Characterization and Distribution of α_2-Adrenergic Receptors in the Human Intestinal Mucosa

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Abstract

The subtype and the expression of the α_2-adrenergic receptor were investigated in the normal mucosa from human intestine by means of radioligand binding, RNase mapping, and measurement of adenylate cyclase activity. The study of the binding of the α_2-adrenergic antagonist, [^{3}H]RX821002, to epithelial cell membranes indicated the existence of a single class of noninteracting sites displaying a high affinity for the radioligand (K_d = 1.1±0.5 nM). The rank order of potency of antagonists to inhibit [^{3}H]RX821002 binding (RX821002 > yohimbine = rauwolscine > phentolamine ≈ idazoxan ≫ chlorpromazine > prazosin) suggested that the receptor is of the α_{2A} subtype. A conclusion which is confirmed by the fact that only α_{2C10} transcripts were found in the human intestine mucosa. Competition curves with (−)-norepinephrine demonstrated that 60% of the receptor population exhibited high affinity for agonists. This high-affinity state was abolished by the addition of GTP plus Na^+ or by prior treatment of the membranes with pertussis toxin indicating it corresponded to G protein–coupled receptors. [^{32}P]ADP-ribosylation and immunoblotting experiments identified two pertussis toxin-sensitive G proteins corresponding to Gi2 and Gi3. The study of the distribution of the receptor indicated that (*a*) the proximal colon is the intestine segment exhibiting the highest receptor density and (*b*) the receptor is predominantly expressed in crypts and is preferentially located in the basolateral membrane of the polarized cell. The distribution of the receptor along the crypt–surface axis of the colon mucosa can be correlated with a higher level of α_{2C10}-specific mRNA and a higher efficiency of UK14304 to inhibit adenylate cyclase in crypt cells. (*J. Clin. Invest.* 1993.91:2049–2057.)

Key words: adenylate cyclase • α_{2C10} subtype • colocyte • enterocyte • Gi protein

Introduction

Several observations indicate that catecholamines play a physiological role in the regulation of water and electrolyte fluxes across the intestinal epithelium (1). The intestinal mucosa is extensively innervated by noradrenergic fibers and high levels of catecholamines can be potentially achieved at the vicinity of the epithelial cells (2). In vitro experiments on isolated intestinal loops demonstrated that the addition of exogenous norepinephrine (3) or the stimulation of endogenous norepinephrine release by tyramine (4) increased the net absorption of Na^+, Cl^-, and fluid. Finally, the diarrhea observed in rats made diabetic with streptozocin is due to a loss of the noradrenergic innervation of the enterocytes (5). The use of selective adrenergic drugs proved the antisecretory action of catecholamines to be at least partially mediated by the stimulation of postsynaptic α_2-adrenergic receptors located on the epithelial cells (6–8). Direct evidence for the presence of α_2-adrenergic receptors on intestinal epithelial cells from different species was subsequently brought by the use of selective radioligands (9–13).

The antisecretory properties of α_2-agonists led to propose these compounds as a promising new class of antidiarrheal drugs in humans (14, 15). In this respect, clonidine and lindamidine were shown to represent a reasonable alternative for the treatment of certain forms of chronic diarrhea (16, 17). However, there are so far no direct data supporting the existence of α_2-adrenergic receptors in epithelial cells from human intestine. Given the pharmacological heterogeneity of α_2-adrenergic receptors (18), the present study was performed in order to define the subtype of the α_2-adrenergic receptor expressed in this tissue. The coupling of the receptor to G proteins and adenylate cyclase was investigated. Moreover, the level of expression of the receptor was studied along the crypt–surface axis of the mucosa and along the oral–aboral axis of the intestinal tract.

Methods

Sources of tissues. Whole intestinal tracts (n = 6) came from irreversibly brain-damaged cadaveric organ donors. These samples were obtained thanks to the collaboration of the French institution France-Transplant (Paris) and were collected within 2 h after death. Additional colon mucosa specimens (n = 21) were obtained from patients who underwent surgical resection of colorectal carcinoma. In this later case, histological examination was performed and only the segments with normal mucosa were retained.

Isolation of epithelial cells and membrane preparation. Epithelial cells were isolated from the intestinal mucosa as previously described by Laburthe et al. (19). Intestine segments were flushed free of content with cold PBS, then everted, and washed twice in the same solution. The following steps of the preparation were carried out at 4°C. Epithelial cells were isolated by shaking the everted intestine in a dispersing solution containing 2.5 mM EDTA and 0.24 M NaCl (pH 7.5). Cells were collected by centrifugation and pellets were washed once in PBS. In some experiments, crypt cells were separated from villus cells by sequential isolation exactly as reported in (12). This procedure generated four fractions (I–IV) displaying different degrees of differentiation. Crude membranes were prepared as in Laburthe et al. (20), and the final membrane pellets were frozen at −80°C until their use for

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binding studies, G-protein identification, or measurement of adenylate cyclase activity.

Purification of brush border and basolateral membranes. Purified membrane fractions were prepared essentially as described by Schmitz et al. (21). Freshly isolated cells were homogenized in 2 mM Tris-HCl buffer (pH 7.4) containing 300 mM mannitol with a Blendor (Waring, New Hartford, CT). The homogenate was chilled at 4°C and $CaCl_2$ was added at a final concentration of 10 mM. After standing for 15 min, the Ca^{++}-treated homogenate was centrifuged 15 min at 3,000 *g*. The resulting pellet (P1) containing the basolateral membranes was washed once and resuspended in Tris-Mg^{++} buffer for determination of binding site number. The supernatant (S1) was collected and centrifuged 10 min at 27,000 *g*. The pellet (P2) containing the brush border membrane vesicles was submitted to two additional cycles of suspension/centrifugation in order to increase the purity of the preparation and then resuspended as above for use in binding experiments.

Binding studies. Binding experiments were conducted as previously described (22). Briefly, 100 μl of membrane suspension was incubated at 25°C in the presence of the radioligand in a 400-μl final volume of Tris-Mg^{++} buffer (50 mM Tris-HCl, 0.5 mM $MgCl_2$, pH 7.5) containing 0.1 mM pyrocatechol, 0.8 mM DTT, and 0.3 mM ascorbic acid. The addition of these protectors was necessary because of high level of monoamine oxidase and catechol-*O*-methyl transferase activity in the preparations. It did not modify the binding parameters of the radioligand (23). After a 45-min period of incubation, membrane-bound radioligand was separated from free by rapid filtration through GF/C filters (Whatman Inc., Clifton, NJ) using a cell harvester (Skatron, Inc., Sterling, VA). Filters were washed with cold Tris-Mg^{++} buffer, air-dried, transferred into vials, and counted for radioactivity by liquid scintillation spectrometry. Specific binding was defined as the difference between total and nonspecific binding determined in presence of 10^{-4} M (−)-epinephrine. For saturation experiments the final concentration of [^{3}H]RX821002 ranged from 0.25 to 10 nM. The values of K_d and B_{max} were calculated from computer-assisted analysis of the data using the equilibrium binding data analysis-LIGAND program (24). Inhibition studies were carried out at a radioligand concentration of 2.5-3 nM. According to the Hill coefficient value (nH), the data were analyzed using either the TWOSITEINHIB or INHIBITION program allowing curve fitting to a two-site or a one-site inhibition model (25). The inhibition constants (K_i) were calculated from IC_{50} values using the equation of Cheng and Prussoff (26).

