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# Functional and molecular evidence for $\beta_1$-, $\beta_2$- and $\beta_3$-adrenoceptors in human colon

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**1** Relaxation of carbachol pre-contracted human colonic muscle to (−)-isoprenaline was examined in circular, longitudinal and taenia coli preparations to determine the $\beta$-adrenoceptor subtypes involved. $\beta_1$-, $\beta_2$- and $\beta_3$-Adrenoceptor mRNAs were also measured in colonic muscle and mucosa.

**2** (−)-Isoprenaline caused relaxation of longitudinal smooth muscle preparations with $pEC_{50} = 7.39 \pm 0.12$, and this response was inhibited by both propranolol (0.1 $\mu$M, $pK_B$ $8.55 \pm 0.12$) and the selective $\beta_1$-antagonist, CGP 20712A (0.1 $\mu$M, $pK_B$ $8.80 \pm 0.20$), while the selective $\beta_2$-antagonist, ICI 118551 (0.1 $\mu$M) failed to inhibit isoprenaline relaxation consistently.

**3** (−)-Isoprenaline caused relaxation of taenia coli with a $pEC_{50}$ of $6.70 \pm 0.17$. Propranolol (0.1 $\mu$M), CGP 20712A (0.1 $\mu$M) and ICI 118551 (0.1 $\mu$M) inhibited the isoprenaline response with similar low affinities ($pK_B$ values 7.93, 7.71 and 7.54, respectively). Carbachol pre-contracted circular smooth muscle preparations failed to relax consistently to isoprenaline and these responses were not characterized.

**4** $\beta_1$- and $\beta_2$-Adrenoceptor mRNAs were present in circular/longitudinal muscle samples and taenia coli samples, and lower levels were detected in mucosa. $\beta_3$-mRNA was also present in both muscle preparations but was not detected in human colonic mucosa.

**5** In summary, $\beta_1$-adrenoceptors are the predominant subtype mediating isoprenaline-induced relaxation of the thin longitudinal smooth muscle of human colon, while $\beta_3$-receptors do not appear to be involved in these responses. However, $\beta_3$-adrenoceptors may play a role in relaxation of the taenia coli as conventional antagonist affinities are low. $\beta_3$-Adrenoceptor mRNA was present in taenia coli and circular/longitudinal smooth muscle but absent from human colonic mucosa.

**Keywords:** $\beta_3$-Adrenoceptors; messenger RNA; human colon; smooth muscle; mucosa

## Introduction

The sympathetic nervous system has an inhibitory effect on human gastrointestinal motility. Previous studies in human isolated gastrointestinal preparations have found that stimulation by $\beta$-adrenoceptor agonists induces relaxation of muscle in ileum (Bennett, 1965), jejunum (Whitney, 1965) and colonic circular (Fishlock & Parks, 1963) and longitudinal smooth muscle (Bucknell & Whitney, 1964). The relative activity of noradrenaline and adrenaline in various human gastrointestinal smooth muscle preparations has suggested that both $\beta_1$- and $\beta_2$-adrenoceptors are present in these tissues (Hedges & Turner, 1969). More recent studies have indicated that atypical $\beta$-adrenoceptors mediate relaxation in gastrointestinal tissues in a variety of species, including guinea-pig ileum and gastric fundus, rat gastric fundus, ileum, jejunum, colon and oesophagus (see Manara *et al*., 1995 for review). In addition to relaxation, atypical $\beta$-adrenoceptor agonists stimulate gastric acid secretion in stomach (Canfield & Paraskeva, 1992), bicarbonate secretion in colon (Canfield & Abdul-Ghaffar, 1992) and have protective effects against indomethacin-induced gastric damage in the rat (Bahl *et al*., 1996). The characteristics of the atypical $\beta$-adrenoceptor in these tissues resemble those of the cloned $\beta_3$-receptor.

The $\beta_3$-adrenoceptor was first isolated and cloned from a human genomic library (Emorine *et al*., 1989) and is 51% and 46% homologous to the human $\beta_1$- and $\beta_2$-adrenoceptors, respectively. The human $\beta_3$-adrenoceptor gene, like that of the rodent, contains several exons which may represent potential splice sites during $\beta_3$-adrenoceptor expression (Granneman *et al*., 1993). There are also sequence differences in the promoter regions of human and rodent $\beta_3$-adrenoceptor genes suggesting the possibility of species differences in the regulation of $\beta_3$ gene expression (Granneman & Lahners, 1994). The human $\beta_3$-adrenoceptor has been extensively characterized in expression systems and differs from the rodent $\beta_3$-receptor with regard to the potency of selective $\beta_3$-agonists. For instance BRL 37344 is less effective in stimulating adenylate cyclase via the human than via the rodent $\beta_3$-adrenoceptor (Liggett, 1992). Also, the $\beta_1$- and $\beta_2$-antagonist, CGP 12177 is an effective agonist at the human $\beta_3$-adrenoceptor whereas it displays poor partial agonist activity at the rat $\beta_3$-adrenoceptor (Liggett, 1992; Granneman *et al*., 1993). Recent studies have detected $\beta_3$-adrenoceptor mRNA in several human tissues including infant perirenal brown adipose tissue (BAT), adult adipose tissue deposits, gall bladder, colon, ileum, stomach and prostate (Granneman *et al*., 1993; Krief *et al*., 1993; Berkowitz *et al*., 1995). $\beta_3$-Adrenoceptor mRNA occurs in human colon and gall bladder independently of the BAT marker uncoupling protein (UCP) (Krief *et al*., 1993).

Although a few studies have investigated the role of $\beta_3$-adrenoceptors in human adipose tissue little has been done to characterize the $\beta$-subtypes mediating responses in human gastrointestinal tissues. Spontaneous activity in human colonic circular smooth muscle is inhibited by isoprenaline and noradrenaline and these effects are blocked by propranolol with an affinity lower than expected for human $\beta_1$- or $\beta_2$-adrenoceptors (McLaughlin *et al*., 1988). However, these preparations do not respond to BRL 37344 and responses to isoprenaline are blocked by the $\beta_1$-selective antagonist, betaxolol (McLaughlin *et al*., 1991). A more recent study with human colonic circular muscle showed that isoprenaline relaxation in the presence of $\beta_1$- and $\beta_2$-antagonists was further inhibited by the $\beta_3$-adrenoceptor antagonist, SR 59230A (De Ponti *et al*., 1996).

