Peptide YY Y1 receptor activates mitogen-activated protein kinase and proliferation in gut epithelial cells via the epidermal growth factor receptor

Peter J. MANNON*†¹ and Jennifer M. MELE†

*Gastroenterology section, Box 3913, Duke University Medical Center, Durham, NC 27710, U.S.A., and †Durham VA, 508 Fulton Street, Durham, NC 27705, U.S.A.

The G-protein-coupled peptide YY (PYY)/neuropeptide Y Y1 receptor (Y1R) subtype is highly expressed in the proliferative zone of human colonic crypt epithelial cells but biochemical and biological support for growth effects have been lacking. Using a model gut epithelial cell system, we have stably expressed the human Y1R in IEC-6 cells and show that the Y1R does couple to mitogen-activated protein kinase (MAPK) phosphorylation and cell growth. This pathway uses pertussis-toxin-sensitive G-proteins and $\beta\gamma$ subunits, inhibited by co-transfected α -transducin. The Src-family tyrosine kinase inhibitor PP1, as well as specific inhibition of the epidermal growth factor receptor tyrosine kinase (EGFR TK) by PD153035, also blocks PYY

stimulation of MAPK. This pathway further requires protein kinase C with EGFR TK inhibition blocking PYY-induced protein kinase C ϵ (PKC ϵ) translocation to the cell membrane. Finally, we show that PYY stimulates growth in Y1R-expressing gut epithelial cells that is dependent on EGFR TK activity. These results demonstrate a novel pathway involving G $_i$ /G $_o$ protein, EGFR and PKC to activate MAPK. Further, they support a role for PYY and the Y1R in regulating growth in human colonic epithelium.

Key words: G-protein, phosphorylation, protein kinase C, tyrosine kinase.

INTRODUCTION

Gut epithelial cell proliferation is regulated in large part by epidermal growth factor (EGF) and related peptides and receptors. However, neither the hierarchy of growth signals in the mucosal crypt nor the mechanism by which environmental exposures such as diet modulate crypt cell proliferation is well known. We have recently shown that Y1 subtype receptors for the abundant gut hormone peptide YY (PYY) are highly expressed in crypt epithelial cells in humans [1], where they are available for paracrine activation after diet-induced PYY release from neighbouring enteroendocrine cells. Furthermore, PYY and the closely related neuropeptide Y ('NPY') have been shown to activate molecules involved in mitogenic pathways and to stimulate DNA synthesis and cell growth in a variety of cell and tissue models [2–6]. In the intestine, PYY increases gut weight and protein content [4], prevents atrophy [7] and might be involved in adaptive responses [8–10]. These results support the notion that in the human colon PYY could exert proliferative effects directly on the colonic mucosa by acting through these epithelial Y1 receptors (Y1Rs); however, a demonstration of the biochemical mechanisms underlying Y1R-mediated mitogenic effects in a relevant gut epithelial cell model has been lacking.

The Y1R is a member of the G-protein-coupled receptor (GPCR) superfamily, signalling predominantly through pertussis-toxin-sensitive G-proteins [2,11–14]. Although many examples of GPCR-activated cell proliferation exist [15–18], cell phenotype is an important determinant of the actual mechanisms underlying GPCR-stimulated mitogenic signalling cascades converging on mitogen-activated protein kinase (MAPK) [19]. For cell models of intestinal lineage, MAPK is a particularly important regulator of growth [20]. To investigate PYY-induced mitogenic effects in gut epithelium, we stably expressed the Y1R

in IEC-6 cells, a non-transformed line derived from rat jejunal crypts. Here we demonstrate that the Y1R activates MAPK in gut epithelial cells and that this pathway requires pertussis-toxin-sensitive G-proteins, $\beta\gamma$ subunits, EGF receptor (EGFR) and Src-family tyrosine kinase activity, and protein kinase C (PKC) in a novel series configuration. Finally we show that PYY stimulates cell proliferation in Y1R-expressing IEC-6 cells; this can be blocked by inhibiting EGFR kinase activity.

