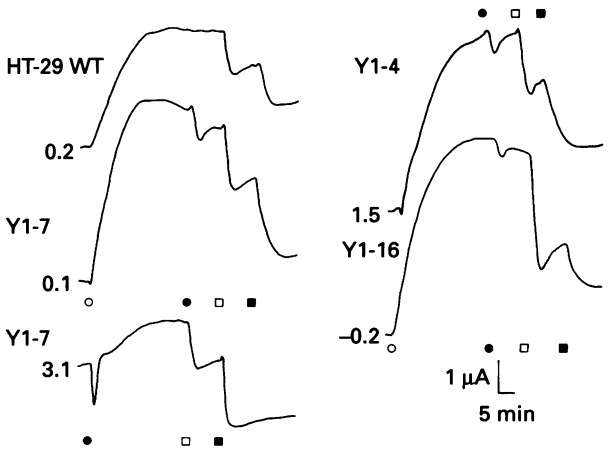

Figure 1 Representative traces from HT-29 wt and the three Y1 cell lines, showing the effects of PYY and Som on VIP-elevated and basal SCC. Agents were added at the following concentrations: VIP (30 nM; ○), PYY (100 nM; ●), Som (100 nM; □) and finally piretanide (200 μM; ■). Responses were obtained from epithelial layers covering an area of 0.2 cm^2, with initial baseline currents (in μA) indicated beside each trace.

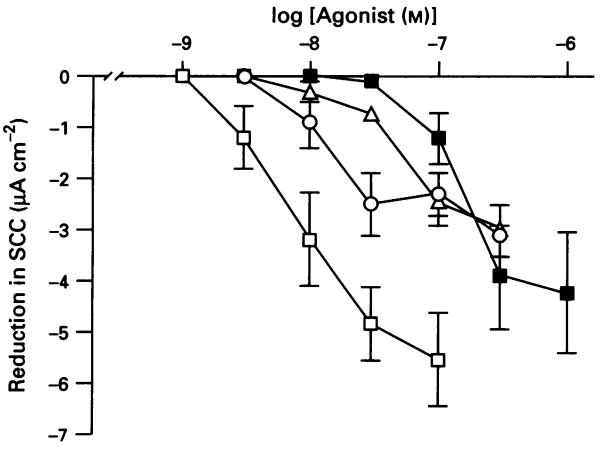

Figure 2 Concentration-response curves showing the effects of PYY in Y1–4 (○; $n=2-8$), Y1–7 (□; $n=2-13$) and Y1–16 cells (△; $n=2-4$), and of [Leu^{31}, Pro^{34}] NPY in the Y1–7 clone (■; $n=3-7$). Cells growing on each filter were stimulated with 30 nM VIP for 30 min, followed by a single application of either PYY or [Leu^{31}, Pro^{34}] NPY. Each point represents the mean ±1 s.e.mean (when $n>2$) of n observations. EC_{50} values were calculated from the pooled data using the curve fitting programme, Graphpad Inplot, and are given in the text.

($n=5$) in Y1–4, -4.2 ± 1.2 μA cm^{-2} ($n=7$) in Y1–7 and -1.6 ± 0.3 μA cm^{-2} ($n=3$) in Y1–16 cells (Figures 4 and 5). Subsequent reductions to PYY (100 nM) were significantly attenuated compared to controls in all three cell lines ($P<0.05$ in each case). The EC_{50} value for [Leu^{31}, Pro^{34}] NPY was 149 nM (111–200, 4 d.f.) in Y1–7 epithelia (Figure 2). VIP-elevated SCC was much less affected by hPP (1 μM; Figure 4), and while responses to PYY (100 nM) applied after hPP were smaller than controls, this difference was not significant. In Y1–7 cells, 1 μM PYY (13-36) reduced VIP-stimulated SCC by -0.7 ± 0.7 μA cm^{-2} ($n=3$) and responses to a further addition of PYY were unaffected (-5.8 ± 2.1 μA cm^{-2}; $n=3$).