[^{32}P]ADP-ribosylation by pertussis toxin. Pertussis toxin–catalyzed [^{32}P]ADP-ribosylation experiments were performed as described by Ribeiro-Neto (27). Frozen cell membranes were suspended in Tris-HCl buffer (70 mM, pH 8.0) containing 1 mM EDTA, 1 mM DTT, and 0.05% lubrol (vol/vol). Aliquots of the suspension (50 μg of protein) were mixed with 60 μl of Tris-HCl buffer (70 mM, pH 8.0) containing 100 ng of pertussis toxin, 0.5 μM NAD^+, 1 μCi[^{32}P]NAD^+, 1 mM ATP, 0.1 mM GTP, 10 mM thymidine, 1 mM EDTA, 0.1 mM MgCl2, 1 mg/ml L-myristyl phosphatidylcholine, 10 mM nicotinamide, and 25 mM DTT. After a 60-min period of incubation at 30°C, the reaction was stopped by the addition of 20 μl of BSA (0.1 mg/ml) in SDS 2%. Proteins were precipitated with 70 μl of TCA 10% and centrifuged for 10 min at 13,000 *g*. Pellets were washed with diethylether, dried, and dissolved in 20 μl of 50 mM Tris-HCl, 10% SDS, and 50 mM DTT (pH 6.8). Samples were heated 5 min at 90°C and treated with 20 μl of *N*-ethylmaleimide 100 mM. [^{32}P]ADP-ribosylated proteins were separated on a 10% SDS-polyacrylamide gel and revealed by autoradiography using Hyperfilm (Amersham International, Amersham, UK).

Immunoblotting of α subunits of G proteins. The method used was that described by Homburger et al. (28). Membrane proteins were separated by electrophoresis on a 10% SDS-polyacrylamide gel and electrotransferred (6 h at 150 mA) on a nitrocellulose membrane (0.45 μm). The nitrocellulose sheet was treated with 10 mM Tris-HCl, 500 mM NaCl, 2% gelatine (pH 7.5) and then incubated overnight in the same Tris-saline buffer containing 1% gelatine and α-subunit antiserum (AS7 or EC2) at a 1:250 dilution. After a series of washes in 50 mM Tris-HCl, 500 mM NaCl, and 0.05% Tween 20, the blots were incubated with ^{125}I-protein A (50 nCi/ml) in 50 mM Tris-HCl, 500 mM NaCl, and 0.02% NaN_3. After an other series of extensive washes, blots were dried and autoradiographied as indicated above.

Extraction of cellular RNAs. Extraction of cellular RNAs was performed using the method described by Chomczynski and Sacchi (29). The cells were homogenized in 4 ml of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, 100 mM 2-mercaptoethanol, 0.5% sarkosyl, pH 7.0). Homogenates were then successively mixed with 0.4 ml of 2 M sodium acetate (pH 4.0), 4 ml of water-saturated phenol, and 0.8 ml of chloroform/isoamylalcohol (24:1). After separation by centrifugation, the aqueous phase was retrieved and mixed with an equal volume of isopropyl alcohol. The mixture was frozen 1 h at −20°C and centrifuged 20 min at 10,000 *g*. The pellet was subjected to an additional cycle of suspension in solution D and precipitation in isopropyl alcohol. The final RNA pellet was washed with 70% ethanol, dried, redissolved in water, and stored at −80°C until use. The integrity of the preparations was assessed by agarose gel electrophoresis and the concentration of RNA determined by UV spectrophotometry.

Construction of the probes α_{2C2}AR221, α_{2C4}AR370, α_{2C10}AR352, and 36B4. The human α_2-adrenergic receptor genes α_{2C2} and α_{2C4} (30, 31) were kindly provided by Drs. R. J. Lefkowitz and J. W. Lomasney (Duke University, Durham, NC). The α_{2C10} gene (32) was purchased from the American Tissue Cell Collection (plasmid p05761). The 36B4 gene (33) was generously given by Dr. P. Chambon (Strasbourg, France). Subcloning of α_{2C2} and α_{2C4} fragments was done as follows. The plasmid pBCα_{2C2} (30) was digested by BamH1 and HindIII. The 221-base fragment corresponding to nucleotides 1311–1531 of the α_{2C2} sequence was ligated to the BamH1-HindIII sites of pBluescriptKS+ to give the plasmid pKSC2-221. The plasmid pSPα_{2C4} (31) was digested by Mae III. The MaeIII-fragment corresponding to nucleotides 457-1382 of α_{2C4} coding sequence was blunt-ended and then ligated into EcoRV-digested pBluescriptKS+. The obtained plasmid was then digested by SmaI and self-ligated. The resulting plasmid pKSC4-370 contained a 0.37-kb SmaI-MaeIII fragment corresponding to nucleotides 1014–1382 of the α_{2C4} coding sequence. The plasmids pKSC10-352 and PKS36B4 contained respectively a 0.35-kb Pst1-Pst1 fragment of the α_{2C10} gene (34) and a 0.7-kb Pst1-Pst1 fragment of the 36B4 gene (33). For synthesis of the radiolabeled probes, the plasmids were linearized with the appropriated restriction enzyme and antisense RNAs were synthesized in the presence of [^{32}P]rNTP using either T3 or T7 polymerase.

Expression of the human α_2-adrenergic receptor subtypes in COS-7 cells. The plasmids which were used to express the α_2-adrenergic receptor genes in COS-7 cells were pBCα_{2C2}, pDPα_{2C10}, and pSG5α_{2C10}. The constructions of pBCα_{2C2} and of pSG5α_{C10} have been described in previous publications (30, 34). The expression vector pDPα_{2C4} contained the cytomegalovirus (CMV) promoter, the entire coding region of the α_{2C4} gene (NcoI-HindIII fragment), and the BamH1-Xho1 fragment of rabbit β-globin genomic sequence (IVS2-β) to increase the stability of the transcripts. COS-7 cells were transfected using the DEAE-dextran method and were collected 48 h after transfection. The study of [^{3}H]RX821002 binding indicated that the α_2-adrenergic receptor density was 9.4 pmol/mg of protein in cells transfected with the α_{2C2} gene, 0.9 pmol/mg of protein in cells transfected with the α_{2C4} gene, and 2.5 pmol/mg of protein in cells transfected with the $\alpha 2_{C10}$ gene.