The aim of the present study was to examine the effects of $\beta$-adrenoceptor stimulation in functional studies with three different muscle preparations of the human colon (circular smooth muscle, longitudinal smooth muscle and taenia coli) to determine the $\beta$-subtypes involved in mediating relaxation of these tissues. In addition these areas of human colon have been

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examined by use of reverse transcription-polymerase chain reaction (PCR) for the presence of mRNA coding for each of the three β-adrenoceptor subtypes. A preliminary account of this work has been presented (Summers *et al*., 1996).

## Methods

### *Organ bath studies of human colonic smooth muscle preparations*

*Tissue preparation* Specimens of human colon were obtained from patients undergoing surgery for cancer of the colon or rectum at the Royal Melbourne or St Vincent's Hospitals, Melbourne, Victoria, Australia. Specimens were collected from the Anatomical Pathology Department having arrived directly from the operating theatres and generally consisted of a section of the whole wall of the colon from a normal region. They were placed immediately into preoxygenated Krebs Henseleit Buffer and transported on ice back to the laboratory. Mucosa and submucosa were gently removed and muscular regions between the taenia coli were cut into strips of approximately 1 mm width along either the circular or longitudinal axis (the circular muscle being the thicker of the muscularis externa at the point of dissection). The taenia coli are three distinct bands of longitudinal muscle that run the entire length of the human colon and terminate at the rectum. When taenia coli were present in the colon specimens they were cut into thin longitudinal strips and the surrounding tissue cut away to provide better perfusion for the thick muscle.

Human colonic muscle strips were mounted on tissue hooks and suspended in jacketed organ baths containing 25 ml Krebs Henseleit solution (composition mM: NaCl 118.4, KCl 4.7, $MgSO_4.7H_2O$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 25, glucose 11 and $CaCl_2$ 2.5) maintained at 37°C and bubbled continuously with 95% $O_2$/5% $CO_2$ (pH 7.4) under 0.5 g tension. Ugo Basile isotonic transducers connected to a MacLab system with an Apple Macintosh Computer were used to measure isotonic changes in the length of the tissues. Tissue strips were allowed to equilibrate for 30–45 min in the presence of desipramine (0.5 μM) to block neuronal uptake and hydrocortisone (30 μM) to block extra-neuronal uptake. Phentolamine (10 μM) was also present to remove any possible contribution from α-adrenoceptors. When antagonists were used they were also present during this equilibration period.

*Concentration-response curves of human colonic smooth muscle relaxation* Concentration-response (C-R) curves were constructed with (−)-isoprenaline in paired tissue strips, from the same patient, in the absence and presence of various concentrations of the $\beta_1/\beta_2$-antagonist, propranolol, the selective $\beta_1$-antagonist, CGP 20712A, or the selective $\beta_2$-antagonist, ICI 118551. Tissues were equilibrated for 45 min in the presence of the antagonists and then precontracted with carbachol (0.3 μM). After 15–20 min, (−)-isoprenaline C-R curves were commenced with only one C-R curve being constructed in each tissue strip. Isoprenaline concentrations (0.1 nM–100 μM) were increased cumulatively in 0.5 log increments. The contact time of each concentration ranged from 3–5 min and was the time required for the response to plateau. Tissue responses were standardized against a final maximum relaxation to papaverine (50 μM). The maximum achievable relaxation obtained with (−)-isoprenaline ranged from 60–75% in relation to papaverine (50 μM).

### *Analysis of organ bath studies*

Relaxation was measured as the reduction of the precontracted baseline tone of the muscle and the activity of the agonists was expressed as a $pEC_{50}$ value (log concentration of the agonist required to produce 50% of the maximal relaxation effect) obtained by analysis with a non-linear curve fitting programme in PRISM (Intuitive Software for Science). Antagonist activity was measured by comparing the shift of the C-R curve in the presence of the antagonist and a dose-ratio (DR) was calculated for each individual antagonist concentration. As single concentrations of antagonist were used 'apparent $pA_2$' (or $pK_B$) values were obtained from a single concentration of antagonist by the equation,

$$pK_B \quad \log \text{ antagonist ratio} \quad 1 \quad \log \text{ antagonist}$$

used according to the method of Furchgott (1972).

C-R curves were plotted as means ± s.e.mean of *n* individual experiments. All values are expressed as means ± s.e.mean. All *n* values refer to the number of individual patients from whom colon specimens were obtained.

### *RNA extractions*

Frozen tissue was ground to a fine powder in a stainless steel mortar and pestle pre-cooled in liquid nitrogen. Total RNA was extracted by the method of Chomczynski & Sacchi (1987). To avoid any cross-contamination, the homogenizer probe was dismantled and washed thoroughly between each sample. The yield and quality of the RNA were assessed by measuring absorbance at 260 and 280 nm, and by electrophoresis on 1.2% agarose gels. Total RNA from each tissue was treated with DNase to remove any possible contaminating genomic DNA. The reaction mix contained 20 μg RNA, 100 mM sodium acetate (pH 7.0), 5 mM $MgSO_4$, 5 mM dithiothreitol, 36 u RNasin (Promega), and 10 u DNase I (Pharmacia) in a total volume of 40 μl. Following digestion at 37°C for 30 min, the solution was diluted to 400 μl with $H_2O$ and extracted with an equal volume of phenol:chloroform (1:1). The RNA was precipitated with 1.0 ml of ethanol and 40 μl of 2 M sodium acetate. The yield and quality of DNase-treated RNA were determined as above.

### *Reverse transcription/PCR*

cDNAs were synthesized by reverse transcription of 1.0 μg of each total RNA with oligo $(dT)_{15}$ as a primer. The RNA in a volume of 7.5 μl was heated to 70°C for 5 min then placed on ice for 2 min before the addition of a reaction mix containing 1× RT buffer supplied by Promega), 1 mM dNTPs, 5 mM $MgCl_2$, 18 u RNasin (Promega), 20 u AMV reverse transcriptase (Promega), and 50 μg $ml^{-1}$ oligo$(dT)_{15}$ in a volume of 12.5 μl. Following brief centrifugation, the reactions were incubated at 42°C for 45 min, then at 95°C for 5 min. The completed reverse transcription reactions were stored at −20°C and used for PCR without further treatment.

