

Alpha₂ adrenoceptors regulate proliferation of human intestinal epithelial cells

S Schaak, D Cussac, C Cayla, J C Devedjian, R Guyot, H Paris, C Denis

Abstract

Background and aims—Previous studies on rodents have suggested that catecholamines stimulate proliferation of the intestinal epithelium through activation of α_2 adrenoceptors located on crypt cells. The occurrence of this effect awaits demonstration in humans and the molecular mechanisms involved have not yet been elucidated. Here, we examined the effect of α_2 agonists on a clone of Caco2 cells expressing the human α_{2A} adrenoceptor.

Methods—Cells were transfected with a bicistronic plasmid containing the $\alpha 2C10$ and neomycin phosphotransferase genes. G418 resistant clones were assayed for receptor expression using radioligand binding. Receptor functionality was assessed by testing its ability to couple Gi proteins and to inhibit cAMP production. Mitogen activated protein kinase (MAPK) phosphorylation was followed by western blot, and cell proliferation was estimated by measuring protein and DNA content.

Results—Permanent transfection of Caco2 cells allowed us to obtain a clone (Caco2-3B) expressing α_{2A} adrenoceptors at a density similar to that found in normal human intestinal epithelium. Caco2-3B retained morphological features and brush border enzyme expression characteristic of enterocytic differentiation. The receptor was coupled to Gi2/Gi3 proteins and its stimulation caused marked diminution of forskolin induced cAMP production. Treatment of Caco2-3B with UK14304 (α_2 agonist) induced a rapid increase in the phosphorylation state of MAPK, extracellular regulated protein kinase 1 (Erk1), and 2 (Erk2). This event was totally abolished in pertussis toxin treated cells and in the presence of kinase inhibitors (genistein or PD98059). It was unaffected by protein kinase C downregulation but correlated with a transient increase in Shc tyrosine phosphorylation. Finally, sustained exposure of Caco2-3B to UK14304 resulted in modest but significant acceleration of cell proliferation. None of these effects was observed in the parental cell line Caco2.

Conclusion—The results obtained in the present study support a regulatory role for α_2 adrenoceptors in intestinal cell proliferation.

(Gut 2000;47:242–250)

Keywords: α_2 adrenoceptor; Caco2; mitogen activated protein kinase; intestinal cell proliferation

The gut mucosa is extensively innervated by noradrenergic neurones, and catecholamines modulate several key functions of the intestinal barrier.^{1,2} Among them, modification of electrolyte movements is undoubtedly the best documented effect. Noradrenaline promotes Na⁺, Cl⁻, and water absorption in both the small intestine and colon.³ In addition, the transepithelial short circuit current is decreased and net HCO₃⁻ secretion is abolished. As shown by the use of selective adrenergic drugs, this antisecretory action is primarily mediated by postsynaptic α_2 adrenoceptors located on epithelial cells.^{4,5} In agreement with this, α_2 adrenoceptors of the α_{2A} subtype were identified on enterocytes and colonocytes isolated from different species, including humans.^{6–9} The precise mechanisms whereby α_2 agonists stimulate net absorption of Na⁺ and Cl⁻ are still debated.¹⁰ Studies on human colonocytes,^{9–11} rat enterocytes,¹² and the adenocarcinoma cell line HT29¹³ have shown that α_2 adrenoceptors are coupled to Gi2/Gi3 proteins and that stimulation inhibits accumulation of intracellular cAMP induced by vasoactive intestinal peptide or forskolin. Consequently, α_2 mediated attenuation of Cl⁻ secretion may, at least partially, result from a direct decrease in the activity of the cystic fibrosis transmembrane conductance regulator. Other pathways independent of cAMP were also proposed. They may include interference with Ca²⁺ dependent mechanisms and/or direct coupling of G proteins to ion channels.^{14,15}

Aside from effects on transepithelial ion transport, several in vivo studies suggest that α_2 adrenoceptors are also responsible for stimulation of proliferation of intestinal crypt cells by catecholamines.¹⁶ So far, the mitogenic effects of α_2 agonists have been observed only in the jejunum and colon of rats and mice. Moreover, the molecular mechanisms whereby they occur

Abbreviations used in this paper: DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate buffered saline; Erk, extracellular regulated protein kinase; FCS, fetal calf serum; MAPK, mitogen activated protein kinase; pCMV, cytomegalovirus early promoter/enhancer; EMCV, encephalomyocarditis virus; PMA, phorbol 12-myristate 13-acetate; PD98059, 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one; RX821002, 2-(2-methoxy-1,4-benzodioxan-2-yl)-2-imidazole; UK14304, 5-bromo-6-(2-imidazole-2-ylamino)-quinoxaline; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PKC, protein kinase C; BSA, bovine serum albumin; RPA, RNase protection assays; DTT, dithiothreitol; RIPA, radio immunoprecipitation; PVDF, polyvinylidene difluoride.

Institut National de la Santé et de la Recherche Médicale, Unité INSERM388, Institut Louis Bugnard, CHU Rangueil, 31403 Toulouse Cedex 4, France
S Schaak
D Cussac
C Cayla
J C Devedjian
R Guyot
H Paris
C Denis

Correspondence to:
Dr C Denis, INSERM U388, Institut Louis Bugnard, CHU Rangueil, Bat. L3, 31403 Toulouse Cedex 4, France. Email: denis@rangueil.inserm.fr

Accepted for publication 8 February 2000

also remain to be elucidated. Putative involvement of α_2 adrenoceptors in the regulation of intestinal proliferative activity in other species however matches several recent findings. Firstly, in rat,⁷ rabbit,⁸ and human⁹ intestinal mucosa, α_2 adrenoceptors are preferentially expressed in crypts where cell division occurs and where most noradrenergic nerve endings project. Secondly, α_2 adrenergic agonists were found to act as co-mitogens and to activate the mitogen activated protein kinase (MAPK) pathway in cells transfected with the gene encoding the human α_{2A} adrenoceptor.¹⁷⁻²⁰ This observation, which was originally restricted to cellular models of fibroblastic origin, was recently extended to epithelial cells. In particular, α_2 adrenoceptor stimulation was reported to increase MAPK activity and DNA synthesis in the OK cell line, derived from the proximal tubule of opossum kidney,²¹ suggesting that such an effect could also take place in epithelial cells of intestinal origin.

The aim of the present study was to examine the putative growth regulating properties of the α_{2A} adrenoceptor in human intestinal cells. To this end, we developed a clone derived from the enterocyte-like differentiated cell line Caco2 that permanently expresses α_{2A} adrenoceptors at a density similar to that noted in normal human intestinal crypt cells. The α_2 adrenoceptor was functionally coupled to Gi2 and/or Gi3 in this model. Stimulation caused activation of MAPK and increased cell growth. Further use of this model may also be helpful for investigating the molecular mechanisms responsible for alteration of transepithelial transport by α_2 agonists.

Materials and methods

DRUGS AND REAGENTS

[³H]2-(2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline (RX821002) (59 Ci/mmol) and [³H]clonidine (66 Ci/mmol) were purchased from Amersham Pharmacia Biotech (Courtaboeuf, France), [³²P]NAD⁺ (800 Ci/mmol) from New England Nuclear (Boston, Massachusetts, USA) and [³²P]UTP (800 Ci/mmol) from ICN (Costa Mesa, California, USA). Phentolamine was donated by Ciba Geigy (Basel, Switzerland), and prazosin hydrochloride and 5-bromo-6-(2-imidazoline-2-ylamino)-quinoxaline (UK14304) tartrate by Pfizer (Sandwich, Kent, UK). Oxymetazoline, idazoxan, chlorpromazine, forskolin, pertussis toxin, GppNHp, phorbol 12-myristate 13-acetate (PMA), and all other chemicals were from Sigma (St Louis, Missouri, USA). Fetal calf serum (FCS) and G418 sulphate were purchased from Gibco BRL (Cergy Pontoise, France). 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059) and genistein were obtained from Calbiochem (La Jolla, California, USA). Radioimmunoassay kits for cAMP determination were from Immunotech (Luminy, France). The human α_{2A} adrenoceptor gene ($\alpha 2C10$) was kindly provided by Dr R J Lefkowitz (Duke University, Durham, North Carolina, USA).