EXPERIMENTAL

Materials

Geneticin, D-erythro-sphingosine, tyrphostins, PD153035, LY294002, pertussis toxin, cytochalasin D and wortmannin were from Calbiochem (San Diego, CA, U.S.A.). Leupeptin, chelerythrine, cAMP, forskolin, 3-isobutyl-1-methylxanthine and PMSF were from Sigma (St Louis, MO, U.S.A.). PP1 was from Biomol (Plymouth Meeting, PA, U.S.A.). [³H]Adenine and [¹⁴C]cAMP were from New England Nuclear (Boston, MA, U.S.A.). All other chemicals were of reagent grade.

Cell culture

IEC-6 cells (A.T.C.C., Manassas, VA, U.S.A.) were transfected with cDNA encoding the human Y1R (a gift from Herbert Herzog, Garvan Medical Research Institute, Sydney, Australia) cloned into the pLNCX vector (Clontech, Palo Alto, CA, U.S.A.). Individual clones from cells surviving 500 μ g/ml geneticin were isolated by limited dilution. The IEC-6-A10 line used in these studies was grown at 37 °C under air/CO₂ (19:1) in DMEM (Dulbecco's modified Eagle's medium) supplemented

Abbreviations used: EGF, epidermal growth factor; EGFR TK, epidermal growth factor receptor tyrosine kinase; GPCR, G-protein-coupled receptor; MAPK, mitogen-activated protein kinase; PI-3K, phosphoinositide 3-kinase; PKC, protein kinase C; PYY, peptide YY; RTK, receptor tyrosine kinase; Y1R, Y1 receptor.

¹ To whom correspondence should be addressed. Present address: Laboratory of Clinical Investigation, Mucosal Immunity section, NIAID, National Institutes of Health, Building 10, Room 11N238, Bethesda, MD 20892, U.S.A. (e-mail pmannon@niaid.nih.gov).

with 5% (v/v) fetal bovine serum (Atlanta Biologicals, Waycross, GA, U.S.A.), 1.0 g/l glucose, 1% (w/v) streptomycin and penicillin G, and 500 µg/ml geneticin.

Adenylate cyclase assay

The accumulation of cAMP in IEC-6-Y1R cells was measured chromatographically by the method of Salomon [21]. Cells seeded at 2×10^5 cells per well in six-well plates were labelled with [^3H]adenine (1 µCi/ml) for 18 h before stimulation. The cells were washed with 1 ml of PBS and preincubated in 1 ml of DMEM containing 10 mM Hepes, pH 7.4, and 1 mM 3-isobutyl-1-methylxanthine, for 30 min at 37 °C. After stimulation with 100 µM forskolin for 10 min in the presence and absence of 0.1 µM PYY, the medium was replaced with 1 ml of ice-cold stop solution [0.1 nM cAMP/4 nCi/ml [^{14}C]cAMP/2.5% (w/v) HClO_4] in each well. After 30 min on ice, the solution was transferred to tubes containing 100 µl of 4.2 M KOH. Tubes were vortex-mixed and stored at -20 °C for cAMP determination by column chromatography. Results are normalized for total cellular [^{14}C]cAMP uptake and column efficiency as described [21].

Cell lysis

IEC-6-A10 cells were seeded at 2×10^5 cells per well in six-well plates and grown for 2 days, with medium exchanged for serum-free medium 24 h and again 1 h before use. After treatment the plates were placed on ice, washed twice with 2 ml of ice-cold PBS and harvested with 60 µl of ice-cold lysis buffer [0.3 M NaCl/25 mM Hepes (pH 7.7)/1.5 mM MgCl_2 /0.2 mM EDTA/0.1% (v/v) Triton X-100/0.5 mM dithiothreitol/20 mM β -glycerol-phosphate/0.1 mM Na_3VO_4 /2 µg/ml leupeptin/100 µg/ml PMSF]. The cells were scraped into chilled 1.5 ml tubes and incubated on a rotary shaker for 30 min at 4 °C; the supernatants were recovered after centrifugation at 14000 g for 10 min at 4 °C. The protein concentration of the lysates was determined by the method of Bradford [22]; the lysates were stored at -80 °C.