RNase protection assay. RNase protection assays were performed by mixing the labeled antisense RNA probe in 30 μl of 0.4 M NaCl, 80% deionized formamide, 1 mM EDTA, 40 mM Pipes, pH 6.7, with 100 μg of cellular RNA. The mixture was heated to 85°C for 5 min and immediately placed at 55°C for 14 h. Samples were digested with 0.5 ml of RNAse A (40 μg/ml) and RNAse T1 (2 μg/ml), in 30 mM NaCl, 5 mM EDTA, 10 mM Tris-HCl (pH 7.5). After 2 h at 37°C, digestion was stopped by the addition of 10 μl of 20% SDS and 5 μl of proteinase K (10 mg/ml), and the samples were further incubated for 15 min at 37°C. Carrier tRNA (10 μg per tube) was added, followed by phenol/chloroform extraction and isopropyl alcohol precipitation. The RNA pellets were dissolved in 20 μl of sample buffer (97% deion-

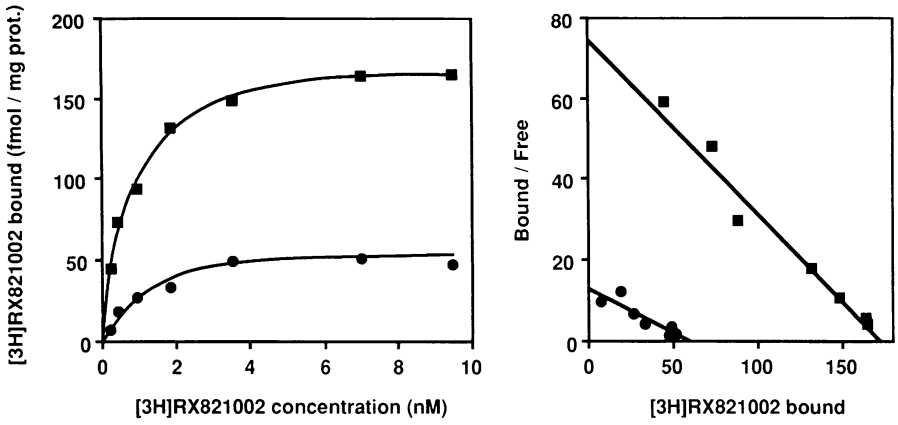

Figure 1. Saturation isotherms (*left panel*) and Scatchard plots (*right panel*) of [^{3}H]-RX821002 binding to epithelial cell membranes. Membranes prepared from human duodenum (●) and proximal colon (■) were incubated in the presence of various concentrations of radioligand. The amount of specifically bound [^{3}H]RX821002 was determined using 10^{-4}M (−)-epinephrine to estimate nonspecific binding. The presented data are from a typical experiment. Computer-assisted analysis of the results from this specific experiment indicated that the B_{max} and K_d values of [^{3}H]RX821002 were, respectively, 177±7 fmol/mg of protein and 0.69±0.11 nM in the colon and 56±4 fmol/mg of protein and 0.99±0.24 nM in the duodenum.

ized formamide, 0.1% SDS, 10 mM Tris-HCl, pH 7.0) and loaded on a 5% acrylamide, 7 M urea gel. The dried gels were exposed to Amersham Hyperfilm at −80°C. When necessary, the intensity of the bands in autoradiograms were quantified by digital analysis of the images using the program IMAGENIA 2000 (Biocom, Les Ulis, France).

Measurement of adenylate cyclase activity. Adenylate cyclase activity was measured according to the method described by Alvarez and Daniels (35). Cell membranes (30 μg of protein) were incubated with 0.1 μCi [α-^{32}P]ATP in 100 μl of Tris-HCl buffer (40 mM, pH 7.4) containing 1.5 mM $MgCl_2$, 5 mM creatine phosphate, 5 U creatine phosphokinase, 1 mM cyclic AMP, 0.5 mM 3-isobutyl-1-methyl-xanthine, 100 μM EGTA, 1 μM GTP, 0.2 mM ATP, and 0.2% bovine serum albumin. Incubations were performed for 15 min at 30°C and were terminated by the addition of 20 μl of a 2.2 N HCl solution containing 10 nCi of [^{3}H]cyclic AMP as an internal standard. The samples were heated 4 min at 95°C and then cooled in ice. The content of each tube was pipetted onto a column of dry neutra alumina (1 g). Cyclic AMP was separated from ATP by elution with 4 ml of 0.1 M ammonium acetate (pH 7). The effluent was collected and counted for ^{3}H and ^{32}P in a liquid scintillation spectrometer. Internal standard recovery ranged from 85% to 90%.

Other methods. Protein concentrations were measured according to Bradford (36). The activity of alkaline phosphatase was estimated using the method of Garen and Levinthal (37).

Drugs and reagents. [^{3}H]RX821002 (57 Ci/mmol) and [α-^{32}P]-UTP (800 Ci/mmol) were obtained from Amersham International. [α-^{32}P]ATP (30 Ci/mmol), [^{32}P]NAD (800 Ci/mmol), and the α-subunit antisera (AS7 and EC2) were from New England Nuclear (Boston, MA). Idazoxan and RX821002 were given by Reckitt and Colman Laboratories (Kingston-upon-Hull, UK). SKF86466 and SKF104078 were donated by Smith Kline and French (Philadelphia, PA). UK14304 was provided by Pfizer (Sandwich, UK). (+)-Epinephrine was a gift from Sterling Winthrop Research Institute (Rensselaer, NY). Prazosin and rauwolscine were respectively obtained from Pfizer (Orsay, France) and Extrasynthèse (Genay, France). Phentolamine and CGP12177 were from CIBA-Geigy (Basel, Switzerland). Pertussis toxin, forskolin, (−)-epinephrine, (−)-norepinephrine, oxymetazoline, yohimbine, chlorpromazine, DTT, EDTA, ascorbic acid, and pyrocatechol were from Sigma Chemical Co. (St. Louis, MO). All other chemicals and biochemicals were reagent or molecular biology grade.