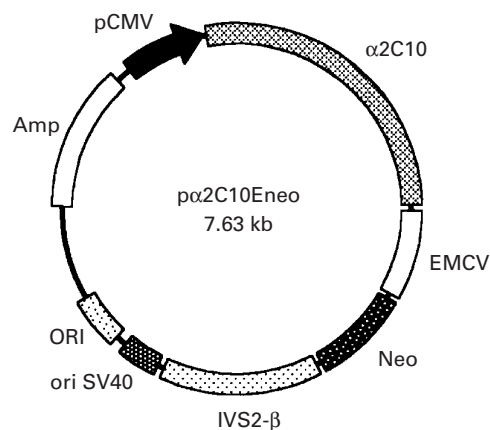


Figure 1 Schematic map of the bicistronic expression vector used for transfection of Caco2 cells. The p $\alpha 2C10Eneo$ vector is 7.63 kb in size. It contains an expression cassette comprising the human cytomegalovirus early promoter/enhancer (pCMV), the gene encoding human α_{2A} adrenoceptor subtype ($\alpha 2C10$), the internal ribosomal entry site derived from the encephalomyocarditis virus (EMCV), the neomycin phosphotransferase gene (Neo), a rabbit β globin genomic fragment containing sequences for mRNA stabilisation and polyadenylation (IVS2- β).

EXPRESSION VECTOR

The expression vector used to transfect Caco2 cells (p $\alpha 2C10Eneo$) was a bicistronic plasmid obtained as follows. The HPalph2GEN construct,²² which contains the BamHI-BamHI fragment (5.5 kb) of the $\alpha 2C10$ gene, was digested with KpnI and HindIII restriction enzymes. The KpnI-HindIII fragment corresponding to nucleotides -1280/+1526 relative to the translation start was subcloned into pKS+ (pBluescriptII KS+, Stratagene, La Jolla, California, USA). This construct was then digested by NheI at position -201 of the $\alpha 2C10$ sequence and NotI in the pKS+ polylinker. The purified insert was cloned into the XbaI and NotI sites of the bicistronic vector, pEN.²³ The resulting p $\alpha 2C10Eneo$ vector (fig 1) contains an expression cassette comprising the human cytomegalovirus early promoter/enhancer (pCMV), the entire ORF encoding for α_{2A} adrenoceptor ($\alpha 2C10$), an internal ribosome entry site derived from encephalomyocarditis virus (EMCV), the neomycin phosphotransferase gene, and a rabbit β globin genomic sequence containing an intron and a polyadenylation signal (IVS2- β).

CELL CULTURE AND TRANSFECTION

The human colon adenocarcinoma cell line, Caco2, was routinely subcultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and non-essential amino acids. Cells were transfected using the calcium-phosphate method. Two days after transfection, they were subcultured in the presence of G418 sulphate (1 mg/ml), and antibiotic resistant clones were collected individually using cloning cylinders.

ENTEROCYtic DIFFERENTIATION AND HYDROLASE ACTIVITIES

Immunofluorescence studies were performed on post-confluent cells cultured on Costar Transwell filters (Dutscher, Brumath, France).

Cell monolayers were fixed with 3.7% paraformaldehyde-30 mM sucrose in phosphate buffered saline (PBS) for 15 minutes and treated for 10 minutes with 50 mM NH_4Cl in PBS to reduce non-specific background. The cells were permeated with 0.05% saponin in PBS containing 1% skimmed milk and all subsequent steps were performed in the same buffer. The filters were cut out and incubated for one hour with phalloidin-FITC conjugate. After extensive washing, samples were mounted with Mowiol and viewed under a confocal laser microscope (Zeiss LSM). Brush border associated enzyme activities were measured on whole cell homogenates. Alkaline phosphatase and dipeptidyl peptidase IV were assayed using *p*-nitrophenyl phosphate and glycyl-L-proline-4-nitroanilide, respectively, as substrates.^{24, 25} Values are expressed as milliunits per mg of protein, one unit being the activity that hydrolyses 1 μmol of substrate per minute at 37°C.

RNA EXTRACTION AND RPA

Total cellular RNAs were isolated using the guanidine isothiocyanate/phenol chloroform extraction method.²⁶ Synthesis of the $\alpha 2\text{C}10$ antisense has been described previously.²⁷ RNase protection assays (RPA) were performed as follows: 100 μg of RNA were added to 30 μl of hybridisation buffer (80% deionised formamide, 0.4 M NaCl, 1 mM EDTA, 40 mM Pipes, pH 6.7) containing an excess of [³²P] labelled riboprobe. Samples were heated to 95°C for five minutes and then immediately kept at 55°C for 14 hours. Non-hybridised probe was eliminated by addition of 0.3 ml of RNase A (40 $\mu\text{g}/\text{ml}$) and RNase T1 (2 $\mu\text{g}/\text{ml}$) in 300 mM NaCl, 5 mM EDTA, and 10 mM Tris HCl (pH 7.5). After two hours at 37°C, digestion was stopped by addition of 5 μl of proteinase K (10 mg/ml) and the samples were further incubated for 15 minutes at 37°C. Carrier tRNA (10 μg) and 0.3 ml of solution D (4 M guanidine isothiocyanate, 25 mM sodium citrate, pH 7.0, 0.1 M 2-mercaptoethanol and 0.5% sarkosyl) were added to each tube and protected hybrids were precipitated with isopropyl alcohol. RNA pellets were washed with 70% ethanol, air dried, taken up in sample buffer (97% deionised formamide, 0.1% sodium dodecyl sulphate (SDS), 10 mM Tris HCl, pH 7.0), and run on a 5% acrylamide gel containing 7 M urea. The gels were exposed for 48 hours at -80°C to x ray film for autoradiography.

RECEPTOR QUANTIFICATION AND G_i PROTEIN IDENTIFICATION

Receptors were quantified on crude membrane preparations using the selective radioligands [³H]RX821002 (α_2 antagonist) and [³H]clonidine (α_2 agonist).²⁸ Specific binding was defined as the difference between total and non-specific binding measured in the presence of 10 μM phentolamine. Saturation isotherms and inhibition curves were analysed using EBDA-LIGAND computer programs.²⁹ ADP ribosylation was carried out essentially as described previously.³⁰ Membranes (50–75 μg of protein) suspended in Tris HCl buffer

containing 1 mM EDTA, 1 mM dithiothreitol (DTT), and 0.05% lubrol (v/v) were incubated for 60 minutes at 30°C in the presence of 1 μCi [³²P]NAD⁺ and 100 ng of preactivated pertussis toxin in a final volume of 60 μl . The reaction mixture contained 70 mM Tris HCl (pH 8.0), 0.5 μM NAD⁺, 1 mM ATP, 10 mM thymidine, 1 mM EDTA, 0.1 mM MgCl₂, 1 mg/ml L-myristyl phosphatidylcholine, 10 mM nicotinamide, and 25 mM DTT. The reaction was stopped by addition of 2 μg of bovine serum albumin (BSA) in 0.04% SDS and the proteins were precipitated with 70 μl of 10% trichloroacetic acid. After centrifugation, pellets were washed twice with diethylether and finally dissolved in Laemmli sample buffer. The labelled proteins were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and revealed by autoradiography.

cAMP MEASUREMENT

Cells were detached in PBS containing 0.6 mM EDTA and collected by gentle centrifugation (400 g, five minutes). The pellet was suspended in DMEM buffered with 25 mM Hepes (pH 7.4). Aliquots of the cell suspension were incubated in a 200 μl final volume of Hepes buffered DMEM containing 0.2 mM IBMX and the indicated concentration of the drug to be tested. After 15 minutes at 37°C, the reaction was stopped by adding 1.8 ml of methanol/formic acid (95/5, v/v). The alcoholic extract was centrifuged (3000 g, 10 minutes, 4°C) and an aliquot of supernatant evaporated. The dry samples were taken up in acetate buffer containing 0.1% NaN₃, and cAMP content was determined by radioimmunoassay.