Western blot analysis for total and phosphorylated MAPK

Samples of cell lysate (10–20 µg per lane) were separated by SDS/PAGE [4–20% (w/v) continuous gradient gel], transferred to PVDF paper, washed with PBS for 5 min and then for 1 h with blocking buffer [PBS containing 0.1% (v/v) Tween 20 and 5% (w/v) non-fat dried milk]. Primary antibody (anti-p42 and p44 MAPK and phospho-specific p44/p42 MAPK antibodies; Santa Cruz Biotechnology and New England Biolabs) was diluted 1:1000 in PBS containing 0.05% (v/v) Tween 20 and 5% (w/v) BSA and incubated with the blot for 18 h at 4 °C. The blot was washed three times for 5 min each with blocking buffer before incubation with secondary antibody (anti-rabbit IgG, alkaline phosphatase-linked, diluted 1:1000 in blocking buffer) for 1 h at 25 °C. The blot was washed three times for 5 min each with blocking buffer and twice with wash buffer [10 mM Tris/HCl (pH 9.5)/10 mM NaCl/1 mM MgCl_2] before development with a chemiluminescent detection method (Phototope-Star; New England Biolabs) and exposure to X-ray film. Films were quantified by laser densitometry analysis performed on a Macintosh computer with the public-domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/ni-image/>). Blots were reprobed after being stripped [30 min at 70 °C in 100 mM 2-mercaptoethanol/2% (w/v) SDS/67.5 mM Tris/HCl (pH 6.7)] so that signals obtained with the phospho-

specific p44/p42 MAPK antibody could be normalized to the total p44/p42 MAPK signal in each lane.

Western blot analysis for PKC isoform translocation

IEC-6-A10 cells were grown to 80% confluence in 10 cm plates and medium was exchanged for serum-free medium 24 h and again 1 h before use. After exposure to various agents, the cells were rinsed with ice-cold PBS and each plate was scraped into 600 µl of homogenization buffer [20 mM Tris/HCl (pH 7.5)/10 mM EGTA/2 mM EDTA/250 mM sucrose/1 mM PMSF/10 µg/ml leupeptin]. The homogenate was sonicated and centrifuged for 1 h at 100000 g and 4 °C. The pellets were solubilized in homogenization buffer containing 0.5% (v/v) Triton X-100 for 30 min at 4 °C and the supernatant was recovered after centrifugation at 14000 g for 10 min at 4 °C. Protein concentrations were determined on the cytosolic and solubilized membrane supernatants; samples (4 µg) were separated by SDS/PAGE [8% (w/v) gel]. Blotting was performed as above with mouse monoclonal anti-PKC antibodies (Santa Cruz Biotechnology), Tris-buffered blocking buffer [Tris-buffered saline containing 0.05% (v/v) Tween 20 and 5% (w/v) non-fat dried milk], horseradish-peroxidase-conjugated rabbit anti-mouse IgG secondary antibodies and the chemiluminescent detection method.

Immunoprecipitation for analysis of protein tyrosine phosphorylation

IEC-6-A10 cells were seeded in 10 cm plates and used when 90% confluent. The medium was exchanged with serum-free medium 24 h and again 1 h before stimulation. Plates were washed twice with ice-cold PBS and cells from two plates were scraped into chilled 1.5 ml tubes with 400 µl of RIPA buffer [150 mM NaCl/50 mM Tris/HCl (pH 7.5)/0.25% sodium deoxycholate/0.1% (v/v) Nonidet P40/1 mM NaF/10 µg/ml leupeptin/10 µg/ml aprotinin/100 µM PMSF/100 µM sodium orthovanadate]. After incubation for 30 s in a 4 °C ultrasonicator water bath, samples were centrifuged at 14000 g for 5 min at 4 °C and protein concentrations were determined on the reserved supernatants.