PCR amplification was carried out on cDNA equivalent to 100 ng of starting RNA, with oligonucleotide primers specific for human $\beta_1$, $\beta_2$ and $\beta_3$-adrenoceptors, adipsin and transferrin receptor (Table 1; synthesized at the Howard Florey Institute, Melbourne or by Life Technologies, Gaithersburg, MD, U.S.A.). PCR mixes contained 1 u of Taq polymerase (Promega), the buffer supplied (10 mM Tris-HCl (pH 9), 50 mM KCl and 0.1% Triton X-100), 200 μM dNTPs, 2 mM Mg-acetate (3 mM for $\beta_2$-receptor), 2.5 pmol of forward primer, 2.5 pmol of reverse primer and cDNA in a volume of 20 μl. For each set of primers, a single reaction mix containing all components except the cDNA was prepared for the entire PCR experiment and placed in aliquots to minimize variation between samples. Each PCR experiment included a negative control consisting of an RT reaction containing no added RNA. PCR was carried out in an FTS-1 capillary thermal sequencer (Corbett Research, Lidcombe, New South Wales, Australia). Following initial heating of samples at 95°C for 2 min, each cycle of amplification consisted of 30 s at 95°C, 30 s at 64°C, and 30 s at 72°C. For each set of primers, the log (PCR product) versus cycle number (20 to 36) was plotted, and a cycle number chosen within the linear portion of the graph (data not shown). Individual cycle numbers were 32 for $\beta_1$-, 30 for $\beta_2$- and 30 for $\beta_3$-adrenoceptors, 24 for adipsin and 30 for transferrin receptor. Following amplification, PCR products

were electrophoresed on 1.3% agarose gels and transferred onto Hybron $N^+$ membranes by Southern blotting in 0.4 M NaOH/1 M NaCl. The membranes were rinsed for 5 min in 0.5 M Tris-HCl (pH 7.5)/1 M NaCl, then in 2× SSC 0.3 M NaCl/30 mM sodium citrate).

### *Detection and measurement of PCR products*

The identity of the PCR products was verified by hybridization to independent probes (Table 1). Oligonucleotide probes (10 pmol) were end-labelled in a 10 μl reaction mix containing 15 μCi [$\gamma$-$^{33}$P]-adenosine 5′-triphosphate (ATP) (2000 Ci mmol$^{-1}$; Bresatec), 1× One-Phor-All Plus buffer (Pharmacia), and 10 u T4 polynucleotide kinase (Pharmacia). Following incubation at 37°C for 30 min, reactions were heated at 90°C for 2 min and diluted to 100 μl with $H_2O$. The labelled probes were separated from unincorporated nucleotide by centrifugation through Chroma-spin 10 columns (Clontech), according to the manufacturers instructions. PCR products were fixed to nylon membranes by exposure to u.v. light for 2 min, and were then pre-hybridized for 2 h at 42°C in a buffer containing 5× SSC, 0.5% SDS, 100 μg ml$^{-1}$ herring sperm DNA, 5× Denhardts solution (1 mg ml$^{-1}$ bovine serum albumin, 1 mg ml$^{-1}$ Ficoll 400 and 1 mg ml$^{-1}$ polyvinylpyrrolidone), and 0.1 mM ATP. Following addition of labelled oligonucleotide probe (equivalent to 5 pmol), hybridization was carried out at 42°C for 16 h. The filters were washed in 2× SSC/0.1% SDS, twice at room temperature for 5 min, then twice at 42°C for 5 min. Radioactivity was detected with a Molecular Dynamics phosphorimager (SI) after exposure to imaging plates for 5–34 h.

To determine the relative levels of β-receptor subtypes and transferrin receptor in regions of human colon, the reverse primer was end-labelled before PCR. The reaction mix contained 250 pmol of oligonucleotide, 250 μCi [$\gamma$-$^{33}$P]-ATP, 1× One-Phor-All Plus buffer (Pharmacia), and 70 u T4 polynucleotide kinase (Pharmacia) in a volume of 100 μl. Incubation and purification of the oligonucleotide were as described above. PCR reactions were carried out with 2.5 pmol of the labelled reverse primer. Following transfer of the PCR products as above, Hybond N+ membranes were dried at room temperature for 30 min, then apposed directly to phosphorimager plates. Exposure times for all quantitative experiments were 8 h.

### *Drugs*

The drugs and reagents used were as follows: (−)-propranolol, (±)-ICI 118551 (erythro-DL-1(7-methylindian-4-yloxy)-3-isopropylaminobutan-2-ol) (Imperial Chemical Industries, Wilmslow, Cheshire, U.K.); (−)-isoprenaline hydrochloride, hydrocortisone, desipramine HCl, carbachol (carbamylcholine chloride), papaverine, (Sigma Chemical Company, St Louis, MO, U.S.A.); (±)-CGP 20712A (2-hydroxy-5(2-((2-hydroxy-3-(4-((1-methyl-4-trifluoromethyl)1H-imidazole-2-yl) phenoxy)propyl) amino)ethoxy) - benzamide monomethane sulphonate), phentolamine HCl (Regitine) (Ciba-Geigy AG Australia); BRL 37344 (Sodium-4-[-2[-2-hydroxy-2-(-3-chlorophenyl) ethylamino] propyl] phenoxyacetate) (Smith Kline Beecham, Great Burgh, Epsom, U.K.); (±)-CGP 12177 hydrochloride ((−)-4-)3-t-butylamino-2-hydroxypropoxy)benzimidazol-2-one) (Research Biochemicals Inc., Massachusetts, U.S.A.); L(+)-ascorbic acid (Merck, Frankfurt, Germany); EDTA (ethylenediaminetetra-acetic acid di-sodium salt) (AJAX Chemicals, Melbourne, Australia).

Stock solutions of CGP 20712A (10 mM), ICI 118,551 (10 mM), desipramine (5 mM), isoprenaline (10 mM) were prepared in 10 mM HCl. Hydrocortisone (25 mM) was prepared in absolute ethanol. All remaining drugs were prepared in distilled water. Dilutions were made with the appropriate buffer. All other chemicals were of analytical grade.

## Results

### *Organ bath studies of human colonic smooth muscle*

Organ bath studies were performed on strips of circular and longitudinal smooth muscle and taenia coli. During the 30–45 min equilibration period in carbogenated buffer at 37°C under 0.5 g tension, most human colonic muscle strips developed varying degrees of spontaneous activity. The pattern of spontaneous activity was very variable between tissues and often variable between different strips from the same colon sample. Some strips displayed a slow phasic contraction with shorter more frequent contractions superimposed on this activity. The slower phasic activity disappeared when the muscle was precontracted and generally the rapid spontaneous activity was stably maintained throughout most of a cumulative isoprenaline C-R curve, then was reduced markedly as the tissue reached approximately 50% of its maximum relaxation. Several extraneuronal uptake inhibitors were tested; the addition of either corticosterone (100 μM), hydrocortisone (30 μM) or phenoxybenzamine (5 μM) caused a decrease in the baseline tone of the circular smooth muscle and corticosterone also appeared to inhibit the spontaneous contractions of the muscle, although this was not quantitated. Hydrocortisone (30 μM) has been used successfully in other studies of atypical β-adre-