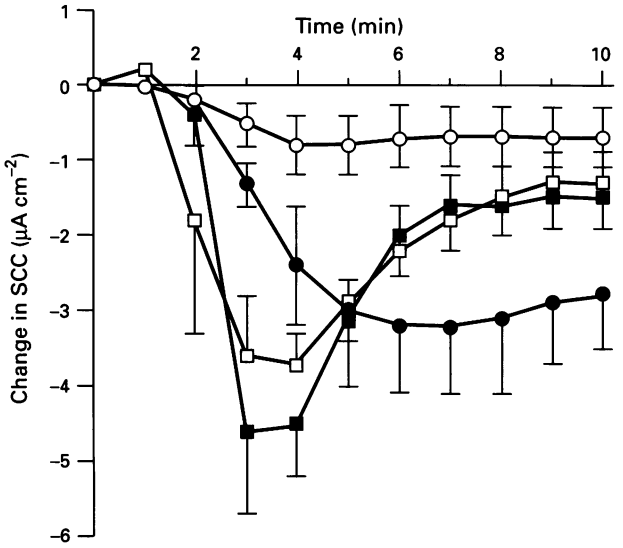

Figure 3 The time-courses of the reductions of VIP-elevated SCC induced by PYY in Y1–7 cells. After epithelial layers had been pretreated with VIP (30 nM) for 30 min, PYY was added at time zero, at one of the following concentrations: 3 nM (○; $n=3$), 10 nM (●; $n=3$), 30 nM (□; $n=7$) and 100 nM (■; $n=8$). Each point shows the mean ± 1 s.e.mean; some error bars have been removed for clarity.

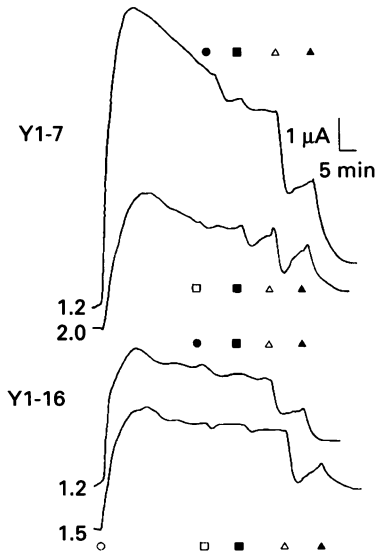

Figure 4 Example traces illustrating the responses to [Leu^{31}, Pro^{34}] NPY and to hPP in the Y1–7 and the Y1–16 clones after stimulation with VIP. Epithelial layers of area 0.2 cm^{-2} were treated sequentially with VIP (30 nM; ○), [Leu^{31}, Pro^{34}] NPY (1 μM; ●) or hPP (1 μM; □), PYY (100 nM; ■), Som (100 nM; △) and piretanide (200 μM; ▲). The initial basal currents are given (in μA) on the left of each trace.

A comparison of agonist time profiles in the Y1–7 clone is shown in Figure 6. Reductions in SCC after hPP (1 μM) were short-lived, returning to baseline after 5 min. [Leu^{31}, Pro^{34}] NPY (1 μM) responses were distinguished principally by their increased latency (3 min) compared to PYY (100 nM; 1–2 min). Time courses for these three agonists were similar in Y1–4 and Y1–16 cells, with the exception that in Y1–16 epithelia, the latency to [Leu^{31}, Pro^{34}] NPY was no higher than for PYY.

Displacement of [^{125}I]-PYY binding in HT-29 wt and Y 1-7 cells

In agreement with previous studies (Mannon *et al.*, 1994), [^{125}I]-PYY binding to HT-29 wt cell membranes was not displaced by unlabelled PYY at concentrations of up to 1 μM. However, [^{125}I]-PYY specific binding was observed in membrane preparations from the Y1–7 clone and was displaced both by unlabelled PYY and by the non-peptide Y_1-receptor antagonist, BIBP 3226 (Figure 7a). The calculated binding