Samples (500–1000 µg) were precleared with 1 µg of normal rabbit serum (Santa Cruz Biotechnology) and 20 µl of Protein A-agarose beads (0.5 ml agarose/2 ml) for 30 min at 4 °C. Precipitating antibody (5 µg/ml) was incubated with the supernatant for 4 h at 4 °C, after which 20 µl of Protein A-agarose beads was added for a further 18 h. The beads were washed three times with RIPA buffer, resuspended in 50 µl of 2% (w/v) SDS/10% (v/v) glycerol/100 mM dithiothreitol/60 mM Tris/HCl (pH 6.8)/0.001% Bromophenol Blue and heated to 100 °C for 10 min. The beads were centrifuged at 14000 g for 5 min at 4 °C; 30 µl of supernatant was separated by SDS/PAGE [8% (w/v) gel]. After transfer to nitrocellulose paper, blotting was performed as above with a mouse monoclonal anti-phosphotyrosine antibody (Santa Cruz Biotechnology).

Cell proliferation assay

IEC-6-A10 cells were seeded at 5000 cells per well in 96-well plates. After 24 h the cells were exposed to medium containing 5% (v/v) dialysed fetal bovine serum; 24 h later they were exposed to various treatments. After 5 days the cells were incubated with the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium/phenazine methosulphate ('MTS/PMS') colorimetric agents (Aqueous Nonradioactive Cell Proliferation Assay; Promega, Madison,

WI, U.S.A.) to determine viable cell mass read as D_{490} in a microtitre plate reader.

RESULTS

The Y1R-expressing IEC-6 cell line used in these studies was derived from a single cell isolated by the limiting dilution technique after selection for stable transformants. Studies were done with cells between passage 10 and 40. Scatchard analysis of equilibrium binding with ^{125}I -PYY showed a K_d of 0.2 nM and approx. 104000 receptors per cell, assuming a 1:1 stoichiometry of ligand to binding site. Consistent with Y1R activity in the butyrate-differentiated HT-29 colon cancer cell line was our observation that PYY significantly blocked forskolin-stimulated cAMP accumulation in IEC-6-Y1R cells (Figure 1) but did not significantly affect basal cellular cAMP levels. Figure 2 shows levels of phosphorylated MAPK stimulated by maximally effective doses of PYY (0.1 μM) and EGF (50 ng/ml). Low levels of phosphorylated MAPK (p42 and p44 proteins respectively) were detectable in unstimulated cells. These basal levels of phosphorylated MAPK were minimized by the incubation in serum-free medium, although the cells were not made quiescent by this treatment. Phosphorylated MAPK levels increased significantly after exposure to PYY or EGF. The time course of

Figure 1 PYY inhibits forskolin-stimulated cAMP accumulation in Y1R-expressing IEC-6 cell

Cells were incubated with 3-isobutyl-1-methylxanthine (1 mM) for 30 min before exposure to forskolin (100 μM) in the presence or the absence of PYY (0.1 μM). After 10 min the cells were harvested for the measurement of cAMP. Results are means $\pm$ S.D., $n = 6$. * $P < 0.01$.

Figure 3 PYY induces a time-dependent increase in MAPK phosphorylation in Y1R-expressing IEC-6 cells

Cells were incubated with 0.1 μM PYY for various durations before measurement of phosphoMAPK levels by quantitative densitometry of Western blots. Results are means $\pm$ S.E.M., $n = 3-6$.

MAPK phosphorylation in the IEC-6 cells after exposure to 0.1 μM PYY is shown in Figure 3. There was a response consisting of an early peak at 3 min and a plateau phase elevated above the baseline for up to 1 h. A prolonged phase of MAPK phosphorylation has been associated with the induction of a proliferative effect [23].

Role of G-protein in Y1R-MAPK signalling

Preincubation with pertussis toxin (200 ng/ml for 20 h) completely blocked the PYY stimulation of MAPK phosphorylation, indicating coupling through pertussis-toxin-sensitive G-proteins G_i or G_o , while leaving EGF stimulation unaffected. We next examined the roles of several signalling molecules known to serve as possible intermediates in the coupling of surface receptors to MAPK. To examine the role of G-protein subunits in coupling the Y1R to MAPK, the IEC-6 cells were transiently co-transfected with α -transducin to sequester free $\beta\gamma$ subunits. Figure 2 shows that α -transducin co-expression was able to attenuate PYY-induced but not EGF-induced MAPK phosphorylation, supporting a role for $\beta\gamma$ subunits' coupling the Y1R, but not the EGFR, to MAPK activation.