DETECTION OF PHOSPHORYLATED Erk1/Erk2 AND Shc

Cells were seeded at low density (10⁵ cells/cm²) in 100 mm culture dishes. Three days later, they were rendered quiescent by incubation in FCS free DMEM for 48 hours. They were then treated for the indicated period of time with the compound to be tested, rapidly rinsed with ice cold PBS, and lysed with 1 ml of radioimmunoprecipitation (RIPA) buffer (10 mM Tris HCl, pH 7.4, 1% Triton X100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride, and 0.5 μM aprotinin). Cell lysates were sonicated, clarified by centrifugation for 15 minutes at 15 000 g, and stored at -80°C until analysis.

The extent of MAPK phosphorylation was measured either on immunoprecipitated tyrosyl phosphorylated proteins using antibodies to extracellular regulated protein kinase 1 (Erk1) and 2 (Erk2) or directly on total cell protein extract using antibody to active MAPK. Tyrosyl phosphorylated proteins were immunoprecipitated (three hours at room temperature) by incubating 2 mg of total cell proteins with protein G-sepharose beads conjugated with an antiphosphotyrosine monoclonal antibody (PY20, Transduction Laboratories, Lexington, Kentucky, USA). The beads were washed four times with RIPA, dried, suspended in 50 μl of Laemmli buffer, boiled for five minutes, and

centrifuged for 10 minutes at 15 000 *g*. Proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Blots were incubated for two hours at room temperature in TBST (10 mM Tris HCl buffer, pH 8.0, 150 mM NaCl, 0.2% Tween 20) containing 5% skimmed milk and then overnight at 4°C in the same buffer containing rabbit polyclonal antibodies to Erk1 and Erk2 (anti-Erk1, 1:250; anti-Erk2, 1:400; Santa Cruz Biotechnologies, Santa Cruz, California, USA). After extensive washing, blots were exposed for one hour to horseradish peroxidase conjugated donkey anti-rabbit IgG. Immunoreactive proteins were visualised by chemiluminescence (ECL Western blotting system, Amersham Pharmacia Biotech, Courtaboeuf, France). The intensity of the bands was analysed using ImageQuant software (Molecular Dynamics, Sunnyvale, California, USA). In some experiments, activated Erk1/Erk2 were detected with a rabbit polyclonal antibody recognising the phosphorylated sequence of the active forms of MAPK (Promega, Madison, Wisconsin, USA). In this case, total cell proteins (30 µg) were separated by SDS-PAGE and transferred onto a Polyscreen/polyvinylidene difluoride (PVDF) membrane (NEN Life Science, Belgium). Blots were treated for one hour in TBST containing 0.1% BSA and then incubated overnight at 4°C in the same buffer containing antiactive MAPK antibody (1:3000). After extensive washing, bound antibody was detected with the horseradish peroxidase conjugated secondary antibody and revealed by chemiluminescence. The membrane was then stripped of Ig and reprobed using anti-Erk2 antibody to assess equal loading. For determination of Shc phosphorylation, 500 µg of cell proteins were immunoprecipitated using 5 µg of rabbit polyclonal Shc antibody (Upstate Biotechnology, Lake Placid, New York, USA). After overnight incubation at 4°C, the immunocomplex was precipitated by addition of 50 µl of protein A-agarose beads (Transduction Laboratories, Lexington, Kentucky, USA). After several washes in RIPA, beads were dried, suspended in Laemmli sample buffer, boiled for five minutes, and centrifuged for 10 minutes at 15 000 *g*. The proteins were subjected to SDS-PAGE, electrotransferred to a PVDF membrane, and probed with an antiphosphotyrosine-horseradish peroxidase conjugated monoclonal antibody (ECL phosphorylation detection system, Amersham Pharmacia Biotech, Courtaboeuf, France). The membrane was stripped of Ig and reprobed using anti-Shc antibody to assess equal loading.

CELL PROLIFERATION ASSAY

Cells were seeded at a density of 10⁵ cells/cm² in 24 well plates. Three days after seeding they were rendered quiescent by incubation in FCS free culture medium for 48 hours. Experiments were started when replacing the cells in DMEM supplemented with 0.5% FCS with or without 1 µM UK14304. The medium was changed every day. At the indicated time following reinitiation of cell proliferation, the effect of the α₂ agonist was estimated by measuring total cellular protein and DNA content.

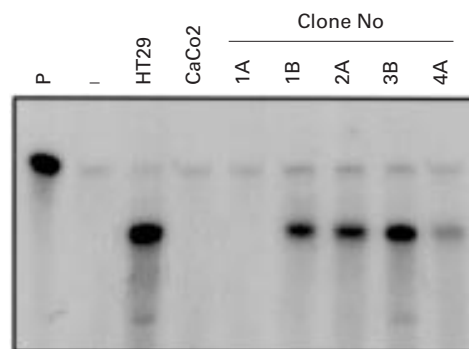


Figure 2 Screening of positive clones by RPA. RNA from HT29 (used as a positive control), Caco2, and G418 resistant clones were hybridised with [³²P]labelled α₂C10 probe. Samples were digested with a mixture of RNases A and T1. The resistant hybrids were analysed by electrophoresis. A representative autoradiogram is shown. Lanes P and (-) correspond to the undigested probe and negative control, respectively, carried out on 100 µg of tRNA.

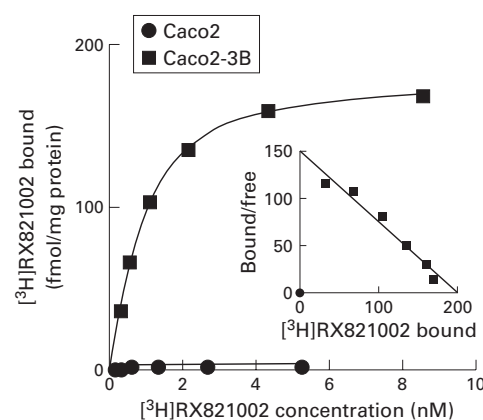


Figure 3 Saturation isotherm of [³H]RX821002 binding to Caco2 and Caco2-3B cell membranes. Membranes prepared from Caco2 and Caco2-3B were incubated in the presence of various concentrations of radioligand. The amount of specifically bound [³H]RX821002 was determined using 10 µM phentolamine to estimate non-specific binding. The data presented are from a typical experiment. The inset shows the corresponding Scatchard plot. Computer assisted analysis of the results from this specific experiment indicated that the B_{max} and K_d values of [³H]RX821002 were, respectively, 198 (12) fmol/mg of protein and 1.05 (0.11) nM in membranes from Caco2-3B. No specific binding was detected on membranes from Caco2 cells.