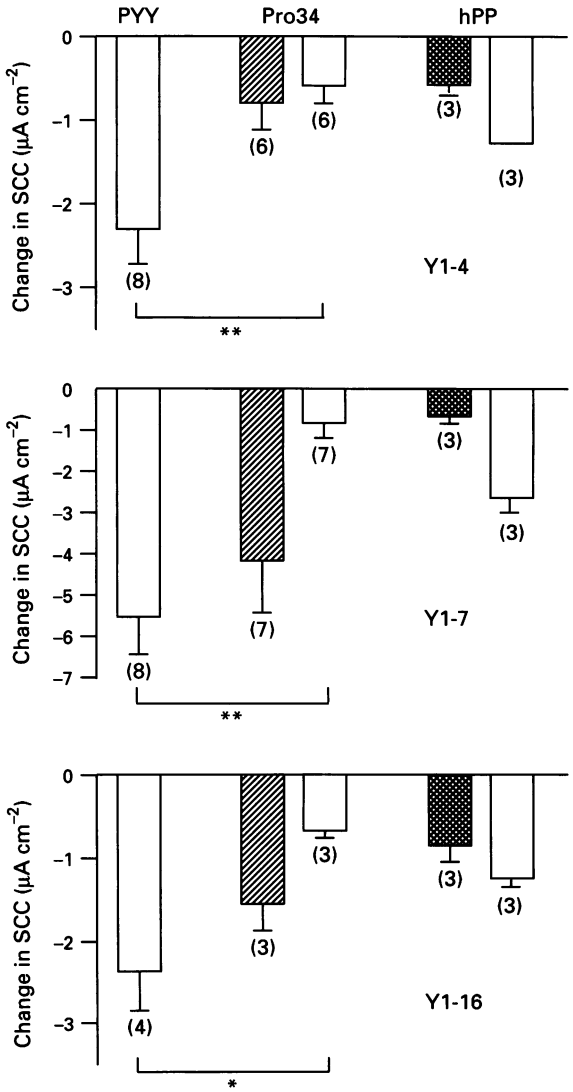

Figure 5 The mean peak reductions in SCC after addition of PYY (100 nM; open columns) or its analogues [Leu^{31}, Pro^{34}] NPY (Pro34; 1 μM; hatched columns) and hPP (1 μM; cross-hatched columns) in Y1–7 cells treated with 30 nM VIP for 30 min. The responses to 100 nM PYY applied 10 min after [Leu^{31}, Pro^{34}] NPY of hPP are shown as the righthand bars; significant differences are indicated by $^{*}P<0.05$ and $^{**}P<0.01$. The number of observations is given in parentheses in each case. Note that the scale of the Y1–7 columns is half that of the histograms showing the Y1–4 and Y1–16 responses.

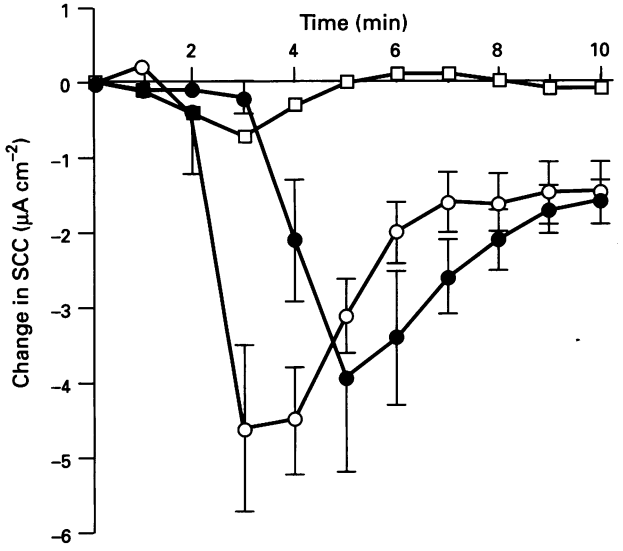

Figure 6 A comparison of the timecourses of the responses to 100 nM PYY (○; $n=8$), 1 μM [Leu^{31}, Pro^{34}] NPY (●; $n=7$) and 1 μM hPP (□; $n=3$) in Y1–7 epithelial layers prestimulated with VIP (30 nM) for 30 min. Basolateral additions of each peptide were made at time zero. Each point represents the mean ± 1 s.e.mean, with the omission of some error bars for clarity.

affinities for PYY and BIBP 3226 were 2.0 ± 1.2 nM (Hill coefficient: −0.7 ± 0.1; 3 experiments) and 3.1 ± 0.4 nM (Hill coefficient: −1.0; $n=3$) respectively.

Effect of BIBP 3226 on PYY responses in Y1–7 cells

The potency of BIBP 3226 was investigated in Y1–7 epithelia prestimulated with VIP (30 nM) for 30 min. BIBP 3226 did not itself alter SCC, but it fully inhibited the responses to PYY (30 nM) added 10 min later with an IC_{50} value of 27.2 nM (16.5–44.9, 4 d.f.; data not shown). After pretreatment of Y1–7 cells with 30 nM BIBP 3226, the concentration-response curve for the effect of PYY on VIP-elevated SCC was shifted to the right without a concomitant decrease in the maximal reduction in SCC afforded by PYY or a significant change in the Hill slope (Figure 7b). The EC_{50} value for PYY in the presence of BIBP 3226 (30 nM) was 34.2 nM (30.5–38.4, 3 d.f.), giving an approximate pK_B for BIBP 3226 of 8.0.