Figure 2 PYY-stimulated, but not EGF-stimulated, MAPK phosphorylation is blocked by pretreatment with pertussis toxin and $\beta\gamma$ subunit sequestration

Cells were incubated in the presence or the absence of pertussis toxin (PTX) (200 ng/ml) for 24 h or transient transfection with α -transducin before stimulation with PYY (0.1 μM for 3 min) or EGF (50 ng/ml for 3 min). These representative Western blots show phosphoMAPK (p44 and p42) levels in replicate samples and the results of reprobing the same blot for phosphorylation-independent MAPK (total p44 and p42).

Figure 4 PKC inhibition blocks PYY-induced MAPK phosphorylation in Y1R-expressing IEC-6 cells

Pretreatment of cells with 25 μM chelerythrine for 30 min (upper panel) or 10–20 μM sphingosine for 10 min (lower panel) significantly blocked phosphoMAPK levels after exposure to PYY (0.1 μM for 3 min).

Role of PKC in Y1R–MAPK signalling

A role for PKC in the Y1R–MAPK pathway was evaluated by first using specific and structurally distinct inhibitors of PKC to disrupt PYY effects. Pretreatment with chelerythrine (25 μM for 30 min) or sphingosine (10–20 μM for 10 min) significantly blocked the effects of PYY on MAPK phosphorylation (Figure 4). The half-maximal effective inhibitory concentration for sphingosine was approx. 10 μM , consistent with a specific inhibitory effect on PKC. To confirm PKC activation by the Y1R and to identify the stimulation of a specific PKC isoform, we used a translocation assay to measure the disappearance of immunoreactive PKC from the cytosolic compartment to the cell membrane after exposure to PYY or EGF (Figure 5, top panel). Figure 5 (middle panel) shows the loss of PKC ϵ from the cytosol after exposure to 0.1 μM PYY, returning to pre-stimulation levels within 10 min of continued exposure. The peak of PKC activation by PYY occurred within 30 s, in advance of peak PYY-induced MAPK phosphorylation and consistent with a role for PKC located between the Y1R and MAPK. PYY-induced PKC ϵ translocation was blocked by preincubation of cells with the EGFR tyrosine kinase (TK) inhibitor PD153035, the Src-like kinase inhibitor PP1 and the PKC inhibitor sphingosine (Figure 5, bottom panel).

Role for tyrosine kinase activity in the Y1R–MAPK pathway

The effects of blocking tyrosine kinase activity on PYY-induced MAPK phosphorylation were studied by treating the cells with various kinase inhibitors; preincubation with tyrphostin A25 (20–100 μM for 20 h) inhibited PYY effects by 40% but the inactive isomer A1 and tyrphostin B42 had no effect. However, pretreating cells with the EGFR TK-specific tyrphostin AG1478

Figure 5 PYY activates PKC in Y1R-expressing IEC-6 cells

Top panel: Western blots showing the levels of PKC ϵ in the cytosolic and cell-membrane compartments of IEC-6-Y1R cells before (Control) and after exposure to PYY (0.1 μM) or EGF (50 ng/ml) for 30 s at 37 $^{\circ}\text{C}$. Each lane contained 5 μg of solubilized protein. Middle panel: representative Western blot showing the time course of PKC ϵ translocation out of the cytosolic compartment after exposure to 0.1 μM PYY. Each lane contained 4 μg of solubilized protein. Bottom panel: densitometric analysis of Western blots measuring PYY-stimulated PKC ϵ translocation out of the cytosol in the presence and the absence of kinase inhibitors. Cells were exposed to 0.1 μM PYY for 30 s with or without preincubation in PD153035 (25 nM for 30 min), PP1 (10 μM for 15 min) and sphingosine (20 μM for 10 min). Results are means $\pm$ S.D., $n = 3$. * $P < 0.05$.