Protein concentration was determined using the Coomassie blue method.³¹ DNA was measured by a fluorometric method using DAPI.³²

STATISTICAL ANALYSIS

Results are expressed as mean (SEM) for the number of observations indicated (n). Data were analysed using the Student's *t* test, and a value of *p*<0.05 was considered statistically significant.

Results

EXPRESSION AND FUNCTIONAL ACTIVITY OF HUMAN α_{2A} ADRENOCEPTOR IN Caco2 CELL TRANSFECTANTS

The Caco2 human colonic adenocarcinoma cell line undergoes enterocytic differentiation under standard culture conditions. This process is growth related. In the latter stages of the

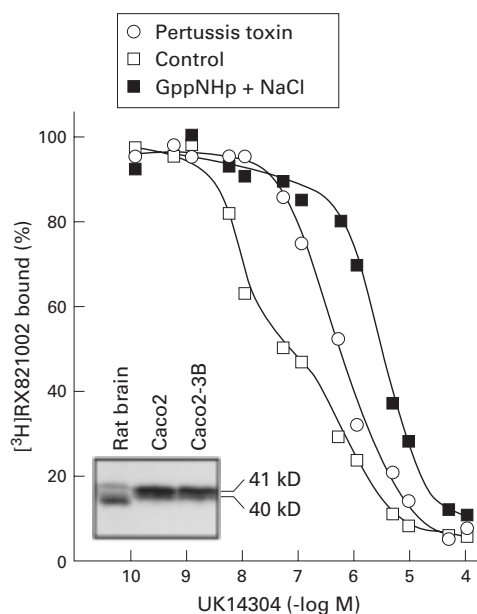


Figure 4 Analysis of receptor coupling and identification of Gi proteins. Caco2-3B cells were or were not treated with 250 ng/ml of pertussis toxin for 16 hours. Cell membrane preparations were incubated in the presence of 3 nM [³H]RX821002 and increasing concentrations of UK14304. The experiments were carried out in the absence or presence of 10 μM GppNHp with 100 mM NaCl. The curves presented are from a specific experiment. Inset: membranes from rat brain, Caco2, and Caco2-3B were ADP ribosylated with pertussis toxin in the presence of [³²P]NAD⁺. The labelled proteins were resolved on SDS-PAGE and autoradiographed as described in materials and methods.

culture, cells are organised as a polarised monolayer with tight junctions and an apical brush border,³³ and form domes that are indicative of transepithelial transport properties.³⁴ RPA and RT-PCR experiments have previously demonstrated that Caco2 cells do not spontaneously contain α₂ adrenoceptors.^{27, 35} In order for this model to express the same receptor subtype as normal human mucosa, the Caco2 cells were transfected with the bicistronic construct pα2C10Eneo (fig 1). Of the 20, G418 resistant clones which were isolated and subcultured, five were submitted to a first round of screening using RPA with a specific riboprobe derived from the α2C10-AR gene. As shown in the autoradiogram in fig 2, one of the five clones (clone 1A) was negative whereas the others contained substantial amounts of transcript. Binding experiments using [³H]RX821002 (α₂ antagonist) were thus performed to check the level of receptor expression. As expected, no specific radioligand binding was detected in the parental cell line or in clone 1A. All other clones displayed radioligand binding sites but at densities ranging from 28 to 300 fmol/mg of membrane protein. The clone 3B (Caco2-3B), which expresses the receptor at a level similar to that in epithelial cells from normal human intestinal mucosa,⁹ was selected for further studies. Data from a typical saturation binding experiment on Caco2-3B membranes is presented in fig 3. The number of [³H]RX821002 binding sites was not dependent on the stage of culture (230 (15) and 195 (23) fmol/mg of protein in pre-confluent and post-confluent

Table 1 Effect of α₂ agonists on basal and forskolin induced cAMP accumulation. Intact cell suspensions prepared from Caco2 and Caco2-3B culture dishes were incubated in serum free Dulbecco's modified Eagle's medium buffered with 25 mM Hepes (pH 7.4) containing vehicle (basal), 1 μM UK14304 (UK), 10 μM forskolin (FK), 10 μM forskolin plus 1 μM UK14304 (FK+UK), 10 μM forskolin plus 10 μM clonidine (FK+clo) or 10 μM forskolin plus 10 μM adrenaline (FK+adre). cAMP was extracted and measured by radioimmunoassay as described in materials and methods. Concentrations of cAMP are expressed as nanomoles of cAMP/mg of cellular protein and are mean (SEM) (n=8)

	cAMP level (nmol/mg of protein)	
	Caco2	Caco2-3B
Basal	0.15 (0.02)	0.20 (0.04)
UK	0.13 (0.03)	0.23 (0.03)
FK	4.62 (0.17)***	5.52 (0.31)***
FK+UK	4.68 (0.11)	2.72 (0.19)†††
FK+clo	4.44 (0.22)	3.12 (0.31)†††
FK+adre	4.78 (0.09)	2.95 (0.22)†††

***Significantly different from corresponding basal condition (p<0.001); †††significantly different from value in Caco2-3B treated with forskolin alone (p<0.001).

cells, respectively) and remained stable over at least 30 successive cell passages. From phase contrast and confocal microscopy, Caco2-3B was seen to exhibit the same pattern of enterocytic differentiation as the parental cell line. In addition, measurement of enzymatic activities in cell extracts showed that brush border associated hydrolases were expressed (alkaline phosphatase 85 (12) mU/mg protein; dipeptidyl peptidase IV, 59 (7) mU/mg protein, n=3). Inhibition of [³H]RX821002 binding by various adrenergic compounds indicated that the K_i values for yohimbine (2.9 (1.3) nM), idazoxan (3.7 (1.2) nM), phentolamine (9.2 (3.1) nM), chlorpromazine (2630 (350) nM), and prazosin (2200 (420) nM) matched the pharmacological properties expected for an α_{2A} adrenoceptor subtype.

The degree of Gi protein coupling was analysed by examining the ability of the receptor to exist under a high affinity state for agonists (fig 4). In contrast with the situation with antagonists, the UK14304 competition curve was shallow and was better fitted to a two site inhibition model. According to computer assisted analysis of the data from three independent experiments, the K_i values for UK14304 for the high and low affinity state receptor were, respectively, 1.05 (0.23) and 108 (30) nM. The proportion with the high affinity conformation for agonist represented 62 (6)% of the whole receptor population. This fraction was abolished in the presence of Gpp(NH)p/Na⁺ or in membranes prepared from cells treated for 16 hours with pertussis toxin (250 ng/ml). Furthermore, [³²P]ADP ribosylation demonstrated that Caco2-3B expressed the same pertussis toxin sensitive G proteins as the parental cell line (fig 4, inset). Two peptides with apparent molecular masses of 40 and 41 kDa were labelled. Immunoblotting with specific antibodies indicated that they corresponded to αi2 and αi3 subunits (not shown). Further evidence for receptor coupling to Gi proteins was provided by the direct study of [³H]clonidine binding. Indeed, this [³H]agonist labelled 122 (18) fmol of sites/mg of protein with high affinity (K_d 2.5 (0.9) nM). As expected, no signifi-