The effect of PYY on SCC stimulated by other secretagogues

Both forskolin (Forsk, 10 μM), a direct activator of adenylyl cyclase, and IBMX (30 μM), an inhibitor of cyclic AMP phosphodiesterase, produced rapid and sustained elevations in SCC in Y1–7 cells of +39.8 ± 4.7 μA cm^{-2} ($n=17$) and +11.1 ± 1.5 μA cm^{-2} ($n=4$) respectively. Figure 8a compares the effect of PYY (100 nM) and Som (100 nM; added after PYY) on VIP-, Forsk- or IBMX-stimulated SCC. Reductions in SCC induced by PYY or Som after a supramaximal concentration of 100 nM VIP (+39.5 ± 3.4 μA cm^{-2}; $n=20$) or after IBMX (30 μM) were similar to those after VIP (30 nM); however, PYY responses after Forsk (10 μM) were significantly attenuated. After stimulation of Y1–7 cells with a lower concentration of Forsk (1 μM), SCC was elevated by +26.3 ± 2.8 μA cm^{-2} ($n=9$) and subsequent peak responses to PYY and Som were restored to the levels observed after 30 nM VIP.

Carbachol (CCh), a muscarinic agonist, elevates SCC in epithelia through a calcium-dependent mechanism. In the Y1–7 clone, CCh (10 μM) produced a complex response consisting of a rapid spike at 30 s (+8.3 ± 1.8 μA cm^{-2}; $n=6$), a transient peak at 2 min (+13.8 ± 3.3 μA cm^{-2}) and a sustained

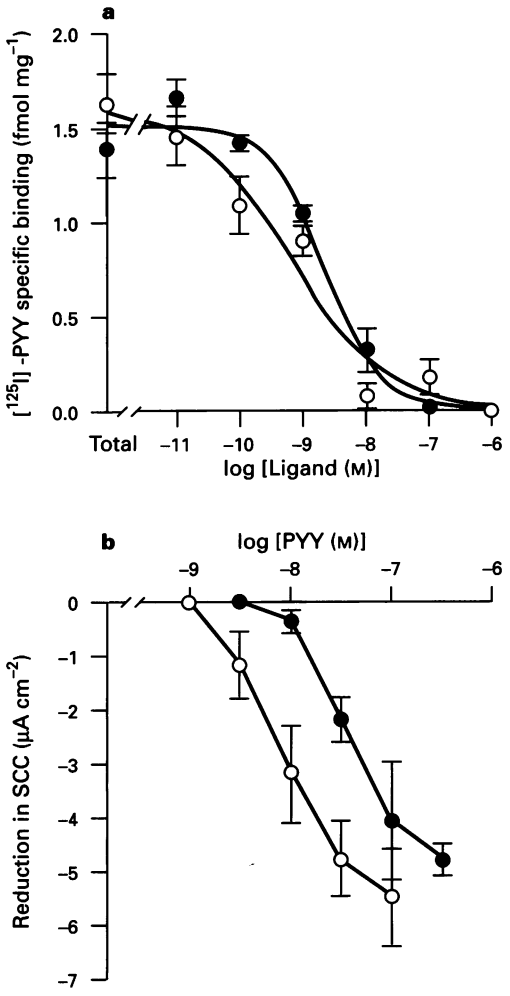

Figure 7 (a) Displacement of [^{125}I]-PYY binding by PYY and BIBP 3226 in Y1–7 membranes. Membranes were incubated for 2 h at 22°C with 19.9 pM [^{125}I]-PYY and either PYY (○) or BIBP 3226 (●; 0.01–1000 nM for each ligand). The data plotted were obtained from one representative experiment; each point shows the mean ± 1 s.e.mean of four replicate observations, in fmol [^{125}I]-PYY bound mg^{-1} membrane protein. Displacement curves were calculated using Graphpad Inplot, with respective Hill coefficients of −0.56 (PYY) and −1.0 (BIBP 3226). Mean PYY and BIBP 3226 K_i values were 2.0 ± 1.2 nM ($n=3$) and 3.1 ± 0.4 nM ($n=3$), respectively. (b) Concentration-response curves showing the effect of PYY on VIP-stimulated SCC in Y1–7 cells, in the absence (○; $n=2$–13) and presence of 30 nM BIBP 3226 (●; $n=4$–7). Cells growing on individual filters were treated with 30 nM VIP for 30 min, followed by BIBP 3226 and a single concentration of PYY at 10 min intervals. Each point represents the mean ± 1 s.e.mean; EC_{50} values were obtained from the curve-fitting programme. Graphpad Inplot, and were used to calculate the approximate pK_B value of 8.0 for BIBP 3226.

elevation in SCC (+5.6 ± 1.5 μA cm^{-2}). The first and second phases of this response were greatly potentiated by pretreatment with Forsk (1 μM) for 30 min, and were significantly attenuated by addition of Som (100 nM; −5.4 ± 2.3 μA cm^{-2}; $n=5$) 3 min beforehand ($P<0.05$ for both; Figure 8b). However prior addition of PYY (100 nM; −4.3 ± 1.2 μA cm^{-2}; $n=6$) 3 min before CCh (10 μM) did not affect these components significantly.