Results

Identification and characterization of the α_2-adrenergic receptor subtype expressed in the human intestinal mucosa. Preliminary studies were carried out in order to assess the binding properties of [^{3}H]RX821002 to human intestinal mucosa. These experiments were performed on epithelial cell membranes prepared from both duodenum and proximal colon. The nonspecific binding of [^{3}H]RX821002 determined in the presence of an excess of (−)-epinephrine was low and linearly correlated to the radioligand concentration. The amount of nonspecific binding measured with the catecholamine was identical to that measured with other competitors, indicating that [^{3}H]RX821002 exclusively labeled adrenergic receptors. Saturation experiments using radioligand concentrations ranging from 0.25 to 10 nM yielded monophasic saturation isotherms (Fig. 1). Analysis of the data revealed the existence of a single class of non-interacting binding sites. The K_d value of [^{3}H]RX821002 for membranes from duodenum (1.22±0.11

Table I. Inhibition of [^{3}H]RX821002 Binding by Adrenergic Drugs

	Duodenum	Colon
	nM	
Antagnoists, K_i		
RX 821002	1.4±0.2	1.1±0.3
Yohimbine	5.9±0.9	3.4±0.3
Rauwolscine	6.1±2.1	ND
Phentolamine	21±3	ND
SKF 86466	26±4	ND
Idazoxan	34±5	37±18
SKF 104078	317±32	ND
Chlorpromazine	1,741±86	4,416±2,421
Prazosin	8,300±124	10,069±7,274
CGP 12177	>10,000	>10,000
Agonists, IC_{50}		
Oxymetazoline	4.0±0.9	2.1±0.7
UK-14,304	80±24	103±15
(−)-Epinephrine	412±73	402±180
(+)-Epinephrine	1,969±101	2,685±231

Membranes prepared from either duodenum or proximal colon mucosa were incubated in the presence of 3 nM [^{3}H]RX821002 and increasing concentrations of the various adrenergic drugs. Inhibition curves were analyzed using INHIBITION, a curve-fitting program for a one-site inhibition model (25). IC_{50} are given for agonists; whereas K_i were calculated from the equation of Cheng and Prussoff (26) for antagonists. The reported values are means±SE from three independent experiments. Abbreviation: ND, not determined.

nM, $n = 6$) was not different from that for membranes from the proximal colon (1.53±0.19 nM, $n = 6$). By contrast, the number of binding sites in the proximal colon (174±34 fmol/mg of proteins) was significantly higher than in duodenum (65±11 fmol/mg of proteins).

The pharmacological profile of the sites labeled by [^{3}H]-RX821002 was delineated by testing the ability of various adrenergic drugs to impair the binding of the radioligand (Table I). Antagonists yielded competition curves with a Hill coefficient near unity and inhibited binding of [^{3}H]RX821002 to duodenal cell membranes with the following rank order of potency: RX821002 > rauwolscine = yohimbine > phentolamine ≈ idazoxan ⩾ chlorpromazine > prazosin > CGP 12177. A similar pharmacological profile was observed in the proximal colon. Inhibition by agonists demonstrated that the α_{2A}-selective agonist, oxymetazoline, exhibited a very high affinity for the receptor. Moreover, the identified receptor displayed stereospecificity, (−)-epinephrine being several times more potent than its (+)-enantiomer. Together these results strongly suggested that the α_2-adrenergic receptor in the human intestinal mucosa is of the α_{2A} subtype. RNase mapping experiments were carried out in order to give further support to binding data (Fig. 2). As shown by the use of COS cells expressing the different subtypes of human α_2-adrenergic receptor, the three probes α_{2C2}AR221, α_{2C4}AR370, and α_{2C10}AR352 are specific. Hybridization of these probes with cellular RNAs prepared from duodenum and colon demonstrated the presence of α_{2C10} transcripts and showed that α_{2C2} and α_{2C4} genes were not expressed in human intestinal mucosa. A similar conclusion can be made for HT29, a cell line known to express exclusively α_{2A}-adrenergic receptors.

Study of the coupling of the α_2-adrenergic receptor to G proteins. The molecular mechanisms by which α_2-adrenergic receptor stimulation inhibits intestinal secretion are not fully understood yet. Experiments were therefore conducted in order to clarify the transduction pathway whereby α_2 agonists may trigger their effects in human intestinal mucosa. Inhibition of [^{3}H]RX821002 binding by the endogenous agonist, (−)-norepinephrine, yielded shallow curves (Fig. 3) indicative of the existence of an heterogeneous population of binding sites for agonists. Computer-assisted analysis of the data obtained in the duodenum, jejunum, ileum, and colon were better fitted with a two-site than with a one-site inhibition model. The calculated values of the inhibition constants of (−)-norepinephrine at the high- and low-affinity state receptor are reported in Table II. The percentage of high-affinity state receptors represented ~ 60% of the whole receptor population. Addition of 10^{-4} M GTP and 100 mM NaCl shifted the inhibition curves to the right (Fig. 3). The Hill coefficient values were increased and the whole receptor population was converted into a low-affinity conformation (Table II). A similar phenomenon was observed in membranes treated with pertussis toxin (data not shown) demonstrating that α_2-adrenergic receptors of human intestinal mucosa are coupled to GTP-binding proteins which are sensitive to pertussis toxin treatment.