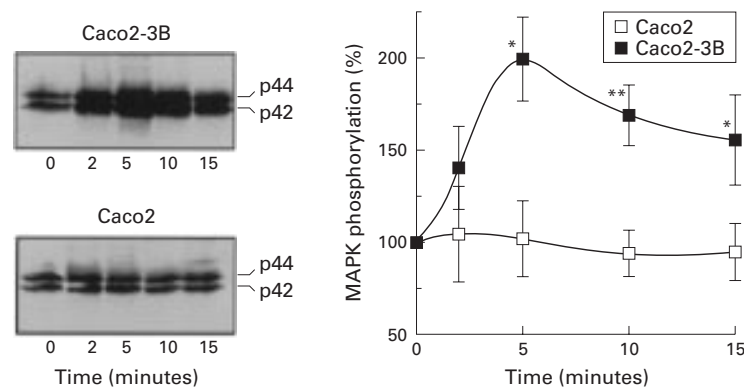


Figure 5 Effect of UK14304 on mitogen activated protein kinase (MAPK) phosphorylation. Caco2 and Caco2-3B cells were seeded at low density (10^5 cells/cm²) and grown for three days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Pre-confluent cell layers were then maintained for 48 hours in serum free medium and treated for the indicated time with 1 μ M UK14304. Left: tyrosine phosphorylated proteins were immunoprecipitated, separated by SDS-PAGE, and MAPK revealed by immunoblotting with anti-Erk1 and anti-Erk2 antibodies as described in materials and methods. Right: the extent of MAPK phosphorylation is expressed as a per cent of that in control cells (untreated). Results are mean (SEM) ($n=3$ for Caco2, $n=7$ for Caco2-3B). Significantly different from untreated cells: * $p<0.05$, ** $p<0.02$.

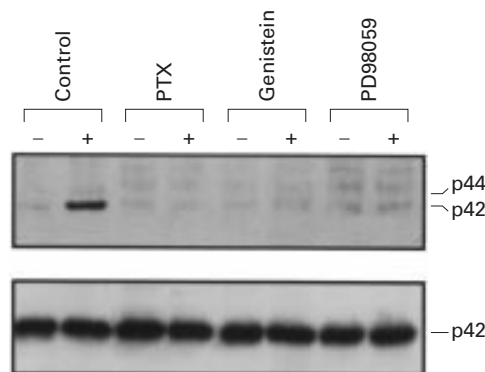


Figure 6 Effect of pertussis toxin (PTX), genistein, and PD98059 on mitogen activated protein kinase (MAPK) phosphorylation. Caco2-3B cells were grown and rendered quiescent as indicated in the legend of fig 5. The cells were treated for 16 hours with pertussis toxin (250 ng/ml) or for 30 minutes with either genistein (25 μ M) or PD98059 (50 μ M). They were then exposed (+) or not (-) to 1 μ M UK14304 for five minutes. Top: MAPK phosphorylation was studied using the anti-active MAPK antibody. Bottom: the membrane was stripped of Ig and reprobed with anti-Erk2 antibody to assess equal protein loading.

cant [³H]clonidine binding was detected in membranes from Caco2-3B cells treated with pertussis toxin. In cells isolated from human and rat colonic crypts,^{11,12} or in the human adenocarcinoma cell line HT29,¹³ α_2 adrenoceptors were negatively coupled to adenylyl cyclase. In the next series of experiments we asked whether this pathway of signal transduction is also efficient in Caco2-3B. Measurement of intracellular cAMP levels indicated that Caco2 and Caco2-3B cells were similarly responsive to forskolin exposure (table 1). UK14304, clonidine, or (-)adrenaline were ineffective in the parental cell line but significantly inhibited forskolin induced cAMP accumulation in Caco2-3B. It is worth mentioning that neither UK14304 nor any of the other tested agonists lowered basal cAMP levels. Further experiments demonstrated that the inhibitory effect of UK14304 on forskolin induced cAMP production was dose dependent (EC_{50} 12 (5) nM) and abolished by addition of α_2 antagonist (not shown).

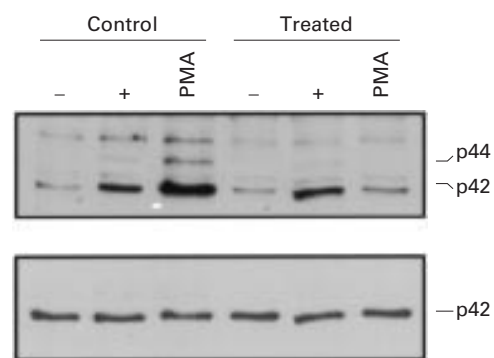


Figure 7 Effect of phorbol 12-myristate 13-acetate (PMA) on UK14304 induced mitogen activated protein kinase (MAPK) phosphorylation. The Caco2-3B cells were grown and rendered quiescent, as indicated in the legend of fig 5, and treated for 16 hours with 2.5 μ M PMA (treated) or vehicle (-), 2.5 μ M PMA (PMA), or 1 μ M UK14304 (+). Top: MAPK phosphorylation was studied using the anti-active MAPK antibody. Bottom: the membrane was stripped of Ig and reprobed with anti-Erk2 antibody to assess equal protein loading.

EFFECT OF α_2 ADRENOCEPTOR STIMULATION ON MAPK ACTIVATION AND CELL PROLIFERATION

The effect of receptor stimulation on MAPK was investigated by immunoprecipitation of tyrosine phosphorylated proteins followed by immunodetection with anti-Erk1/Erk2 antibodies (fig 5). In Caco2 and Caco2-3B, these antibodies recognised two proteins with apparent molecular masses of 44 and 42 kDa. Treatment of Caco2-3B with UK14304 resulted in a rapid increase in the extent of phosphorylation of the two forms of MAPK. Phosphorylation was observed as early as two minutes after treatment, was maximal at five minutes, and persisted for at least 15 minutes. On the basis of the analysis of seven independent experiments, the maximal effect observed after five minutes of exposure represents a two-fold increase in phosphorylation. No significant change in phosphorylation of MAPK was detected in Caco2 cells, demonstrating that the effect of UK14304 was primarily due to stimulation of α_2 adrenoceptors. The mechanisms whereby stimulation of α_{2A} adrenoceptor increases MAPK phosphorylation were then investigated directly using antiactive MAPK antibody on total cellular extracts. Unlike anti-Erk antibodies on immunoprecipitated phosphoproteins (as in fig 5), the antiactive MAPK antibody revealed a major band corresponding to phosphorylated Erk2 (see figs 6, 7). The western blots presented in fig 6 clearly show that the increase in Erk2 phosphorylation following exposure to UK14304 was totally abolished by pretreatment of the cells with pertussis toxin (250 ng/ml, 16 hours). Thus the integrity of Gi proteins is necessary for the effect of the α_2 agonist to occur. In the same manner, addition of the inhibitor of protein tyrosine kinases, genistein (25 μ M), or addition of the inhibitor of the MEK1 form of MAPK kinases, PD98059 (50 μ M), prevented the effect of UK14304. As shown in fig 7, acute stimulation of protein kinase C (PKC) by addition of 2.5 μ M PMA for five minutes also caused marked activation of MAPK. Long term treatment of cells with PMA totally abol-

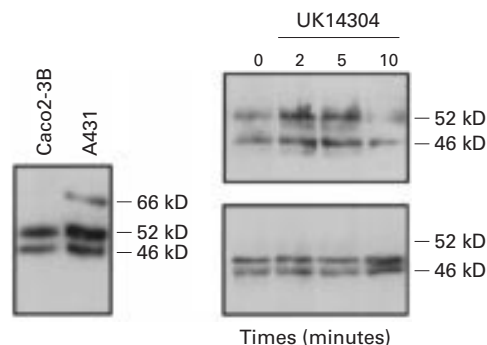


Figure 8 Identification of Shc isoforms and effect of UK14304 on phosphorylation. Left: identification of the Shc isoforms expressed in Caco2-3B cells. Protein extracts from Caco2-3B and A431 cells (used here as positive control) were subjected to SDS-PAGE and Shc isoforms were revealed by western blot using anti-Shc antibody. Right: UK14304 induced phosphorylation of Shc. The Caco2-3B cells were grown, rendered quiescent as indicated in the legend of fig 5 and then treated for the indicated time with 1 μ M UK14304. Shc proteins were immunoprecipitated with anti-Shc antibody and the extent of their phosphorylation estimated by western blot with an antiphosphotyrosine antibody (right upper panel). The membrane was stripped of Ig and reprobed using anti-Shc antibody to assess equal protein loading (right lower panel).