**Table 1** Oligonucleotides used as PCR primers and hybridization probes

| *Name* | *Length* | *Strand* | *Sequence (5′→3′)* | $T_m$ (°C)[a] | *Location*[b] |
|---|---|---|---|---|---|
| *Primers* | | | | | |
| hb1.157 | 22 | for | CTGTCTCAGCAGTGGACAGCGG | 68 | J03019: 243–264 |
| hb1.510 | 22 | rev | CAGCAGGCTCTGGTAGCGGAAG | 68 | J03019: 575–596 |
| hb2.2116 | 25 | for | ACCCACCAGGAAGCCATCAACTGCT | 72 | M15169: 2116–2140 |
| hb2.2532 | 31 | rev | GCCTATCCAATTTAGGAGGATGTAAACTTCC | 67 | M15169: 2502–2532 |
| hb3.1112 | 25 | for | TGCCCTGAACTGGCTAGGTTATGCC | 70 | X70811: 1112–1136 |
| hb3.1479 | 25 | rev | CCCAGTCGTCAGGTTCTGGAGGGTA | 70 | X70811: 1455–1479 |
| had.92 | 22 | for | CCTACATGGCGTCGGTGCAGCT | 71 | M84526: 92–113 |
| had.603 | 23 | rev | GGAGTCACCCTTGCAGCTGTCCC | 71 | M84526: 581–603 |
| htr.1738 | 24 | for | GCAACTTCAAGGTTTCTGCCAGCC | 69 | X01060: 1738–1761 |
| htr.2208 | 25 | rev | AGTCTCCACGAGCAGAATACAGCCA | 68 | X01060: 2184–2208 |
| *Probes* | | | | | |
| hb1.pro | 22 | + | CATGGGTCTGCTGATGGCGCTC | 72 | J03019: 266–278 |
| hb2.pro | 21 | + | AGCCAGGTGGAGCAGGATGGG | 71 | M15169: 2323–2343 |
| hb3.pro | 30 | − | TCTGATCAACAGAGTTGTTGCTTCTTGTCC | 69 | X70811: 1360–1389 |
| had.pro | 22 | − | GGTCCAGCACTGGCAAGAGCAC | 69 | M84526: 484–505 |

[a] $T_m$ determined by 'PRIMER' (Version 0.5, Whitehead Institute).
[b] Genbank accession number and nucleotide numbers within corresponding entry.

noceptors and was used to maintain consistency with previous studies performed in rat gastrointestinal tissue.

Since all three muscle preparations failed to develop sufficient spontaneous tone to construct full concentration-relaxation curves, tissues were precontracted with carbachol. C-R curves to carbachol were performed and a concentration of 0.3 $\mu$M caused a contraction of the circular and longitudinal smooth muscle of 60–70% of maximum (data not shown).

*Effect of (−)-isoprenaline in circular smooth muscle preparations* Initially, C-R curves to (−)-isoprenaline were performed on circular smooth muscle preparations with the spontaneously developed tone of the tissue as a baseline. Table 2 shows the $pEC_{50}$ values of (−)-isoprenaline in two circular smooth muscle preparations in which full C-R curves were obtained and the corresponding $pK_B$ values for propranolol of approximately 8. However, the spontaneous tone of the muscle was not consistently maintained in all muscle strips and, unlike previous studies of human circular smooth muscle (McLaughlin *et al*., 1988; 1991; De Ponti *et al*., 1996) which have relied on a stable basal tone, we found this to be insufficient to allow the successful completion of a full (−)-isoprenaline C-R curve. Attempts to perform (−)-isoprenaline C-R curves in circular smooth muscle preparations after precontraction with carbachol were unsuccessful as the contracted muscle developed an increased amplitude of spontaneous contractions and no subsequent relaxation with increasing amounts of (−)-isoprenaline. This phenomenon was particularly evident in experiments performed in the presence of the neuronal and extraneuronal uptake blockers. Due to the limited availability of human colonic tissue no further β-adrenoceptor subtype characterization was attempted in circular smooth muscle preparations.

*Effect of (−)-isoprenaline on carbachol-precontracted longitudinal smooth muscle* Tissue strips of longitudinal smooth muscle lying between the taenia coli provided a preparation with high reproducibility which showed spontaneous contractions and maintained a stable baseline after carbachol (0.3 $\mu$M) precontraction. Clear concentration-dependent relaxation of the muscle was observed to cumulative additions of (−)-isoprenaline with $pEC_{50}$ values of $7.39 \pm 0.12$ ($n=8$). Table 2 shows the $pEC_{50}$ values of (−)-isoprenaline for each individual muscle preparation.

(−)-Propranolol (0.1 $\mu$M) was an effective inhibitor of (−)-isoprenaline relaxation and caused rightward shifts in the C-R curves with a $pK_B$ value of $8.55 \pm 0.12$ ($n=8$; Table 2). CGP 20712A (10–300 nM) also caused rightward shifts in the (−)-isoprenaline C-R curves with $pK_B$ values in the range of 8.3 to 9.5 (Table 2). The mean $pK_B$ value for 0.1 $\mu$M CGP 20712A was $8.80 \pm 0.20$ ($n=5$). In contrast, the selective $\beta_2$-adrenoceptor antagonist ICI 118551 (0.1 $\mu$M) had low affinity in inhibiting (−)-isoprenaline-induced relaxation and caused only small shifts in 4 ($pK_B$ $7.98 \pm 0.13$) of 7 tissues and no significant shift in the remaining preparations (Table 2). The mean data for antagonist effects on (−)-isoprenaline C-R curves in longitudinal smooth muscle is shown in Figure 1.

*Effect of (−)-isoprenaline on carbachol-precontracted human taenia coli* Taenia coli were present in five of the colon specimens received and muscle strips from these preparations showed lower levels of spontaneous activity than the longitudinal smooth muscle preparations described above. (−)-Isoprenaline relaxed carbachol-precontracted preparations with a reduced potency in taenia coli compared to longitudinal smooth muscle ($pEC_{50}$ $6.70 \pm 0.17$, $n=5$; Table 2).