The nature of the Gi protein expressed in epithelial cell

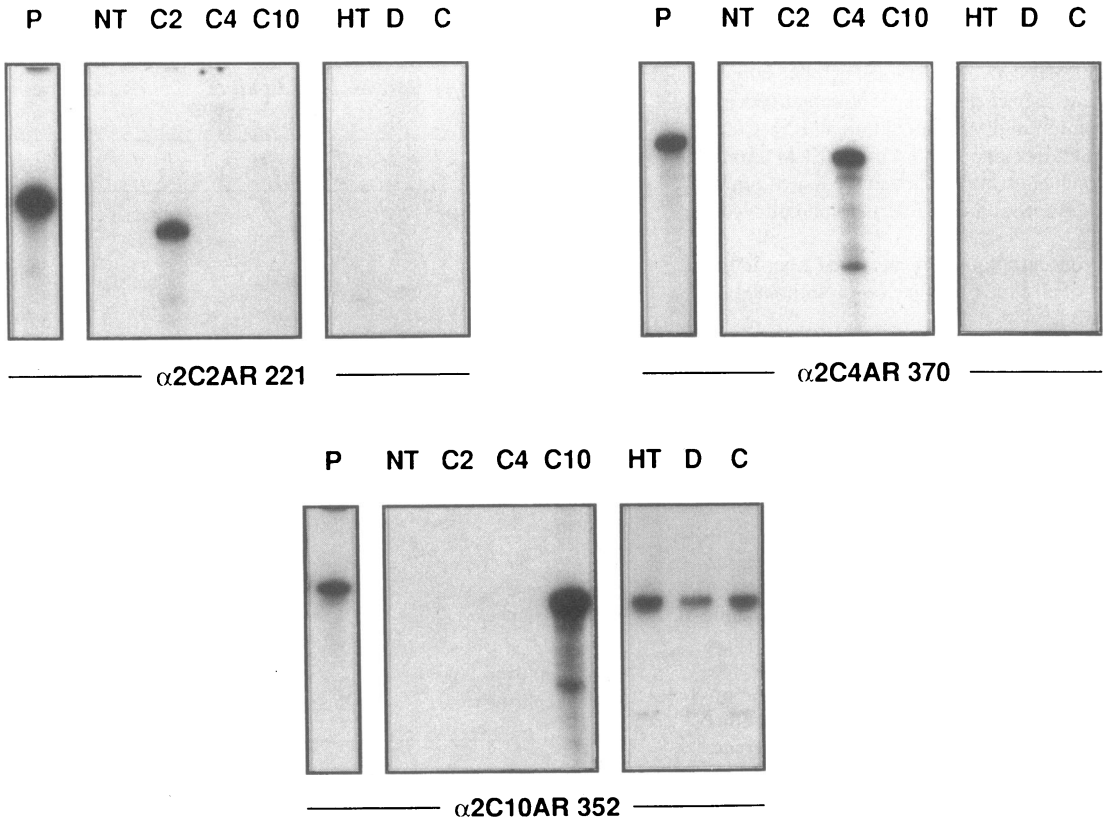

Figure 2. RNase protection analysis of α_2-adrenergic receptor mRNAs with the probes α2C2AR221, α2C4AR370, and α2C10AR352. Each of the labeled probes was hybridized with either 10 μg of RNA from COS-7 cells untransfected (*NT*), transfected with pCBα2C2 (*C2*), pDPα2C4 (*C4*), and pSG5α2C10 (*C10*); or 50 μg of RNA from HT29 cells (*HT*) or 100 μg of RNA from duodenum (*D*) and colon (*C*) mucosa. Samples were digested with a mixture of RNases A and T1. The resistant hybrids were electrophoresed and gels were autoradiographied as described in Methods. Lanes *P* correspond to undigested probes.

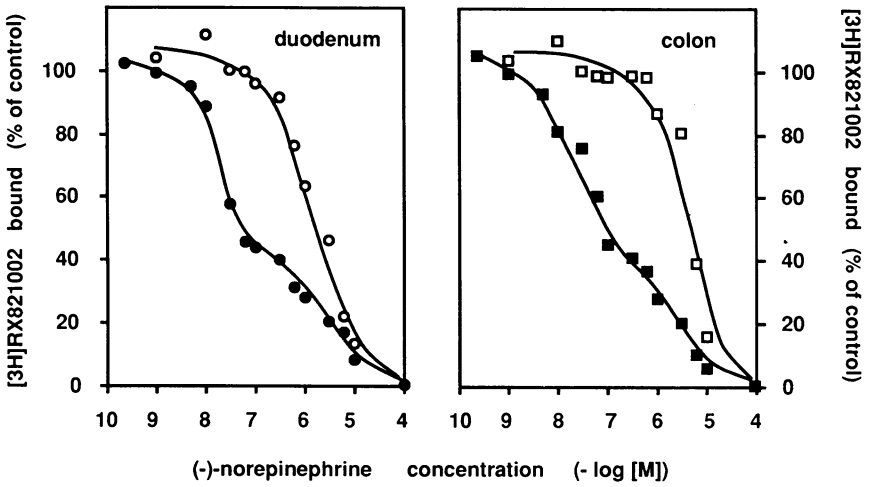

Figure 3. Inhibition of [^{3}H]RX821002 binding by the endogenous agonist, (−)-norepinephrine. Membranes from duodenum and colon mucosa were incubated in the presence of 2.5 nM [^{3}H]RX821002 and increasing concentrations of (−)-norepinephrine. The experiments were carried out either in the absence (*solid symbols*) or in the presence of 10^{-4} M GTP plus 100 mM NaCl (*open symbols*). The presented curves were from a specific experiment. The binding parameters of (−)-norepinephrine calculated from computerized analysis of the curves are summarized in Table II.

membranes was next examined. Pertussis toxin–catalyzed [^{32}P]ADP-ribosylation of membranes from duodenum or colon revealed the presence of two distinct peptides with apparent molecular masses of 40 and 41 kD (Fig. 4). Comparison of the relative mobility of these proteins with that of the proteins labeled in membranes from rat jejunum mucosa (a tissue which expresses $\alpha i2$ and $\alpha i3$) and from bovine brain (a tissue which expresses $\alpha i1$, $\alpha i2$, $\alpha i3$, and $\alpha 0$) indicated that the band of greater mobility corresponded to $\alpha i2$. Since there are two 41-kD peptides ($\alpha i1$ and $\alpha i3$) which are substrates for pertussis toxin, the exact nature of the second band needed to be identified by immunodetection with specific antibodies (Fig. 5). Immunoblotting with the anti-$\alpha i1/\alpha i2$ antiserum (AS7) revealed the presence of a single immunoreactive peptide corresponding to $\alpha i2$ and suggested that the second polypeptide labeled by pertussis toxin is $\alpha i3$. The presence of $\alpha i3$ was confirmed by the use of an anti-$\alpha 0/\alpha i3$ antiserum (EC2). Thus, human enterocytes and colonocytes express Gi2 and Gi3, but not Gi1 and G0.

Distribution and subcellular localization of the α_2-adrenergic receptor. Previous reports on rat jejunum (12) and on rabbit colon (13) have shown the existence of an heterogenous distribution of the α_2-adrenergic receptor along the villus–crypt axis of the intestinal mucosa. Further experiments were performed to see whether this was also the case in humans. In this series of experiments, we also examined the level of expression of the receptor in the different segments of the intestinal tract as well as its subcellular localization in the polarized cell.

Results from the quantification of the receptor in the different segments of the gut are summarized in the Table III. As already noted in experiments dealing with the characterization of the receptor, it is obvious that the level of expression of the receptor widely varies according to the segment considered. In the small intestine, duodenum was the segment expressing the highest level of receptors. A twofold lower density was found in jejunum and only trace amounts were detectable in ileum. In the large bowel, the density of receptor which was maximal in the proximal segment, then slowly decreased in transverse and distal segments. Whatever the segment considered there was no change in the K_d value of the radioligand and yohimbine was a thousandfold more potent than prazosin to inhibit [^{3}H]-RX821002 binding indicating that the same receptor subtype was expressed through the whole intestinal tract.