showed near complete inhibition while use of the PDGFR-specific tyrosine kinase inhibitor tyrphostin AG1295 failed to show any decrease in PYY-induced MAPK phosphorylation (Figure 6, upper panel). Inhibition of non-receptor tyrosine kinase activity by pretreatment with PP1 (5 μM for 15 min) also significantly blocked PYY-induced MAPK phosphorylation, which supports a role for Src-like kinases in the Y1R–MAPK pathway. PP1 had similar inhibitory effects on EGF-stimulated MAPK phosphorylation, suggesting a common role distal to both receptors (Figure 6, lower panel). The use of a second EGFR TK inhibitor, PD153035, also blocked PYY-induced MAPK phosphorylation with a half-maximal inhibitory concentration of between 10 and 50 nM, a dose range consistent with specific inhibition when added extracellularly [24] (Figure 7). In contrast, inhibiting focal adhesion kinase activity by disrupting the cytoskeleton scaffold with cytochalasin D (10 μM for 30 min) did not inhibit effects of PYY, suggesting no role for this mechanism. The phosphoinositide 3-kinase (PI-3K) inhibitors wortmannin (100 nM for 30 min) and LY294002

Figure 6 Effects of inhibition of tyrosine kinase activity on PYY-induced MAPK phosphorylation in Y1R-expressing IEC-6 cells

Upper panel: densitometric analysis of Western blots measuring the effect of various kinase inhibitors on PYY-induced MAPK phosphorylation. Cells were preincubated with various inhibitors before exposure to PYY (0.1 μ M for 3 min). Abbreviations and conditions: A1, tyrphostin A1 (100 μ M, 20 h); A25, tyrphostin A25 (100 μ M, 20 h); B42, tyrphostin B42 (100 μ M, 20 h); AG1295 (20 μ M, 30 min); AG1478 (20 μ M, 30 min); Cyto D, cytochalasin D (10 μ M, 30 min); PP1 (10 μ M, 15 min). Results are means $\pm$ S.D., $n = 2$ or 3. * $P < 0.05$ compared with control. Lower panel: representative Western blots measuring phosphoMAPK levels in cells stimulated with PYY or EGF in the presence and the absence of PP1.

Figure 7 Inhibition of the EGFR TK blocks PYY-induced MAPK phosphorylation in Y1R-expressing IEC-6 cells

Densitometric analysis of Western blots showing the inhibition of PYY-induced phosphoMAPK levels in the presence of increasing amounts of EGFR TK inhibitor PD153035. Results are means $\pm$ S.D., $n = 3$. * $P < 0.05$ compared with PYY alone.

Figure 8 PYY and EGF increase tyrosine phosphorylation of the EGFR

Cells incubated for the indicated durations in the presence or the absence of PYY or EGF were solubilized; the EGFR was immunoprecipitated for measurement of phosphorylated tyrosine content. Abbreviation: Cont, control.

Figure 9 PYY- and EGF-stimulated proliferation in Y1R-expressing IEC-6 cells

Cells were grown in medium containing 5% (v/v) dialysed fetal bovine serum in the absence and the presence of PYY (0.1 μ M) or EGF (50 ng/ml) (see the Experimental section). The change in cell number was determined by a colorimetric MTS/PMS assay; the number of viable cells per well was measured as D_{490} (OD 490 nm). Results are means $\pm$ S.D., $n = 8$. * $P < 0.05$ compared with basal.

(100 μ M for 30 min) also had no inhibitory effects on PYY-induced MAPK phosphorylation (results not shown). To examine whether PYY could transactivate the EGFR, we measured the phosphotyrosine content of the EGF receptor protein after exposure to PYY and EGF. Both peptides caused an increase in phosphorylation of the EGFR, with a substantially higher signal after stimulation with EGF (Figure 8).

Stimulation of IEC-6-A10 cell proliferation by PYY

Lastly, we compared the proliferative response of IEC-6-Y1R cells to both EGF and PYY. Cells were seeded in wells at subconfluence and incubated with medium containing 5% (v/v) dialysed fetal bovine serum to control the quantity of exogenous growth factors added during the study. After 48 h of incubation to achieve quiescence, the cells were exposed for a further 3 days to medium alone, to EGF (50 ng/ml) or to PYY (0.1 μ M). Both peptides induced equivalent significant increases in viable cell numbers reflected by the amounts of chromogenic metabolite produced in each well and measured as the change in D_{490} (Figure 9). The proliferative effects of both PYY and EGF were abolished by co-incubation with AG1478.