(−)-Propranolol (0.1 $\mu$M) caused rightward shifts of the (−)-isoprenaline C-R curves but with rather lower affinity than in longitudinal smooth muscle preparations (Table 2). The $pK_B$ values for propranolol ($7.93 \pm 0.25$; $n=4$) were low for typical β-adrenoceptor values ($P<0.05$ for comparison between longitudinal muscle and taenia coli) but significantly higher than the affinity of propranolol expected at atypical β-adrenoceptors. The selective antagonists, CGP 20712A (0.1 $\mu$M) and ICI 118551 (0.1 $\mu$M), both caused similar shifts of the (−)-isoprenaline C-R curves with $pK_B$ values of $7.71 \pm 0.08$ ($n=3$) and $7.54 \pm 0.48$ ($n=4$) respectively, lower affinities ($P<0.005$ for comparison between longitudinal muscle and taenia coli for CGP 20712A) than expected for their respective subtypes, indicating the possible involvement of another receptor. (−)-Isoprenaline C-R curves in taenia coli are shown in Figure 2. BRL 37344 was tested in one tissue and found to cause concentration-dependent relaxation of the taenia coli but with low potency ($pD_2$ 5.49).

## *Detection of $\beta_1$-, $\beta_2$- and $\beta_3$-adrenoceptor mRNA in human colon samples*

We used RT-PCR to detect β-adrenoceptor and adipsin mRNA in taenia coli, circular/longitudinal smooth muscle and mucosa obtained from samples of human colon, and in a sample of white adipose tissue (WAT) from the epicardium of

**Table 2** Summary of results from individual organ bath experiments

| *Disease* | *Tissue region* | *Patient sex (age)* | *Isoprenaline ($pEC_{50}$)* | *Propranolol ($pK_B$)* | *CGP 20712A ($pK_B$)* | *ICI 118551 ($pK_B$)* |
|---|---|---|---|---|---|---|
| Circular muscle | | | | | | |
| Rectal polyps | Proximal | m (64) | 6.74 | 8.00, 8.07 | – | – |
| Ca rectum | Distal | N/A | 7.39 | 8.04 | – | – |
| Longitudinal muscle | | | | | | |
| Ca rectum | Distal | f (69) | 7.23 | 8.08, 8.02 | – | – |
| Ca rectum | Distal | f (40) | 7.51 | – | 8.41[b], 9.26 | 8.28, no shift |
| Rectosigmoid Ca | Distal | f (62) | 7.37 | 8.70 | 8.54 | no shift |
| Ca right colon | Proximal | m (66) | 7.70 | – | 8.48[b], 8.66, 7.30[c] | 7.65 |
| Ca rectum | Distal | f (46) | 7.31 | 8.76 | 8.26 | 8.02 |
| Rectosigmoid Ca | Distal | m (74) | 6.66 | 8.39, 8.31 | – | no shift |
| Ca colon | Proximal | f (79) | 7.57 | 8.78 | 9.04[b], 9.26, 9.54[a] | 7.95 |
| Ca rectum | Distal | m (70) | 7.75 | 8.63 | – | – |
| Taenia coli | | | | | | |
| Ca colon | Distal | m (56) | 6.42 | 7.78 | 7.73 | 7.10 |
| Ca rectum | Distal | m (70) | 7.34 | 8.66 | – | – |
| Ca sigmoid colon | Sigmoid | f (74) | 6.72 | – | 7.84 | 8.49 |
| Ca rectum | Sigmoid | f (35) | 6.43 | 7.47 | 7.49 | – |
| Chronic constipation | Distal | f (39) | 6.61 | 7.83 | 7.77 | 7.03 |

Antagonist concentration is 0.1 $\mu$M unless otherwise indicated: [a] CGP 20712A 10 nM, [b] CGP 20712A 30 nM, [c] CGP 20712A 300 nM. Ca: cancer; N/A: information not available.

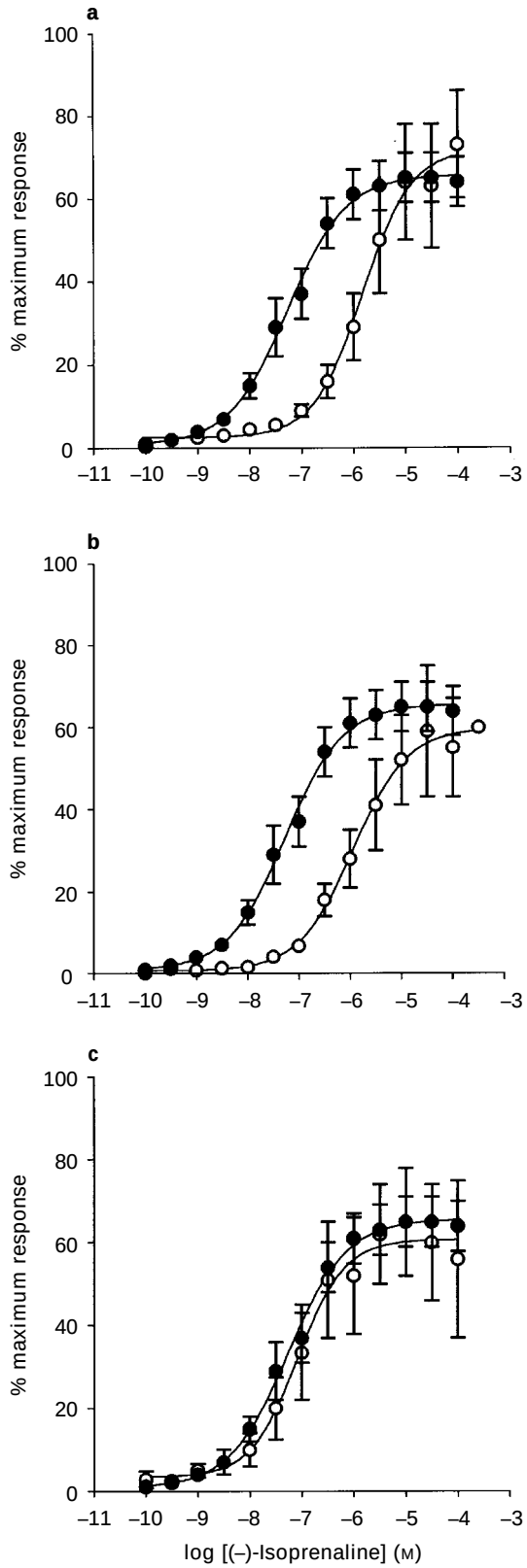


**Figure 1** Mean (−)-isoprenaline concentration-relaxation response curves of carbachol-precontracted, human colonic longitudinal smooth muscle strips. Each graph shows (−)-isoprenaline concentration-response curves in the absence (●) and presence (○) of a β-adrenoceptor antagonist. In (a) the β-adrenoceptor antagonist propranolol (0.1 μM), (b) the selective $\beta_1$-adrenoceptor antagonist CGP 20712A (0.1 μM) and (c) the selective $\beta_2$-adrenoceptor antagonist ICI 118551 (0.1 μM). Points show mean and vertical lines indicate s.e.mean ($n = 5-6$).