There is so far no data dealing with the subcellular distribution of the α_2-adrenergic receptor in polarized cells. The localization of the receptor was studied by measuring the number of [^{3}H]RX821002 binding sites in basolateral and brush border membranes prepared from epithelial cells of the proximal colon mucosa. The results from this study are summarized in the Table IV. As expected the brush border membrane-enriched fraction exhibited a very high level of alkaline phosphatase activity. This increase in the specific activity of the enzyme was associated with a decrease in capacity to bind the labeled α_2 antagonist. Conversely, the basolateral membrane fraction displayed a reduced phosphatase activity and a higher binding site number. The α_2-adrenergic receptor is therefore preferentially located in the basolateral compartment of the polarized intestinal cell.

The distribution of α_2-adrenergic receptors along the crypt to surface axis was investigated in duodenum and proximal colon by studying [^{3}H]RX821002 binding on four different membrane fractions generated by sequential isolation of the epithelial cells (Fig. 6). The alkaline phosphatase activity was measured as an index of cell differentiation. In both segments, the activity of the enzyme was higher in fractions I and II corresponding to surface cells than in fractions III and IV which are enriched in crypt cells. The analysis of [^{3}H]RX821002 saturation isotherms indicated that the α_2-adrenergic receptor density decreased from the crypt to the surface cell fractions. Again, no significant difference in K_d values was observed between the fractions.

α_{2C10} mRNA level and α_2-adrenergic receptor-mediated inhibition of adenylate cyclase along the surface–crypt axis of the colon mucosa. The last part of this study was designed in order to better understand the mechanisms leading to higher receptor expression in crypt cells and to examine the functional conse-

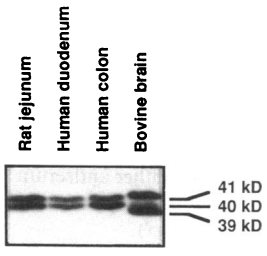

Figure 4. [^{32}P]ADP-ribosylation of G proteins by pertussis toxin. Membranes of epithelial cells from rat jejunum, human duodenum, human proximal colon, and membranes from bovine brain were ADP-ribosylated with thiol-preactivated pertussis-toxin and [^{32}P]NAD$^+$. The labeled proteins were resolved on SDS-PAGE and the gel was autoradiographied as described in Methods.

Table II. Inhibition of [^{3}H]RX821002 Binding by Norepinephrine in the Absence or in the Presence of GTP and Na^+

Intestine segment	Without GTP/Na				With GTP/Na	
	nH	K_{iH}	K_{iL}	Percent RH	nH	K_i
		nM	*nM*	%		*nM*
Duodenum	0.64	7.5±2.4	826±319	62±15	0.95	492±104
Jejunum	0.71	7.8±1.5	931±214	63±18	0.98	808±144
Ileum	0.75	6.1±1.3	387±98	60±7	0.97	775±309
Colon	0.59	9.9±2.3	582±158	59±14	1.02	823±114

Membranes prepared from duodenum, jejunum, ileum, and colon epithelium were incubated with [^{3}H]RX821002 and increasing concentrations of (−)-norepinephrine as described in the Fig. 2. According to the Hill coefficient value (nH), inhibition curves in the absence of GTP and Na^+ were better fitted by a two-site inhibition model and constant values were calculated using TWOSITEINHIB (25). Inhibition data in the presence of GTP plus Na^+ were consistent with the existence of a single class of sites and were analyzed using INHIBITION (25). Inhibition constant values (K_i), K_{iH} and K_{iL}, represent, respectively. The inhibition constant at the high- and low-affinity state receptor. The proportion of receptor under the high-affinity state (RH) is also given. The reported values are means ± SE from three experiments.

quences of this heterogenous distribution. All these experiments were performed on cell fractions isolated from the proximal colon. RNase mapping experiments were carried out using the radioprobes α2C10AR352 to quantify the α_{2A}-adrenergic receptor mRNA and 36B4 as an internal standard to verify the quality of the RNA preparations (Fig. 7). Analysis of the autoradiograms from five independent experiments clearly showed that the amount of α_{2C10} transcripts was higher in crypt than in surface cells. There is therefore a correlation between the level of expression of the receptor and the amount of its mRNA. The biological efficacy of the receptor was estimated in cell fractions I (surface cells) and IV (crypt cells) by measuring the inhibitory effect of UK14304 on forskolin-stimulated adenylate cyclase. As reported in the Fig. 8, the basal activity of the enzyme was similar in both membrane preparations (5.6±0.3 pmol of cAMP produced per mg/min). Forskolin induced a five- and threefold increase in activity in crypt and surface cells, respectively. The α_2 agonist, UK14304, was able to significantly reduce the forskolin-stimulated adenylate cyclase activity in a dose-dependent manner in crypt cells. It was by contrast inefficient in surface cells.

Discussion

Several open clinical trials have demonstrated that α_2 agonists may represent a promising new class of antidiarrheal agents. Clonidine efficiently inhibited secretory diarrhea in patients with lung cancer (16) and with insulin-dependent diabetes (17). Unfortunately, the currently available α_2 agonists have undesirable central side effects and the therapeutic use of these compounds for treating diarrheal diseases obviously requires the development of drugs displaying a higher gut selectivity. Molecular biology having brought evidence for the existence of α_2-adrenergic receptor subtypes with different pharmacology in human (30–32), the development of such drugs depends upon the subtype of receptor expressed in the gut. Surprisingly, there are so far no data concerning the pharmacological properties of this receptor. The aim of the present study was therefore to characterize the receptor expressed in human intestinal mucosa. Its distribution and coupling to Gi proteins were also examined.