ished any further response to the phorbol ester but did not affect the response to UK14304, suggesting that α_2 adrenoceptors trigger their effects through a PKC independent pathway. As activation of MAPK by several G protein coupled receptors was shown to correlate with Shc phosphorylation in Rat-1 fibroblasts,³⁶ we examined if such a phosphorylation also occurs on α_{2A} adrenoceptor stimulation in Caco2-3B cells. The use of anti-Shc antibody on extracts prepared from A431 and Caco2-3B cells (fig 8) indicated that our model predominantly expressed the 46 and 52 kDa isoforms of Shc. Furthermore, treatment of the cells with 1 μ M UK14304 caused increased phosphorylation of both isoforms of Shc. Activation was notable after two minutes of treatment with the α_2 agonist and was transient, a decrease in phosphorylation being observed after 10 minutes. The time course of Shc phosphorylation was thus compatible with activation of Shc preceding that of MAPK.

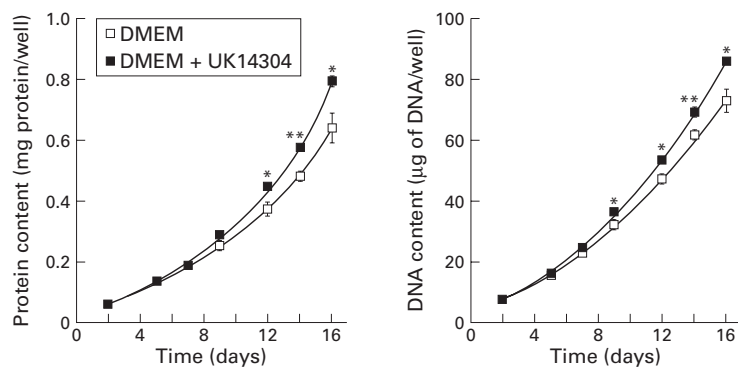


Figure 9 Effect of UK14304 on cell growth. Caco2-3B cells were seeded at low density (10^3 cells/cm²). Three days after seeding, they were rendered quiescent in serum free medium for 48 hours. Cellular growth was then reinitiated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.5% fetal calf serum with or without 1 μ M UK14304. Protein content (left) and DNA content (right) per well were determined as described in materials and methods. Data presented are from a typical experiment and the results are mean (SEM) (n=3). Significantly different from untreated cells: *p<0.05, **p<0.01.

Activation of MAPK being generally associated with cellular proliferation, we finally examined the effect of α_2 adrenoceptor stimulation on the growth rate of Caco2-3B. No effect was detected when UK14304 was tested in serum free or in 10% FCS medium. However, an accelerated proliferation was repeatedly observed when assays were carried out in culture medium containing 0.5% FCS. The proliferative effect of the α_2 agonist was modest but significant. As estimated by measurement of total cellular protein and DNA content, it represented a 20% increase in cell proliferation after 16 days of culture (fig 9). Furthermore, this effect was solely due to α_2 adrenoceptor activation because it was not observed in the parental cell line Caco2 (not shown).

Discussion

The α_2 adrenoceptors regulate a number of physiological functions including neurotransmitter release, insulin secretion, vasoconstriction, platelet aggregation, renal Na⁺ reabsorption, and intestinal Cl⁻ secretion.³⁷ In addition to these actions, recent findings have demonstrated that these G protein coupled receptors can also activate the MAPK pathway and behave as mitogens or co-mitogens on transfected cells.¹⁷⁻²¹ This latter effect is reminiscent of former observations which indicated that administration of noradrenaline to rodents activated proliferation of intestinal crypt cells via stimulation of α_2 adrenoceptors.¹⁶ As yet the occurrence of this mitogenic effect awaits demonstration in other species and the molecular mechanisms involved remain to be elucidated. As it is impossible to carry out in vivo studies in humans or to maintain crypt cells isolated from normal mucosa in culture, the putative action of α_2 adrenoceptors on proliferation can only be investigated in transformed cells. The human colon adenocarcinoma HT29, which spontaneously expresses α_2 adrenoceptors, may have represented an alternative model to approach this question. However, receptor expression is growth related in this cell line.³⁸ More precisely, receptor density is very low during the exponential phase of proliferation and increases at confluence, making HT29 inadequate. To circumvent this problem, we developed a model in which receptor expression was independent of culture conditions. The host cells we retained for this purpose were Caco2 cells. The reason for this choice lies in the fact that in addition to proliferation studies, this spontaneously differentiated model represents a valuable in vitro system to investigate the regulatory effects of α_2 adrenoceptor on intestinal ion transport mechanisms.

Transfection of Caco2 cells gave a permanent clone (Caco2-3B) expressing α_{2A} adrenoceptors at a density similar to that of human intestinal epithelium.⁹ As in normal enterocytes or in HT29,^{9, 39} the receptor is coupled to the pertussis toxin sensitive G proteins Gi2 and/or Gi3, and stimulation caused a marked reduction in forskolin stimulated adenylyl cyclase. Exposure to UK14304 induced an increase in the phosphorylation state of Erk1

and Erk2. As expected, this effect was blocked by addition of RX821002 (α_2 antagonist). This was also observed with the endogenous catecholamine (–)adrenaline (not shown). MAPK phosphorylation was rapid (peak at five minutes) and similar to that found in other cell systems in which α_2 adrenoceptors had mitogenic properties.^{19–21} Previous studies have established that the mechanisms whereby G protein coupled receptors activate MAPK vary according to the receptor or cell type examined. Indeed, Erk1/2 phosphorylation may be mediated by either Gq or Gi/o and be dependent on PKC or Ras activation.^{20–40} As α_2 adrenoceptors stimulate PKC via a Gi dependent pathway in OK cells or in human platelets,^{41–42} and as they increase phospholipase C activity and intracellular Ca⁺⁺ in HEL or transfected CHO cells,⁴³ we investigated if the effect of UK14304 involved PKC activation. Short term exposure of Caco2-3B cells to the PKC activator PMA resulted in an increase in Erk phosphorylation. However, the effect of UK14304 was not significantly influenced after desensitisation of PKC activity. In contrast, it was totally abolished by pertussis toxin treatment, indicating that it is fully dependent on activation of Gi proteins. Nevertheless, it is clear that inhibition of adenylyl cyclase is not responsible for the action of the α_2 agonist because under conditions where effects on Erk are observed (that is, in the presence of UK14304 alone) intracellular levels of cAMP are unchanged. In contrast, the change in MAPK phosphorylation was abolished by genistein and PD98059, indicating that tyrosine kinases and MEK 1 are necessary for UK14304 to act. To further elucidate the signalling pathway leading to MAPK activation, phosphorylation of Shc was measured. The two major isoforms of Shc (46 kDa and 52 kDa), which are expressed in Caco2-3B, are transiently phosphorylated following agonist exposure. As in transfected COS7 cells,⁴⁴ it is therefore likely that α_{2A} adrenoceptors in Caco2-3B stimulate a cascade of events comprising recruitment of Gi proteins, G $\beta\gamma$ subunit mediated formation of Shc-Grb2-SOS complex, and subsequent activation of MEK 1 and MAPK.