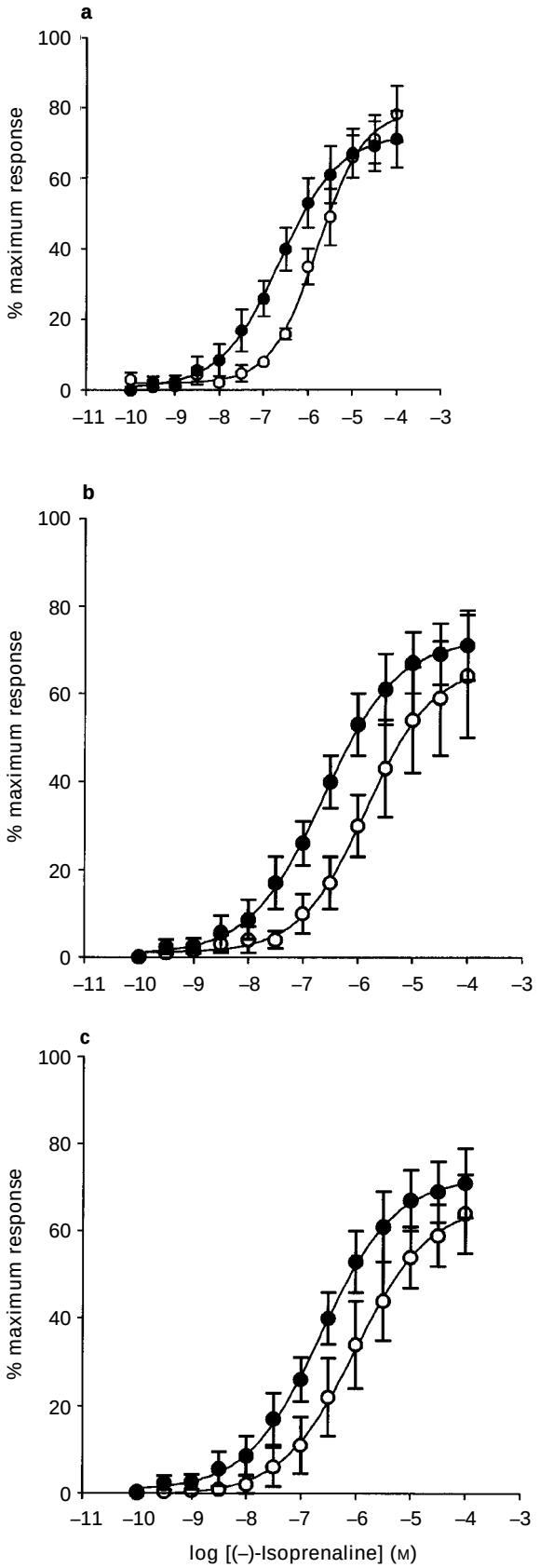


**Figure 2** Mean (−)-isoprenaline concentration-relaxation response curves of carbachol-precontracted, human colonic taenia coli muscle strips. Each graph shows (−)-isoprenaline concentration-response curves in the absence (●) and presence (○) of a β-adrenoceptor antagonist. In (a) the β-adrenoceptor antagonist propranolol (0.1 μM), (b) selective $\beta_1$-adrenoceptor antagonist, CGP 20712A (0.1 μM) and (c) the selective $\beta_2$-adrenoceptor antagonist ICI 118551 (0.1 μM). Points show mean and vertical lines indicate s.e.mean ($n = 3-4$).

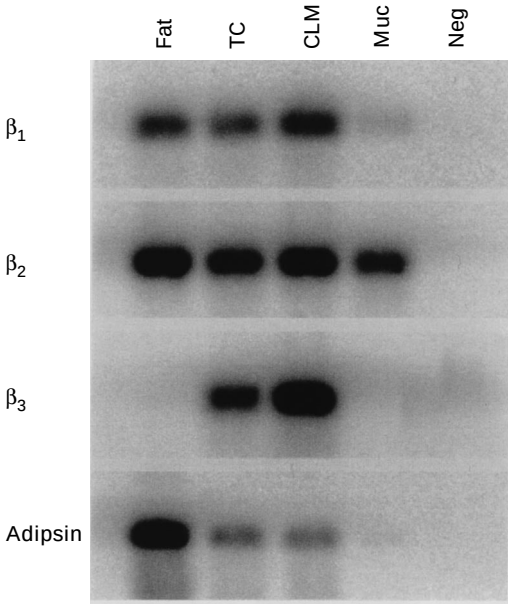


**Figure 3** β-Adrenoceptor and adipsin mRNA in human tissues. RT-PCR was done on RNA from single samples of human fat and colon taenia coli (TC), circular/longitudinal smooth muscle (CLM), and mucosa (Muc), or a negative control (Neg) containing no RNA, with unlabelled primers. Following electrophoresis and transfer to nylon membranes, the PCR products were hybridized with $^{33}$P-labelled independent probes (Table 1). Sizes of the products were checked on the ethidium bromide-stained gels by comparison with 100 bp DNA ladder (Pharmacia). The blots were exposed to phosphorimager plates for varying times before being scanned: 34 h for $\beta_1$-adrenoceptor and adipsin, 5 h for $\beta_2$- and 17 h for $\beta_3$-adrenoceptor mRNA.

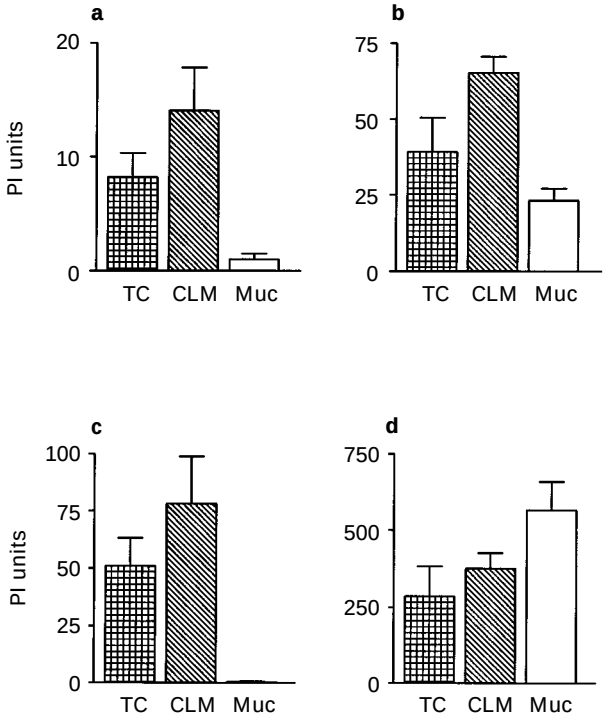


**Figure 4** Relative levels of β-adrenoceptor (a) $\beta_1$, (b) $\beta_2$ and (c) $\beta_3$ and (d) transferrin receptor mRNA in regions of human colon: taenia coli (TC), circular/longitudinal smooth muscle (CLM), and mucosa (Muc). All PCR experiments were carried out on a single set of reverse transcription reactions. In addition, the PCR with each set of primers was done in a single run, ensuring valid comparison between tissues. Differences both in levels of expression of the different mRNAs and in their efficiency of amplification are reflected by variations in the y-axes. Numbers refer to arbitrary phosphorimager (PI) units ($\times 10^{-4}$) obtained from the 'volume report' function of ImageQuaNT software (Molecular Dynamics). Columns show the mean ± s.e.mean ($n = 5$).