Whatever the intestinal segment considered, the selective α_2-adrenergic antagonist, [^{3}H]RX821002, bound to a single class of sites. Inhibition experiments with a series of adrenergic drugs demonstrated that the rank order of potency of antagonists to inhibit [^{3}H]RX821002 binding was RX821002 > rauwolscine = yohimbine > phentolamine ≈ idazoxan ⩾ chlorpromazine > prazosin. The labeled sites exhibited moreover a high affinity for oxymetazoline, suggesting that the human intestinal receptor is of the α_{2A} subtype. This conclusion based on binding data was further supported by RNase mapping experi-

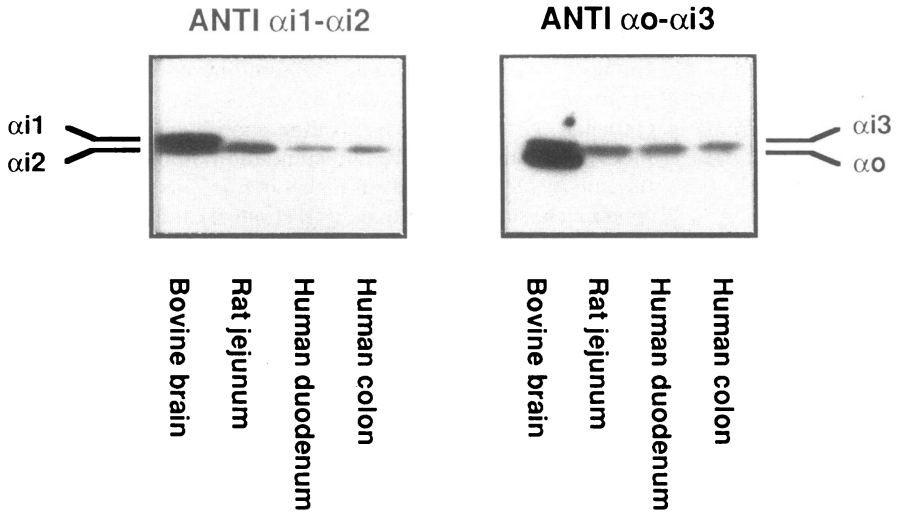

Figure 5. Immunoblot analysis of the α subunits of Gi proteins with specific antisera. Membrane proteins from bovine brain, rat jejunum, human duodenum, and human proximal colon were separated by SDS/PAGE and transferred on nitrocellulose. Immunoblottings were performed as described in Methods with either antiserum AS7 (anti αi1-αi2, *left panel*) or antiserum EC2 (anti α0-αi3, *right panel*).

Table III. Level of Expression of the α_2-Adrenergic Receptor in the Different Segments of the Intestinal Tract

Intestine segment	B_{max}	K_d
	fmol/mg protein	*nM*
Duodenum	65±11	1.22±0.11
Jejunum	31±4	1.22±0.26
Ileum	13±4	0.78±0.18
Proximal colon	174±34	1.53±0.19
Transverse colon	139±17	0.94±0.17
Distal colon	51±9	1.25±0.14

Intestinal tracts coming from six organ donors were cut into segments. Membranes prepared from the mucosa were incubated with concentrations of [^{3}H]RX821002 ranging from 0.25 to 8 nM. Specific binding was determined using 10^{-4} M (−)-epinephrine as competitor. Saturation isotherms were analyzed using the EBDA-LIGAND program (24). K_d and B_{max} values are respectively expressed in nanomolar and in femtomoles of binding sites per milligram of protein. Reported values are means±SE of individual determinations on six samples of each segment.

ments which demonstrated that the α_{2C10} gene, but not the α_{2C2} or α_{2C4}, was expressed in intestinal mucosa.

The biochemical mechanisms whereby α_2-adrenergic receptor stimulation inhibits intestinal secretions are still matter of controversy. The receptor was shown to be negatively coupled to adenylate cyclase in human colonic crypts (38) and in the colon adenocarcinoma cell line HT29 (39). Nakaki and co-workers (9) proposed the effect of α_2 agonists on secretions to be triggered by cAMP-independent mechanisms. Other mechanisms that were proposed include interference with Ca^{++}-dependent pathways (40) and a direct coupling of G proteins to a ion channel (1). Interestingly, a GTP-sensitive Cl^- channel distinct from the cAMP-dependent Cl^- channel was recently characterized in the apical membranes of differentiated HT29 cells (41). As shown by the present data, the human intestinal α_2-adrenergic receptor is coupled to pertussis toxin-sensible G proteins which were identified as Gi2 and Gi3. Experiments on reconstituted systems (42) or on native membranes (43) have demonstrated that the α_2-adrenergic receptor can interact with multiple Gi proteins, each playing an individual function in signal transduction. According to this later point, Gi2 is thought to be preferentially responsible for the inhibition of adenylate cyclase (44), whereas Gi3 would be more specifically involved in the regulation of ion-channel activity (45). The discrete role of Gi2 and Gi3 in the human mucosa needs complementary work to be elucidated, but the preferential coupling of Gi3 to a Cl^- channel could represent an interesting working hypothesis for the future. Also, recent data from our groups have demonstrated that in the rat jejunum mucosa the expression of both Gi2 and Gi3 is higher in villi than in crypts (46). The possibility of a similar distribution remains to be established in human.

Table IV. Subcellular Distribution of the α_2-Adrenergic Receptor

Membrane preparation	α_2-Adrenergic receptor	Alkaline phosphatase activity
	fmol/mg protein	*mU/mg protein*
Crude membrane fraction	127±17	520±105
Basolateral membrane	364±53	220±70
Brushborder membrane	24.7±9.1	9,850±1,750

Epithelial cells were isolated from the proximal colon mucosa, then crude and purified membranes were prepared as described in Methods. The density of α_2-adrenergic receptor was determined using [^{3}H]RX821002 and alkaline phosphatase activity measured using p-nitrophenyl phosphate as substrate (37). Reported values are means±SE from three independent experiments.

The second aim of this work was to study the distribution of α_2-adrenergic receptors in the intestinal mucosa. In the small intestine, α_2-adrenergic receptor number decreases from duodenum to ileum. A similar decrease was also observed from the proximal to the distal colon. Moreover, in proximal and transverse colon the density in α_2-adrenergic receptors was at least threefold higher than in small bowell. The physiological relevance of such a distribution remains unclear since a wide number of factors are involved in the regulation of water and electrolytes absorption. The abundance of receptor in the proximal colon is, however, consistent with the crucial role of this large bowel segment in the regulation of water absorption.

The study of the distribution of the α_2-adrenergic receptor within duodenal and colonic mucosa showed that the receptor