Sidedness of responses to VIP and PYY in Y1–7 epithelia

The responses to VIP (30 nM) and PYY (100 nM) applied to either the basolateral (bl) or apical (ap) cell surface were in-

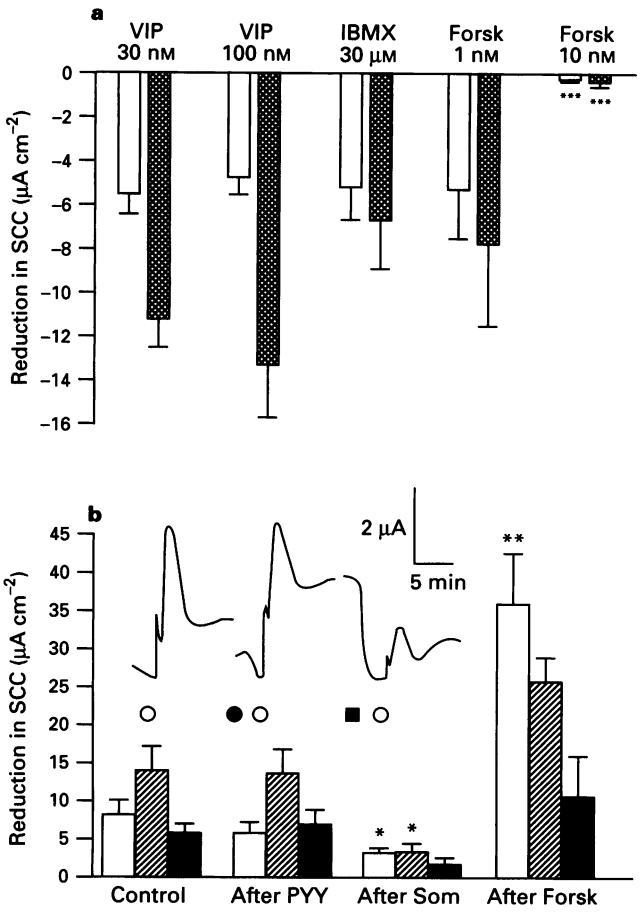

Figure 8 (a) A comparison of the peak responses to PYY (100 nM; open columns) and Som (100 nM added 10 min after PYY; cross-hatched columns) in the Y1–7 clone, after SCC had been elevated by VIP (30 nM or 100 nM; $n=8$ for both), IBMX (30 μM; $n=3$) or forskolin (Forsk; 1 μM or 10 μM; $n=6-8$). Reductions in SCC that are significantly smaller than those after VIP (30 nM) are indicated by $^{***}P<0.001$. (b) The mean increases in SCC induced by carbachol (CCh; 10 μM) in Y1–7 cells receiving no previous addition (Control; $n=6$), pretreated for 3 min with PYY (100 nM; $n=6$) or Som (100 nM; $n=5$), or pre-stimulated for 30 min with 1 μM Forsk. The three columns represent the 30 s spike (open), 2 min peak (hatched) and sustained component (solid) of the CCh response in each case. Significant differences from control values are indicated by $^{*}P<0.05$ and $^{**}P<0.01$. Example traces showing the effect of PYY (100 nM; ●) or Som (100 nM; ■) on the subsequent CCh secretory response (10 μM; ○) are given in the inset. Initial SCC readings were 2.0 μA (Control), 3.2 μA (PYY-treated) and 5.2 μA (Som-treated).

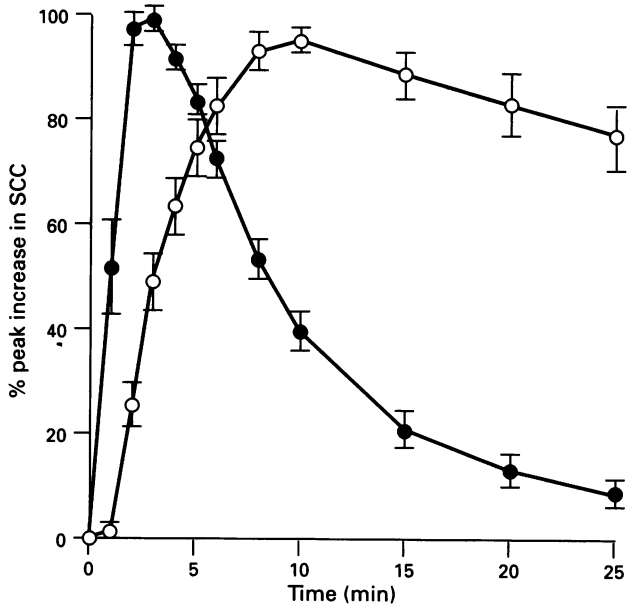

Figure 9 The time-courses of the secretory responses following addition of basolateral (○; $n=8$) or apical (●; $n=3$) VIP (30 nM) in Y1–7 epithelial layers. Points show the mean ± 1 s.e.mean. Peak absolute changes in SCC were $+40.8\pm1.9$ μA cm^{-2} ($n=160$) after VIP (bl) and $+47.6\pm2.4$ μA cm^{-2} ($n=4$) after VIP (ap).