human right ventricular free wall. The identity of the β-adrenoceptor and adipsin products was confirmed by hybridization of unlabelled PCR products with independent oligonucleotide probes (Figure 3). To ensure that the PCR products were derived exclusively from mRNA and not contaminating genomic DNA, we treated all total RNA samples with DNase. In addition, we used intron-spanning primers to detect $\beta_3$-adrenoceptor and adipsin mRNA. The observed product sizes were found to correspond to the expected 368 and 512 bp, whereas any contaminating DNA containing introns would have given PCR products of 1393 bp ($\beta_3$-adrenoceptor) and 921 bp (adipsin). We did not observe these larger bands in any samples of cDNA used for the subsequent measurement of β-adrenoceptor or adipsin mRNA.

We used semi-quantitative RT-PCR to determine the relative levels of β-adrenoceptors and transferrin receptor mRNA in 5 samples of each colon region (Figure 4). For each type of mRNA all samples were processed within a single PCR experiment, ensuring valid comparison between tissues. However, we could not make any direct comparisons between the β-adrenoceptor subtype mRNAs, since they were found to amplify with differing efficiencies. In particular, $\beta_1$-adrenoceptors amplified at low efficiency as found by other groups (Krief *et al*., 1993; Rodriguez *et al*., 1995). The mRNAs for all three β-subtypes were present at relatively high levels in circular/longitudinal smooth muscle and in taenia coli, whereas mucosa contained moderate levels of $\beta_2$-adrenoceptor, very low levels of $\beta_1$-adrenoceptor and undetectable amounts of $\beta_3$-adrenoceptor mRNA. Low levels of β-adrenoceptor mRNA in mucosal samples were not due to low amounts of input RNA, as the level of transferrin receptor mRNA was higher in mucosa than in the other regions. To ensure that the $\beta_3$-adrenoceptor mRNA detected in smooth muscle was not due to contaminating fat, we compared levels of adipsin mRNA (Evans *et al*., 1996) in all colon samples with that in human WAT (100%). The mean values were 5.4 ± 2.2% in taenia coli, 11.3 ± 5.6% in circular/longitudinal smooth muscle, and 4.1 ± 1.4% in mucosa. When we analysed all 15 colon samples individually, there was no correlation between levels of $\beta_3$-adrenoceptor and adipsin mRNA. Low levels of adipsin mRNA and the lack of correlation with $\beta_3$-adrenoceptor mRNA indicate that the presence of the $\beta_3$-adrenoceptor mRNA in taenia coli and circular/longitudinal smooth muscle is not due to fat. Interestingly, the sample of human WAT showed substantial levels of $\beta_1$- and $\beta_2$-adrenoceptor mRNA, but undetectable $\beta_3$- mRNA, reflecting the variable expression of $\beta_3$-adrenoceptor mRNA between different fat deposits in man (Krief *et al*., 1993).

## Discussion

β-Adrenoceptor stimulation in a range of human gastrointestinal smooth muscle preparations consistently produces a relaxation response. The order of potency of a range of agonists in ileum, colon and rectum suggests differences in the β-adrenoceptors in longitudinal compared to circular smooth muscle (Hedges & Turner, 1969). The present studies of longitudinal muscle strips from human colon showed the high affinity of (−)-propranolol in shifting the (−)-isoprenaline C-R curves, suggesting that the receptor mediating this relaxation is a typical β-adrenoceptor. The high affinity of CGP 20712A and the relative lack of effect by ICI 118551 indicate that the $\beta_1$-adrenoceptor is the predominant subtype involved in the (−)-isoprenaline stimulated relaxation of human longitudinal smooth muscle. In addition, higher levels of $\beta_1$-adrenoceptor mRNA were detected in the circular/longitudinal muscle preparations compared to taenia coli and mucosa.

These findings are consistent with studies by Lyrenas (1985), who concluded that $\beta_1$-adrenoceptors are physiologically important in the motility of smooth muscle based on the increased colonic motility and pressure observed in man *in vivo* after administration of a selective $\beta_1$-antagonist. However, in contrast to the studies by Lyrenas (1985), the present study did not show the additional involvement of $\beta_2$-adrenoceptors.

Although the functional studies of McLaughlin *et al*. (1991) showed little evidence for a subtype other than $\beta_1$ in circular smooth muscle preparations, more recent studies have provided evidence, with the selective $\beta_3$-antagonist, SR 59230A, for the presence of $\beta_3$- as well as $\beta_1$ and/or $\beta_2$-adrenoceptors in human circular muscle (De Ponti *et al*., 1996). This is consistent with the detection of high levels of $\beta_1$-, $\beta_2$- and $\beta_3$-adrenoceptor mRNA in the combined longitudinal/circular preparations in the present study. Unfortunately, we were unable to separate the circular and longitudinal muscle layers and the molecular evidence is provided for a mixed muscle coat. The circular smooth muscle makes up the bulk of these mixed muscle preparations and it is possible that responses of muscle strips cut in the longitudinal orientation could involve the circular muscle coat. However, our longitudinal muscle responses showed no evidence of $\beta_3$-adrenoceptor involvement in comparison to studies performed on circular smooth muscle by De Ponti *et al*. (1996). In addition, any possible $\beta_3$-adrenoceptor synthesized in the longitudinal muscle does not appear to contribute to the isoprenaline-induced relaxation of the longitudinal preparations.