DISCUSSION

Our results support a role for PYY as a growth factor or growth promoter in gut epithelium by demonstrating that PYY can activate molecules known to be involved in mitogenic pathways

and can stimulate cell proliferation via the Y1R. PYY-induced MAPK phosphorylation is entirely dependent on several signalling molecules, including the $\beta\gamma$ subunits of pertussis-toxin-sensitive G-proteins, PKC and the tyrosine kinase activity of the EGF receptor and non-receptor Src family. Each of these components is required to complete a signalling pathway in series that couples the Y1R to MAPK. A novel aspect of this signalling pathway is the fact that PKC has such a prominent role in coupling a G_i -associated GPCR to MAPK, especially when the PKC activation is dependent on receptor tyrosine kinase (RTK) and Src-like kinase activity.

GPCRs offer alternative mechanisms to classical RTK-stimulated mitogenesis (such as EGF, fibroblast growth factor and transforming growth factor α) in response to the binding of cell-surface peptide ligand. Pertussis-toxin-sensitive G-proteins are involved in stimulating cell proliferation [15–18], with the general pattern of the signalling pathway involving the $\beta\gamma$ subunit (activating a receptor and/or non-receptor tyrosine kinase to initiate Ras activation via the Shc–Grb2–Sos complex [25,26]) or possibly the α subunit (to stimulate PKC for the activation of Raf [27]). The $\beta\gamma$ subunits clearly mediate Y1R-stimulated MAPK activation in our model of the gut epithelial cell. Established mechanisms for $\beta\gamma$ subunit involvement in MAPK activation pathways include the stimulation of PI-3K [28], the activation of Ca^{2+} -dependent (via phospholipase C activity) and PKC-dependent non-receptor tyrosine kinases such as Pyk2 [29,30] and coupling to Src or Src-like kinases. Theoretically, PI-3K could have a role upstream of the EGFR in our Y1R–MAPK, activated by $\beta\gamma$ subunits as a link to Src activation [31], or downstream of the EGFR with the non-enzymic adaptor p85 subunit of PI-3K interacting with the activated EGFR [32]; however, our results show that there is no effect of PI-3K inhibition on the Y1R–MAPK pathway in IEC-6 cells. Similarly, $\beta\gamma$ subunits acting through Pyk2 to stimulate MAPK in an EGFR-dependent [30] or EGFR-independent [19] manner is also an unlikely mechanism in our cell model, given the inability of cytoskeleton disruption by cytochalasin D to inhibit the Y1R-mediated MAPK phosphorylation. Finally, a possible role for $\beta\gamma$ -subunit-activated Src is supported by the blockade of the Y1R–MAPK pathway by the Src family tyrosine kinase inhibitor PP1; however, our results suggest that Src kinase activity is located distal to the EGFR TK and ‘upstream’ of PKC activity. In other GPCR-stimulated MAPK pathways involving RTK transactivation, Src activity can have a role either upstream [25] or downstream of the RTK [19]. The $\beta\gamma$ subunits could activate other tyrosine kinases such as the non-receptor Src-family kinases, which could in turn phosphorylate the EGFR or adaptor proteins such as Shc directly, thereby activating a cascade that converges on MAPK; however, because Src inhibition equally blocks PYY- or EGF-stimulated MAPK in our model, a more likely position for Src activity is distal to the EGFR. The present results cannot resolve whether Src has a role both upstream and downstream of the EGFR but this issue of Src’s coupling the Y1R to EGFR transactivation in our cell model will be explored in future studies.