A recent study on a subclone of HT29 cells (HT29-N2) showed that a decrease in MAPK activity plays a critical role in the biochemical and morphological differentiation of intestinal cells.⁴⁵ It was thus conceivable that the reverse may hold true and that activation of MAPK by UK14304 enhances proliferation of Caco2-3B. In fact, no stimulatory effect of UK14304 was observed when proliferation tests were conducted in either the presence or absence of high concentrations of FCS. However, a significant increase in growth was found when cells were placed in medium containing trace amounts of growth factors (0.5% FCS). Thus if the α_2 agonist is inefficient in quiescent cells and cannot be considered per se as a mitogen, it acts on slowly growing cells and therefore behaves as a co-mitogen on Caco2-3B cells. In this respect, our results differ from those found in transfected 3T3F442A cells or in OK cells in which

α_2 agonists accelerate the growth rate, even in the absence of FCS.^{19–21} They are, however, consistent with earlier observations on CCL39 cells transfected with the human α_{2A} adrenoceptor.¹⁷ Indeed, α_2 agonists cannot stimulate the proliferation of these fibroblastic cells when tested alone, but they do stimulate [³H]thymidine incorporation when assayed in combination with fibroblast growth factor. The reason why UK14304 does not increase Caco2-3B proliferation in serum free medium is unclear. It is likely that MAPK activation is not persistent enough and/or not sufficient to result in exit from the Go phase and entry into the cell cycle.⁴⁶ It is also noteworthy that in rat aortic smooth muscle cells,⁴⁷ activation of MAPK by an α_2 agonist is not associated with an increase in cellular proliferation but rather results in cell migration due to F-actin depolymerisation. Conversely, α_2 agonists were demonstrated to promote actin polymerisation and cell spreading in mouse preadipocytes.⁴⁸ In this latter case, the effect of α_2 agonists on cell adhesion correlated with activation of the RhoA/FAK pathway.⁴⁹ Interestingly, a previous study on Caco2 cells showed that Rho proteins play a crucial role in the mechanisms whereby growth factors stimulate migration of intestinal cells and thus contribute to the maintenance of epithelium integrity.⁵⁰ The question of whether α_2 agonists also stimulate migration or attachment of Caco2-3B deserves further attention. Finally, the clone Caco2-3B retained the morphological features of a polarised epithelium and in the future may represent a suitable model to study the effects of α_2 adrenoceptor on transepithelial transport. Preliminary results indicate that α_2 agonists increase the activity of the peptide transporter PepT1 in these cells,⁵¹ suggesting that at least part of the effects of clonidine observed in vivo⁵² are due to direct stimulation of α_2 adrenoceptors located on intestinal epithelial cells.

In conclusion, the present work shows that α_2 adrenoceptors activate the MAPK pathway and act as co-mitogens on a cell line derived from human intestinal epithelium. Further studies are necessary to clarify the precise molecular mechanisms responsible for this action and to assess the action of these receptors on other epithelial functions. However, together with previous findings, our results support the participation of the α_2 adrenoceptor in the regulation of intestinal cell proliferation.

This work was supported by the BIOMED 2 Programme PL963373 (European Commission, Brussels, Belgium) and by a grant from the Fondation pour la Recherche Médicale (Paris, France). During the time of his participation in this study, JCD was the recipient of a fellowship from the Fondation IPSEN (Paris, France). We thank F Quinchon and E Fonta for helpful technical assistance.

- 1 Gershon MD, Erde SM. The nervous system of the gut. *Gastroenterology* 1981;**80**:1571–94.
- 2 Wu Z, Gaginella TS. Release of [³H]norepinephrine from nerves in rat colonic mucosa: effects of norepinephrine and prostaglandin E₂. *Am J Physiol* 1981;**241**:G416–21.
- 3 Tapper EJ, Broon AS, Lewand DL. Endogenous norepinephrine release induced by tyramine modulates intestinal ion transport. *Am J Physiol* 1981;**241**:G264–9.
- 4 Chang EB, Field M, Miller RJ. Alpha2-adrenergic receptors regulation of ion transport in rabbit ileum. *Am J Physiol* 1982;**242**:G237–42.