vestigated in the Y1–7 clone. The effect of PYY on VIP-elevated SCC when it was added apically (-1.7 ± 0.3 μA cm^{-2}; $n=3$) was significantly smaller than after basolateral application (-5.5 ± 0.9 μA cm^{-2}; $n=8$; $P<0.05$). The reductions in SCC to a further addition of PYY (100 nM; bl) were unaffected (-10.3 ± 1.5 μA cm^{-2}; $n=3$; $P=0.32$); in addition piretanide (200 μM, ap) had no effect on SCC, indicating that no exchange of drugs could take place between the apical and basolateral reservoirs. Surprisingly VIP (30 nM, ap) elicited a large secretory response ($+47.6\pm2.4$ μA cm^{-2}; $n=4$; compared with an initial VIP (30 nM, bl) response of $+40.8\pm1.9$ μA cm^{-2} A; $n=160$). This increase in SCC was very rapid and transient, returning to baseline levels within 30 min (Figure 9). Subsequent responses to VIP (30 nM, bl) were of a similar size ($+31.1\pm6.2$ μA cm^{-2}; $n=3$) and profile to controls. The VIP (ap) EC_{50} value was 1.4 nM (1.1–1.7, 5 d.f.; $n=4$ for each concentration). Similar transient elevations in SCC after VIP (30 nM, ap) were also observed in HT-29 wt epithelia ($+25.9\pm6.2$ μA cm^{-2}; $n=4$).

Discussion

Stable transfection of the neuropeptide Y Y_1 receptor cDNA into the human adenocarcinoma cell line HT-29 has generated a number of clones which show high levels of specific [^{125}I]-PYY binding. Both the HT-29 wt and the Y1 cell lines are capable of functional responses to secretagogues, such as VIP, and to the antisecretory agents Som and Clon when changes in electrogenic ion transport are measured in cultured epithelial layers. However, in contrast to wt cells, PYY reduces both basal and VIP-stimulated SCC in all three Y1 clones. The Y_1-selective analogue, [Leu^{31}, Pro^{34}] NPY is also an effective antisecretory agonist. The inhibition of VIP-induced secretion, which is cyclic AMP-dependent (Turner *et al.*, 1986), indicates that PYY is likely to exert its effect through an inhibition of adenylyl cyclase; there was no evidence of an increase in cytosolic calcium after activation of the receptor, which in HT-29 cells produces a transient increase in SCC. Apical additions of PYY were associated with much smaller responses in Y1–7 cells, suggesting that, at least in this clone, the transfected receptor is targeted to the basolateral membrane.

The wt HT-29 cell line is rather undifferentiated in character, and hence has been relatively little used in electrophysiological experiments in favour of a number of subpopulations, such as colony 19A (Augeron & Laboisse, 1984; Morris *et al.*, 1994). Nevertheless, we have demonstrated that when grown on collagen-coated permeable supports, these cells can undergo some degree of polarization, since electrogenic chloride secretion in response to agonists such as VIP requires subcellular organisation into apical and basolateral domains (Binder & Sandle, 1994). A second difficulty associated with the undifferentiated phenotype is that the transfected clones might show morphological and biochemical characteristics that are not shared by the parent HT-29 cells. In the three Y1 clones studied, no substantive differences in morphology could be observed under the light microscope. The Y1–4 and Y1–16 clones were also similar to wt in measurements of basal transepithelial resistance and SCC, and in the size of their secretory responses. In contrast, Y1–7 epithelial layers exhibited significantly higher basal resistance compared to wt and larger changes in SCC after addition of either secretagogues or antisecretory agonists. These properties

indicate the existence of a greater number of tight junctions between the cells, and possibly also an increased expression of functional apical chloride channels (Morris *et al.*, 1994). Thus the Y1–7 cell line shows features characteristic of a more differentiated phenotype.