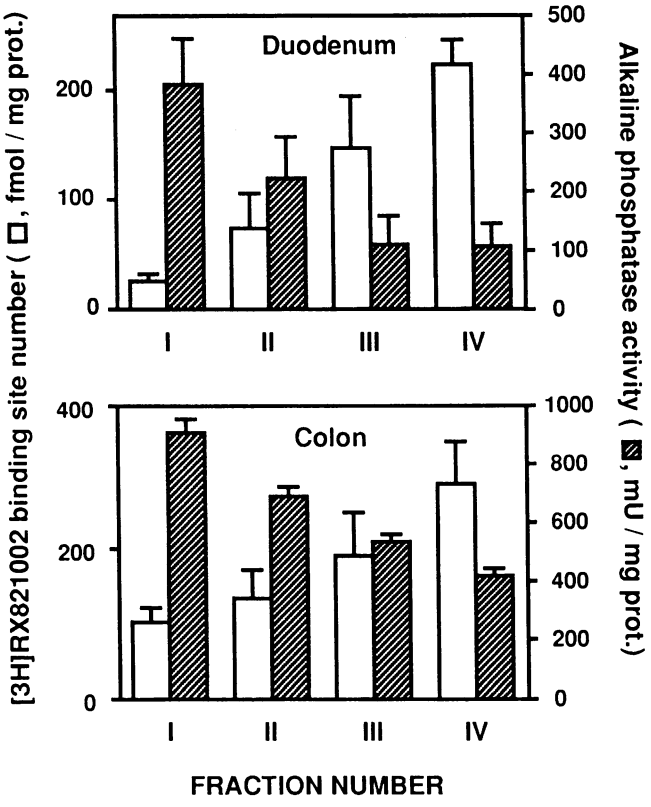

Figure 6. Distribution of α_2-adrenergic receptors along the villus–crypt axis. Cells fractions from duodenum and proximal colon mucosa were separated by sequential isolation as described in Methods. This procedure generated four fractions (I–IV), which were tested for their capacity to bind [^{3}H]RX821002 and for their alkaline phosphatase activity. The density of α_2-adrenergic receptors is expressed in femtomoles per milligram of protein (*open bars*), whereas alkaline phosphatase activity is in milliunits per milligram of protein (*hatched bars*). Reported values are means±SE from six independent experiments.

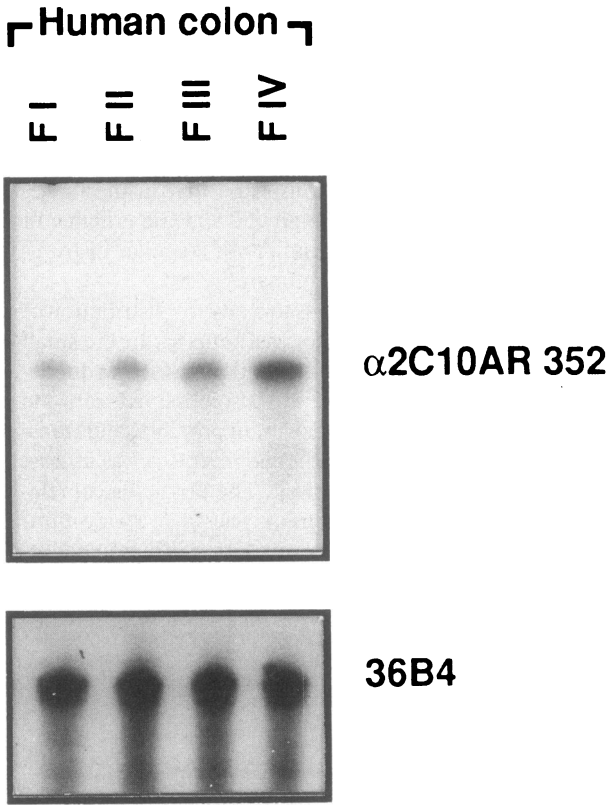

Figure 7. Quantification of α_{2C10}-adrenergic receptor mRNA in cell fractions isolated from proximal colon. 100 or 10 μg of RNA prepared from fractions I, II, III, and IV of the proximal colon were respectively hybridized with the labeled probes, α2C10AR352 (*upper autoradiogram*) and 36B4 (*lower autoradiogram*). All the samples were digested with a mixture of RNases A and T1. The resistant hybrids were electrophoresed and gels were autoradiographied as described in Methods. The results shown are representative of five experiments.

is preferentially expressed in crypts, where it is negatively coupled to adenylate cyclase. A similar pattern of distribution was also found in rat jejunum (12) and rabbit colon (13). The mechanisms responsible for the loss of receptor during cell differentiation are unknown. The present results show for the first time that the gradient of receptor expression reflects changes at its mRNA level suggesting that the regulation occurs at the transcriptional level. However, further studies will be necessary to confirm this later point. The distribution of the α_2-adrenergic receptor in human intestinal mucosa is identical to that of the EGF receptor (47). By contrast, it strictly differs from that of the vasoactive intestinal peptide receptor which is more abundant in villus cells than in crypts (Salomon et al., unpublished results). As previously underscored, the distribution of α_2-adrenergic receptor strongly suggests that the antisecretory action of α_2 agonists takes place in crypts where Cl^- secretion is thought to occur. It is also in agreement with electron-microscopy observations indicating that noradrenergic fibers preferentially innervate the basal granulated crypt cells (48). The subcellular distribution of [^{3}H]RX821002 binding sites indicates that α_2-adrenoceptors are located in the basolateral membrane of the polarized cells. Such a localization is consistent with the availability of its natural agonists at this site and with a coupling of the receptor to Gi2 and Gi3 which are predominantly located in this membrane fraction (49).

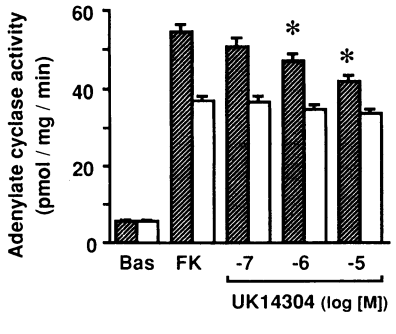

Figure 8. Inhibition of forskolin-stimulated adenylate cyclase activity by the α_2 agonist, UK14304. The activity of adenylate cyclase was measured in fraction I (*open bars*) and fraction IV (*hatched bars*) isolated from proximal colon mucosa. The production of cAMP was determined under basal conditions (*Bas*), in the presence of 10 μM forskolin (*FK*) or in the presence of 10 μM forskolin plus various concentrations of UK14304. The enzyme activity is expressed in picomoles of cAMP produced per milligram of protein per minute. Results are means±SE from six independent experiments. Statistical analysis was performed using the Student's *t* test (asterisk indicates a significant inhibition at $P < 0.05$ of the forskolin-stimulated cAMP production).

The presence of α_2-adrenergic receptors on crypt cells which are immature proliferating cells also raises the question of a possible involvement of this receptivity in the regulation of their mitogenic activity. Such a role has not yet been explored in detail. Three pieces of data may, however, support this hypothesis. First, low levels of cyclic AMP have been correlated with the high rate of cell division in crypt cells (50). Secondly, α_2 agonists were shown to act as co-mitogen in fibroblasts transfected with the α_2-adrenergic gene α_{2C10} (51). Finally, in vivo studies on rat have shown that infusion of noradrenaline accelerates crypt cell proliferation in jejunum (52). This effect was, moreover, demonstrated to be triggered through the stimulation of receptors displaying an α_2-adrenergic pharmacology.

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