The present study of human taenia coli revealed a more complex response as the affinities of (−)-propranolol, ICI 118551, and CGP 20712A were all somewhat lower than expected for $\beta_1$- and $\beta_2$-adrenoceptors but still higher than values considered appropriate for the atypical β-adrenoceptor. These results indicate that there is a heterogeneous population of β-receptors in human taenia coli. The lower than expected affinity of (−)-propranolol may indicate that an atypical β-adrenoceptor population is present. A mixed population of subtypes was consistent with the presence of mRNA for all three β-adrenoceptors in taenia coli. The lack of activity by BRL 37344 in the taenia coli preparations was consistent with the findings of others studying human colon relaxation (McLaughlin *et al*., 1991; De Ponti *et al*., 1996; J Kelly, personal communication) and supported previous observations that BRL 37344 is a poor partial agonist at the human $\beta_3$-adrenoceptor. In comparison, the $\beta_1$-/$\beta_2$-antagonist and $\beta_3$-agonist, CGP 12177 has been shown to relax both human colonic circular smooth muscle (De Ponti *et al*., 1996) and human taenia coli (J Kelly, personal communication). Studies of the human adipocyte $\beta_3$-adrenoceptor have also shown that CGP 12177 is a more effective agonist than BRL 37344 (Lonnqvist *et al*., 1993).

Additional factors that must also be considered in studies with human tissues are the various medications taken by the patients for gastrointestinal or other unrelated conditions and the level of severity of these conditions. Although colon specimens were taken from macroscopically normal regions of the tissue, patient medication and history were not available and other underlying variations between the samples could be present. Chronic treatment with β-antagonists has been shown to enhance β-adrenoceptor-mediated responses without altering β-adrenoceptor density in rat heart (Tan & Summers, 1995). However, the sensitivity to isoprenaline of the various muscle preparations used in this study did not appear to vary noticeably between different patients (see Table 2).

Abnormalities of colonic motility are believed to play a major role in many alimentary disorders but suitable treatment has not evolved due in part to a lack of understanding of the complex interactions between extrinsic and intrinsic nerves, endocrine and paracrine factors and other biologically active molecules present in the human colon (Camilleri & Ford, 1994). Sympathetic nerves reach all regions of the human gastrointestinal tract and nerve stimulation causes relaxation of human ileum and of both ascending and sigmoid colon (Bennett & Stockley, 1975). Little is known about the physiological role of β-adrenoceptors in the human gastrointestinal tract but it has been suggested that $\beta_1$-receptors are located within ganglionic plexuses while $\beta_2$-receptors are on smooth muscle cells (Ek *et al*., 1986). β-Adrenoceptor stimulation is known to relax smooth muscle, and sympathetic hyperactivity and increased circulating catecholamines during surgery inhibit gastrointestinal motility in the stomach and colon. The precise mechanism of this condition (postoperative ileus) is unknown. However, β-adrenoceptor stimulation is thought to play a role, as treatment with the non-selective β-antagonist propranolol shortens but does not obliterate the period of postoperative ileus in patients after colonic surgery (Glise & Hallerback, 1983).

There are also indications that the colonic motility dysfunction of irritable bowel syndrome may be due in part to a decrease in sympathetically mediated β-adrenergic activity (Lyrenas *et al*., 1985) and non-selective β-adrenoceptor blockade enhances sigmoid colon motility and aggravates these symptoms (Abrahamsson *et al*., 1983). Knowledge of the β-adrenoceptor subtypes activated by sympathetic nerves in human colon could also be beneficial for the autonomic neuropathy of Crohn's disease which has been determined as predominantly a sympathetic dysfunction (Lindgren *et al*., 1991). An absent postprandial colonic response in diabetic patients also appears to be related to an autonomic neuropathy and the smooth muscle appears to remain normally responsive to direct stimulation (Battle *et al*., 1980). The therapeutic benefits of $\beta_3$-adrenoceptor agonists to improve glucose tolerance in diabetic patients (Cawthorne *et al*., 1992) may need to be considered in view of the additional effects these compounds may have on human gastrointestinal smooth muscle.

β-Adrenoceptors are known to influence gastrointestinal peptides and secretions and the present study found moderate levels of $\beta_2$- and lower levels of $\beta_1$-adrenoceptor mRNA in human mucosa compared with their respective levels in circular/longitudinal smooth muscle preparations. Isoprenaline has been shown to induce gastrin secretion in man (Bransborg *et al*., 1976) and human intestinal mucosal absorption of electrolytes such as sodium, chloride and water is also increased by intravenous administration of isoprenaline (Morris & Turnberg, 1981). Propranolol reduced these effects suggesting the involvement of β-adrenoceptors but the subtypes involved were not investigated. Several studies performed to examine gastrointestinal secretory processes have considered the potential involvement of the atypical β-adrenoceptor. Gastric acid secretion in the rat stomach (Canfield & Paraskeva, 1992) and bicarbonate secretion in rat caecum (Canfield & Abdul-Ghaffar, 1992) are stimulated by the $\beta_3$-agonists, SR 58611A and BRL 37344. In addition, the $\beta_3$-agonists BRL 37344 and CL 316243 have recently been shown to be potent inhibitors of indomethacin-induced gastric ulceration (Bahl *et al*., 1996). Daly (1984) proposed that β-adrenoceptor involvement in gastrin release and gastric acid secretion may only be important under conditions of severe stress. This favours the argument for a mucosal $\beta_3$-adrenoceptor as the $\beta_3$-subtype has been shown to be less susceptible to desensitization and down-regulation during periods of increased sympathetic drive when high circulating catecholamine levels cause rapid desensitization of $\beta_1$- and $\beta_2$-receptors (Granneman, 1992).

Although the detection of $\beta_3$-adrenoceptor mRNA has previously been demonstrated in mucosal samples from rat colon (Evans *et al*., 1996), the present study shows moderate levels of both $\beta_1$- and $\beta_2$-adrenoceptor mRNA but a lack of $\beta_3$-adrenoceptor mRNA in human colonic mucosa. However, previous (−)-[$^{125}$I]-cyanopindolol binding studies in human colon homogenates revealed an atypical β-adrenoceptor binding site and autoradiographic studies showed high levels of propranolol-resistant 'atypical' β-adrenoceptor in the mucosal layer of the human colon. The regional distribution of the propranolol-resistant binding site was similar to the distribution of binding of a $\beta_3$-adrenoceptor riboprobe demonstrated by *in situ* hybridization (Summers *et al*., 1995). The

absence of $\beta_3$-adrenoceptor mRNA in human samples may be explained by species variation of $\beta_3$-receptor distribution between rodents and man, but the conflicting evidence for a mucosal $\beta_3$-adrenoceptor from binding, functional and molecular studies may be resolved if further novel subtypes of the atypical $\beta$-adrenoceptor are identified. Speculation on possible clinical advantages of $\beta_3$-adrenoceptor agonists on human mucosal function should be made with caution at this stage.

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