Although the transfected clones appear to differ in the extent to which they polarize, the ability to compare PYY responses across the Y1 cell line depends upon the extent to which they differ in their biochemical characteristics, for example in the expression of different receptors, effectors and their associated G-proteins. In this study, we have obtained no evidence to suggest that these differences exist. All the cell lines responded to a similar range of secretory and antisecretory agents. Indeed, the VIP EC_{50} values for the HT-29 wt (11.7 nM) and Y1 clones are remarkably similar and correlate well with those for stimulation of anion secretion in the rat jejunum (12 nM; Cox & Cuthbert, 1989) and for stimulation of adenylyl cyclase activity in HT-29 wt (2.9 nM; Turner *et al.*, 1986). The Som EC_{50} values (13–42 nM) are more variable, although with the exception of the Y1–16 clone they are not significantly different from each other. They also fall within the same range as the EC_{50} calculated in the rat colon (15 nM; Ferrar *et al.*, 1990).

Our use of the rat Y_1 receptor cDNA sequence (which is 93% identical to its human counterpart; Larhammar *et al.*, 1992) for transfection is supported both by *in situ* hybridization studies, demonstrating the presence of this receptor subtype in human colonic crypt cells (Wharton *et al.*, 1993), and by the recent identification of two colonic cell lines that are responsive to PYY. Butyrate-treated (BT) HT-29 cells endogenously express Y_1 receptors (Mannon *et al.*, 1994), whilst Colony 6 cells, a subpopulation derived from the HCA-7 adenocarcinoma cell line, appear to express both Y_1-like and PP-specific receptors (Cox & Tough, 1995; Tough & Cox, 1996).

PYY EC_{50} values for the inhibition of VIP-stimulated SCC varied between the Y1 clones, possibly due to differences in the level of receptor expression (e.g. see Kim & Milligan, 1994). Only small reductions in SCC were obtained after addition of high concentrations of hPP or the Y_2-selective agonist PYY (13-36) (in Y1–7 cells), consistent with the low affinity of these peptides for the rat Y_1 receptor (Krause *et al.*, 1992). Surprisingly, there was a greater disparity between the potencies of PYY and [Leu^{31}, Pro^{34}] NPY in the Y1–7 cell line than observed in other cell types transfected with the Y_1 receptor (binding studies; Krause *et al.*, 1992) and in HT-29 BT cells (inhibition of adenylyl cyclase activity; Mannon *et al.*, 1994). Although [Leu^{31}, Pro^{34}] NPY was a full agonist in the Y1–7 clone, the EC_{50} calculated for the inhibition of VIP-elevated SCC was 18.6 fold higher than the corresponding value for PYY. In epithelial Y receptor systems, however, PYY is often more potent than NPY, for example in small intestine (Y_2-like; Cox *et al.*, 1988; Servin *et al.*, 1989), the renal proximal tubule cell line PKSV-PCT (Y_2-like; Voisin *et al.*, 1993) and in the HCA-7 colony 6 cell line (Cox & Tough, 1995). Preliminary data including [Leu^{31}, Pro^{34}] PYY and NPY from Y1–7 cells suggests the following order of agonist potency: PYY > [Leu^{31}, Pro^{34}] PYY > NPY > [Leu^{31}, Pro^{34}] NPY, with the difference in EC_{50} values between PYY and NPY being approximately 10 fold (data not shown). Thus the Y_1 receptor transfected into HT-29 cells appears to display a PYY-preferring phenotype.

In each Y1 cell line, addition of higher concentrations of PYY was associated with an increase in the transient nature of the responses to it, and after application of [Leu^{31}, Pro^{34}] NPY, reductions of PYY were significantly attenuated. Both these features indicate that the transfected receptor is capable of desensitization when it is expressed in HT-29 cells. There is strong evidence that the initial event in the desensitization of G-protein coupled receptors is the phosphorylation of serine and/or threonine residues in the third intracellular loop and the C-terminal region of the protein (for review, see Kobilka, 1992). The phosphorylation of agonist-occupied receptors is mediated by G-protein receptor kinases (GRKs), of which the best characterized are the β-adrenoceptor kinases 1 and 2 (also known as GRK2 and GRK3; Premont *et al.*, 1995). Phosphorylation of the β_2-adrenoceptor by βARK is in itself not sufficient for desensitization, but the phosphorylated receptor is recognized by an additional protein, arrestin, which disrupts activation of G_s (Benovic *et al.*, 1987a). Despite its name, βARK has also been shown to target other adrenoceptor subtypes (Benovic *et al.*, 1987b; Freedman *et al.*, 1995), muscarinic receptors (Kwatra *et al.*, 1989) and the substance P receptor (Kwatra *et al.*, 1993). Thus individual GRKs may recognise several G-protein coupled receptors. Our observation that the Y_1 receptor appears to desensitize in an agonist-dependent manner supports this conclusion, since it implies that either wt HT-29 cells express a Y receptor-specific GRK (and arrestin-like protein) without the corresponding receptor, or more plausibly that GRKs that phosphorylate endogenous receptors are also capable of recognising the transfected Y_1 subtype.