- 5 Cox HM, Cuthbert AW. Antisecretory activity of the α_2 -adrenergic receptor agonist, xylazine in rat jejunal epithelium. *Naunyn Schmiedeberg's Arch Pharmacol* 1989; **339**:669-74.
- 6 Chang EB, Field M, Miller RJ. Enterocyte α_2 -adrenergic receptors: yohimbine and p-aminoclonidine binding relative to ion transport. *Am J Physiol* 1983; **244**:G76-82.
- 7 Paris H, Voisin T, Remaury A, et al. Alpha2-adrenergic receptor in rat jejunum epithelial cells: characterization with [³H]RX821002 and distribution along the villus-crypt axis. *J Pharmacol Exp Ther* 1990; **254**:888-93.
- 8 Senard JM, Langin D, Estan L, et al. Identification of the α_2 -adrenergic receptor and non-adrenergic idazoxan binding sites in rabbit colon epithelial cells. *Eur J Pharmacol* 1990; **191**:59-68.
- 9 Valet P, Senard JM, Devedjian JC, et al. Characterization and distribution of α_2 -adrenergic receptors in the human intestinal mucosa. *J Clin Invest* 1993; **91**:2049-57.
- 10 Pamukcu R, Chang EB. Alpha2-adrenergic agonists as anti-diarrhoeal agents: a review of physiological and cellular mechanisms. In: Lebenthal E and Duffey M, eds. *Textbook of secretory diarrhoea*, vol. 28. New York: Raven Press, 1990:383-93.
- 11 Boige N, Munck A, Laburthe M. Adrenergic versus Vipergic control of cyclic AMP in human colonic crypt. *Peptides* 1984; **5**:379-83.
- 12 Laburthe M, Amiranoff B, Boissard C. Alpha-adrenergic inhibition of cyclic AMP accumulation in epithelial cells isolated from rat small intestine. *Biochem Biophys Acta* 1982; **721**:101-8.
- 13 Remaury A, Larrouy D, Daviaud D, et al. Coupling of the α_2 -adrenergic receptor to the inhibitory G-protein Gi and adenylate cyclase in HT29 cells. *Biochem J* 1993; **292**:283-8.
- 14 Chang EB, Gill AJ, Wang NS, et al. Alpha2-adrenergic inhibition of Ca-dependent intestinal secretion. *Gastroenterology* 1984; **85**:1044-51.
- 15 Tilly BC, Kansan M, Van Gageldonk PGM, et al. G-proteins mediate intestinal chloride channel activation. *J Biol Chem* 1991; **266**:2036-40.
- 16 Tutton PJM, Barkla DH. Biogenic amines as regulators of the proliferative activity of normal and neoplastic intestinal epithelial cells. *Anticancer Res* 1987; **7**:1-12.
- 17 Seuven K, Magnaldo J, Kobilka BK, et al. Alpha2-adrenergic agonists stimulate DNA synthesis in Chinese hamster lung fibroblasts transfected with a human α_2 -adrenergic receptor gene. *Cell Regul* 1990; **1**:445-51.
- 18 Ablas J, Van Corven EJ, Hordijk PL, et al. Gi-mediated activation of the p21ras-mitogen-activated protein kinase pathway by α_2 -adrenergic receptors expressed in fibroblasts. *J Biol Chem* 1993; **268**:22235-8.
- 19 Bouloumié A, Planat V, Devedjian JC, et al. Alpha2-adrenergic stimulation promotes preadipocyte proliferation. Involvement of mitogen-activated protein kinases. *J Biol Chem* 1994; **269**:30254-9.
- 20 Della Rocca GJ, Van Biesen T, Daaka Y, et al. Ras-dependent mitogen activated protein kinase activation by G protein-coupled receptors. Convergence of Gi- and Gq-mediated pathways on calcium/calmodulin, Pyk2 and Src kinase. *J Biol Chem* 1997; **272**:19125-32.
- 21 Kribben A, Herget-Rosenthal S, Lange B, et al. Alpha2 adrenoceptors in opossum kidney cells couple to stimulation of mitogen-activated protein kinase independently of adenylyl cyclase inhibition. *Naunyn Schmiedeberg's Arch Pharmacol* 1997; **356**:225-32.
- 22 Kobilka BK, Matsui H, Kobilka TS, et al. Cloning, sequencing, and expression of the gene coding for the human platelet α_2 -adrenergic receptor. *Science* 1987; **238**:650-6.
- 23 Maret A, Galy B, Arnaud E, et al. Inhibition of fibroblast growth factor 2 expression by antisense RNA induced a loss of the transformed phenotype in a human hepatoma cell line. *Cancer Res* 1995; **55**:5075-9.
- 24 Garen A, Levinthal C. A fine structure genetic and chemical study of the enzyme alkaline phosphatase of E. coli. I. Purification and characterization of alkaline phosphatase. *Biochim Biophys Acta* 1960; **38**:470-83.
- 25 Svensson B, Danielsen M, Staun M, et al. An amphiphilic form of dipeptidyl peptidase IV from pig small-intestinal brush-border membrane. Purification by immunoadsorbent chromatography and some properties. *Eur J Biochem* 1978; **90**:489-98.
- 26 Chomczynski P, Sacchi N. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; **162**:156-9.
- 27 Devedjian JC, Fargues M, Denis-Pouxviel C, et al. Regulation of the α_2 A-adrenergic receptor in the HT29 cell line, effects of insulin and growth factors. *J Biol Chem* 1991; **266**:14359-66.
- 28 Langin D, Lafontan M, Stillings MR, et al. [³H]RX821002: a new tool for the identification of α_2 A adrenoceptors. *Eur J Pharmacol* 1989; **167**:95-104.
- 29 Mc Pherson GA. Analysis of radioligand binding experiments: a collection of computer programs for the IBM PC. *J Pharmacol Methods* 1985; **14**:213-28.
- 30 Ribeiro-Neto FAP. ADP-ribosylation of membrane components by pertussis and cholera toxins. *Methods Enzymol* 1985; **109**:566-72.
- 31 Bradford MM. A rapid sensitive method for the quantitation of protein utilizing the principle of protein dye binding. *Anal Biochem* 1976; **72**:248-54.
- 32 Kapuscinski J, Skoczylas B. Simple and rapid fluorimetric method for DNA microassay. *Anal Biochem* 1977; **83**:252-7.
- 33 Pinto M, Robine-Leon S, Appay MD, et al. Enterocytic differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol Cell* 1983; **47**:323-30.
- 34 Grasset E, Bernabeu J, Pinto M. Epithelial properties of human colonic carcinoma cell line Caco-2: effect of secretagogues. *Am J Physiol* 1985; **248**:C410-18.
- 35 Schaak S, Cayla C, Blaise R, et al. HepG2 and SK-N-MC: two human models to study α_2 -adrenergic receptors of the α_2 C subtype. *J Pharmacol Exp Ther* 1997; **281**:983-91.
- 36 Luttrell LM, Daaka Y, Della Rocca GJ, et al. G protein-coupled receptors mediate two functionally distinct pathways of tyrosine phosphorylation in Rat1a fibroblasts. Shc phosphorylation and receptor endocytosis correlate with activation of Erk kinases. *J Biol Chem* 1997; **272**:31648-56.
- 37 Ruffolo Jr RR, Nichols AJ, Stadel JM, et al. Pharmacologic and therapeutic applications of α_2 adrenoceptor subtypes. *Annu Rev Pharmacol Toxicol* 1993; **32**:243-79.
- 38 Paris H, Bouscarel B, Cortinovic C, et al. Growth-related variation of α_2 -adrenergic receptivity in the HT29 adenocarcinoma cell-line from human colon. *FEBS Lett* 1985; **184**:82-6.
- 39 Bouscarel B, Cortinovic C, Carpenne C, et al. Alpha2-adrenoceptors in the HT29 human colon adenocarcinoma cell line: characterization with [³H]clonidine; effects on cyclic AMP accumulation. *Eur J Pharmacol* 1985; **107**:223-31.
- 40 Luttrell LM, Hawes BE, Van Biesen T, et al. Role of c-Src tyrosine kinase in G protein-coupled receptor and G $\beta\gamma$ subunit-mediated activation of mitogen-activated protein kinases. *J Biol Chem* 1996; **271**:19443-50.
- 41 Wang TT, Lachance S, Delalandre A, et al. Alpha-adrenoceptors and angiotensinogen gene expression in opossum kidney cells. *Kidney Int* 1995; **48**:139-45.
- 42 Nieuwland R, Wijburg OL, van Willigen G, et al. Alpha2A-adrenergic receptors activate protein kinase C in human platelets via a pertussis toxin-sensitive G-protein. *FEBS Lett* 1994; **339**:79-83.
- 43 Dorn GW, Oswald KJ, McCluskey TS, et al. Alpha2A-adrenergic receptor stimulated calcium release is transduced by Gi-associated G $\beta\gamma$ -mediated activation of phospholipase C. *Biochemistry* 1997; **36**:6415-23.
- 44 Van Biesen T, Hawes BE, Luttrell DK, et al. Receptor-tyrosine-kinase- and G $\beta\gamma$ -mediated MAP kinase activation by a common signalling pathway. *Nature* 1995; **376**:781-4.
- 45 Taupin D, Podolsky DK. Mitogen-activated protein kinase activation regulates intestinal epithelial differentiation. *Gastroenterology* 1999; **116**:1072-80.
- 46 Lavoie JN, Rivard N, L'Allemain G, et al. A temporal and biochemical link between growth factor-activated MAP kinases, cyclin D1 induction and cell cycle entry. *Prog Cell Cycle Res* 1996; **2**:49-58.
- 47 Richman JG, Regan JW. Alpha2-adrenergic receptors increase cell migration and decrease F-actin labeling in rat aortic smooth muscle cells. *Am J Physiol* 1997; **274**:C654-62.
- 48 Bétuing S, Daviaud D, Valet P, et al. Alpha2-adrenoceptor stimulation promotes actin polymerization and focal adhesion in 3T3F442A and BFC-1 β preadipocytes. *Endocrinology* 1996; **137**:5220-9.
- 49 Bétuing S, Daviaud D, Pagés C, et al. G $\beta\gamma$ -independent coupling of α_2 -adrenergic receptor to p21rhoA in preadipocytes. *J Biol Chem* 1998; **273**:15804-10.
- 50 Santos MF, McCormack SA, Guo Z, et al. Rho proteins play a critical role in cell migration during the early phase of mucosal restitution. *J Clin Invest* 1997; **100**:216-25.
- 51 Berlioz F, Maoret JJ, Paris H, et al. Alpha2-adrenoceptors modulate the di- and tripeptide transporter function in Caco2-3B cells. *Gastroenterology* 1999; **116**:A860 abstract G3741.
- 52 Berlioz F, Julien S, Tsocas A, et al. Neural modulation of cephalaxin intestinal absorption through the di- and tripeptide brush border transporter of rat jejunum in vivo. *J Pharmacol Exp Ther* 1999; **288**:1037-44.