Although a role for PKC activity has been recognized in mitogenic responses, the mechanism of PKC involvement in pathways involving GPCR transactivation of the EGFR for MAPK stimulation has not been clearly defined. A study of GPCR–MAPK pathways shows that PKC activation is typically associated with signals mediated by the pertussis-toxin-insensitive G_q protein [33], although at least one report demonstrates PKC activation via G_o protein [27]. PKC activity has been shown to mediate carbachol-induced EGFR phosphorylation via the M1 muscarinic receptor [34] and P_{2Y2} purinoceptor [30], to block

angiotensin II-induced EGFR phosphorylation [35] or to act in parallel with EGFR to mediate bradykinin-stimulated MAPK activation while acting neither upstream nor downstream of EGFR [36]. In contrast with these reports, our results show that PKC activity is required for MAPK stimulation in a pathway position distal to EGFR TK. One possible mechanism for this EGFR-induced PKC activation is the stimulation of phospholipase $C\gamma$, producing diacylglycerol to activate PKC, which is consistent with the observed PYY-induced translocation of the phorbol-responsive isoform PKC ϵ . PKC in turn can couple to MAPK activation by phosphorylating Raf-1 [36–38], seemingly independently of Ras. However, newer evidence suggests that Ras can have an essential role in PKC-mediated MAPK activation [39] rather than being bypassed for direct Raf stimulation, and we have shown that both PKC and Ras are necessary for MAPK activation in Y1R-transfected Chinese hamster ovary cells [2]. It is not unexpected that PKC should be involved in a mitogenic pathway in IEC-6 cells because PKC has been seen to regulate gut epithelial proliferation, promoting as well as negatively modulating growth. For instance, PKC ϵ overexpression can be tumorigenic for non-transformed colonic epithelial cells [40], whereas the expression of other isoforms such as PKC α is associated with cell cycle arrest [41] or the isoforms have their expression restricted to terminally differentiated, non-proliferating cells [42,43].

GPCRs can transactivate the EGFR by a variety of mechanisms, nearly all involving kinase activity. In addition to the pathways mentioned above, two potential mechanisms include a phosphorylated GPCR– β -arrestin complex activating Src for EGFR stimulation [44] and another mechanism showing that the proteolytic release of an EGFR ligand can be stimulated by GPCR [45], although the intracellular effector molecules have not been identified. Despite the essential role of EGFR TK in the Y1R–MAPK pathway in IEC-6 cells, the results show that there is little tyrosine phosphorylation of the EGFR by PYY, particularly in comparison with EGF stimulation. The differential phosphorylation of the EGFR by PYY and EGF might be related to EGFR activation by autophosphorylation versus transphosphorylation by non-receptor tyrosine kinases, for instance. For the most part, transactivation of the EGFR by a GPCR results in less receptor phosphorylation; at least one report demonstrates that the pattern of phosphorylation might be different [25]. In our model, $\beta\gamma$ subunits are early effectors in the Y1R–MAPK pathway, probably acting upstream of the EGFR because EGFR-stimulated MAPK phosphorylation is unaffected by $\beta\gamma$ -subunit sequestration.

In summary, these results support a role for PYY in the regulation of proliferation in gut epithelial cells with implications for the human colon, where Y1Rs and PYY ligand can be found in relative abundance. We have shown that PYY can exert as much of a proliferative effect as maximal doses of EGF even in a cell line that is known for its limited proliferative response to standard peptide growth factors [46]. The pathway in IEC-6 cells from the Y1R to MAPK activation involves a PKC isoform that has been characterized for its proliferative effect in gut epithelial cells and requires the tyrosine kinase activity of the EGF receptor, itself considered a pre-eminent regulator of intestinal epithelial growth. Evidence suggests that intestinal epithelial cells have a tissue-specific biology that might make their growth more sensitive to MAPK stimulation [20], less susceptible to transformation by Raf versus Ras than fibroblasts [47], and probably have characteristic properties that determine the coupling of GPCRs to MAPK and RTKs [19]. Future studies will need to address the following: (1) how Y1R transactivation of the EGFR affects its autophosphorylation and specific signalling

events that are coupled to its phosphorylation state, (2) whether Y1R second messenger coupling changes with the differentiation state of the epithelial cell, (3) how dietary exposure that modulates PYY release can affect EGFR expression and activity in the gut, and (4) to define more precisely the physiological role of PYY in normal crypt cell proliferation and maturation. An ultimate aim is to test how PYY and interactions with its gut epithelial receptor might be a biological link between the epidemiological association of colon cancer risk and the same dietary factors that release the highest levels of PYY, namely high-calorie and high-fat meals.

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