The non-peptide Y_1 receptor antagonist, BIBP 3226, inhibited PYY responses in the Y1–7 clone with an IC_{50} of 27.2 nM. The approximate pK_B value (8.0), calculated from the parallel rightward shift of the PYY concentration-response curve in the presence of 30 nM BIBP 3226, is similar to the estimate of its binding affinity (K_i = 3.1 nM). In comparison, Rudolf *et al.* (1994) showed that BIBP 3226 bound to rat cortex with a K_i value of 7.7 nM and inhibited the NPY-induced increase in perfusion pressure in the rat kidney with an IC_{50} of 26 nM. Our results are therefore in accordance with other binding and functional studies in the rat, and confirm that BIBP 3226 is an effective antagonist at the rat Y_1 receptor.

Responses to PYY and Som were much reduced in the Y1–7 cell line when these agonists were applied after a maximal concentration of Forsk (10 μM) rather than after VIP or IBMX, although in all three cases secretion is stimulated by elevated cyclic AMP levels. To some extent, these results reflect those from *in vitro* experiments. For example in the rat jejunum, NPY inhibits Forsk-stimulated SCC, but these responses are approximately 50–60% as large as those after VIP (Cox & Cuthbert, 1988). In mucosal preparations, this discrepancy might arise from the restriction of NPY receptor expression to a smaller proportion of the cells stimulated by Forsk than those responsive to VIP. In the Y1–7 clone, however, the Y_1 receptor is ubiquitously expressed and maximal concentrations of VIP and Forsk elevate SCC to a similar extent, suggesting that an equivalent proportion of cells is involved in each case. On the other hand it is possible that after 10 μM Forsk, reductions in cyclic AMP levels do occur following PYY or Som receptor inactivation, but do not cause changes in SCC because of the saturation of downstream pathways. This may be supported by the fact that PYY and Som responses reappear after addition of a 10 fold lower (and submaximal) concentration of Forsk. However, a similar attenuation of antisecretory responses after maximal or supramaximal concentrations of VIP is not observed. At present, therefore, our results do not exclude a second possibility, namely that PYY and Som are more effective inhibitors of responses initiated through receptors than those mediated via direct activation of adenylyl cyclase.

The transient increases in SCC after addition of CCh in the Y1–7 clone resemble those observed in other adenocarcinoma cell lines (Cuthbert *et al.*, 1987) and are broadly similar to those in the rat jejunum (Cox & Cuthbert, 1988). CCh responses in Y1–7 cells were potentiated by elevated cyclic AMP levels induced by Forsk, and were substantially inhibited by pretreatment with Som; however pretreatment with PYY had no effect. In contrast, NPY significantly reduces the size of subsequent CCh and other Ca^{2+}-mediated responses in the rat jejunum (Cox & Cuthbert, 1988). This difference in potency may arise from the fact that NPY in the rat small intestine and Som in the Y1–7 clone both produce sustained decreases in basal SCC. On the other hand, the PYY response in unstimulated Y1–7 cells is transient, so that basal SCC is returned to original levels within 5–6 min of its addition. Thus

the increases in SCC after addition of CCh at the point of maximal PYY inhibition may be relatively unaffected because of its short-lived nature.

The finding that both HT-29 wt and Y1–7 cells respond to apical application of VIP was surprising, particularly as the response profile observed was markedly different from that after basolateral addition of the peptide. Conceivably, apical and basolateral responses could be mediated by distinct receptor subtypes. Although only a single population of binding sites had been identified in wt HT-29 cells (Couvineau *et al.*, 1985), VIP can be covalently cross-linked to two distinct proteins in normal human colonic epithelial membranes, in both a specific and GTP-sensitive manner (Couvineau & Laburthe, 1985). Alternatively, the same receptor might be coupled to different downstream pathways dependent on its subcellular localization, for example to different second messenger systems (Sreedharan *et al.*, 1994).

Stable expression of the rat Y_1 receptor cDNA in the human adenocarcinoma cell line HT-29 has therefore provided a number of clones which respond to PYY and its analogues in the manner expected for that receptor subtype. In particular, the Y1–7 cell line will be a valuable pharmacological tool in assessing the effectiveness of novel Y_1-specific agonists and antagonists, by virtue of its enhanced secretory and antisecretory responses. These studies also provide the basic groundwork for the analysis of mutated Y_1 receptors at both the functional and biochemical levels.

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