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Characterization of β-adrenoceptor mediated smooth muscle relaxation and the detection of mRNA for β_1-, β_2- and β_3-adrenoceptors in rat ileum

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1 Functional and molecular approaches were used to characterize the β-AR subtypes mediating relaxation of rat ileal smooth muscle.

2 In functional studies, (−)-isoprenaline relaxation was unchanged by CGP20712A (β_1-AR antagonist) or ICI118551 (β_2-AR antagonist) but shifted by propranolol ($pK_B = 6.69$). (±)-Cyanopindolol, CGP12177 and ICID7114 did not cause relaxation but antagonized (−)-isoprenaline relaxation.

3 BRL37344 (β_3-AR agonist) caused biphasic relaxation. The high affinity component was shifted with low affinity by propranolol, (±)-cyanopindolol, tertatolol and alprenolol. CL316243 (β_3-AR agonist) relaxation was unaffected by CGP20712A or ICI118551 but blocked by SR58894A (β_3-AR antagonist; $pA_2 = 7.80$). Enhanced relaxation after exposure to forskolin and pertussis toxin showed that β_3-AR relaxation can be altered by manipulation of components of the adenylate cyclase signalling pathway.

4 The β_1-AR agonist RO363 relaxed the ileum ($pEC_{50} = 6.18$) and was blocked by CGP20712A. Relaxation by the β_2-AR agonist zinterol ($pEC_{50} = 5.71$) was blocked by SR58894A but not by ICI118551.

5 In rat ileum, β_1-, β_2- and β_3-AR mRNA was detected. Comparison of tissues showed that β_3-AR mRNA expression was greatest in WAT > colon = ileum > cerebral cortex > soleus; β_1-AR mRNA was most abundant in cerebral cortex > WAT > ileum = colon > soleus; β_2-AR mRNA was expressed in soleus > WAT > ileum = colon > cerebral cortex.

6 These results show that β_3-ARs are the predominant β-AR subtype mediating rat ileal relaxation while β_1-ARs may produce a small relaxation. The β_2-AR agonist zinterol produces relaxation through β_3-ARs and there was no evidence for the involvement of β_2-ARs in relaxation despite the detection of β_2-AR mRNA.

Keywords: β-adrenoceptors; β_3-adrenoceptors; gastrointestinal smooth muscle; relaxation; rat ileum; messenger RNA

Abbreviations: AR, adrenoceptor; C-R, concentration response; CYP, cyanopindolol; ISO, isoprenaline

Introduction

Although the intestinal β-AR was originally described as a β_1-AR (Lands *et al*., 1967) atypically low affinities of β-AR antagonists and propranolol-resistant relaxation responses observed in gastrointestinal smooth muscle preparations from several species indicated that other β-AR subtypes were involved in these responses (see Arch & Kaumann, 1993, for review). Atypical β-AR responses that were resistant to blockade by propranolol and selectively stimulated by a novel group of β-AR agonists were also described in adipocytes. The β_3-AR was subsequently cloned and sequenced (Emorine *et al*., 1989) and found to share many of the pharmacological characteristics of the atypical β-AR. β_3-ARs are highly expressed in adipose tissue and β_3-AR agonists such as BRL 37344, SR 58611A and CL 316,243 are potent stimulants of lipolysis and thermogenesis, cause marked weight loss in obese animals, increase insulin sensitivity and improve glucose tolerance in diabetic animals. Whilst the human β_3-AR is emerging as an exciting target for the development of selective stimulants as potential treatments for obesity and diabetes (Strosberg & Pietri-Rouxel, 1996), these same compounds may also be useful in the treatment of irritable bowel syndrome. β_3-ARs are also highly expressed in regions of the gastrointestinal tract such as colon, ileum and stomach in many species including human (Berkowitz *et al*., 1995; Evans *et al*., 1996; Roberts *et al*., 1997). β_3-AR mediated relaxation in gastrointestinal smooth muscle is resistant to blockade by propranolol and selective β_1- and β_2-AR antagonists such as CGP 20712A and ICI 118551 (for review see Manara *et al*., 1995).

Earlier studies of gastrointestinal smooth muscle used non-selective β-AR or selective β_1- or β_2-AR agonists to characterize β-AR responses in various animal models. For example, studies using non-selective β-AR or selective β_2-AR agonists in diabetic animal models have shown a marked reduction in β-AR responsiveness in the intestine (Ozturk *et al*., 1996). Decreased responses to the selective β_2-AR agonist salbutamol are of particular note as the low potency of salbutamol strongly suggests that it is acting through β_3-ARs rather than β_2-ARs. In addition, a recent binding study mapping β-AR subtypes throughout the diabetic rat gastrointestinal tract used a concentration of the radioligand [^{125}I]-CYP (20 pM) so low that it would label only 1.5% of β_3-ARs in each sample (Yu & Ouyang, 1997). The findings from these studies therefore offer limited information on the changes in β-

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AR density and responsiveness as the tools/compounds used were not appropriate for examination of all three β-AR subtypes. It is necessary to clearly establish the role and contribution of each β-AR subtype to gastrointestinal relaxation and as selective compounds for each subtype are now available, a more comprehensive study is clearly possible. A highly selective β_3-AR agonist, CL 316243, has been developed that has at least 10,000 fold selectivity for the β_3-AR compared with the β_1- and β_2-ARs respectively (Dolan *et al*., 1994). More recently the first selective β_3-AR antagonists were developed and these new pharmacological tools have enabled a clear demonstration of β_3-AR mediated functional responses in rat colon and rat brown adipose tissue (Manara *et al*., 1996).

Previous studies of the β_3-AR in rat ileum have been carried out in the presence of β_1- and β_2-AR blockade and the relative contribution of β-AR subtypes has not been examined using agonists and antagonists selective for each of the three β-AR subtypes. Studies of functional responses of rat ileum relaxation (Growcott *et al*., 1993; Hoey *et al*., 1996) have shown that stimulation by selective β_3-AR agonists produces a relaxation response in the presence of β_1- and β_2-AR blockade. The aim of this study therefore was to utilize selective β_3-AR compounds as well as selective β_1- and β_2-AR agonists and antagonists to characterize the functional β-AR subtypes present in rat ileum and assess the relative contribution of these subtypes to β-AR mediated relaxation of the rat ileal smooth muscle. We have also measured mRNA levels of the three β-AR subtypes in ileal smooth muscle.

Methods

Tissue preparation and incubation

Male Sprague Dawley rats (250–300 g) were anaesthetized with 80% CO_2/20% O_2 and decapitated. A 12–15 cm segment of the small intestine was removed from 2 cm above the ileocaecal junction and the intraluminal contents flushed out with Krebs Henseleit buffer (composition:- (mM) NaCl 118.4, KCl 4.7, $MgSO_4.7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11, $CaCl_2$ 2.5) containing ascorbic acid (0.1 mM) and EDTA (0.04 mM). Segments of 1.5–2 cm length were mounted on tissue hooks and suspended in jacketed organ baths containing Krebs Henseleit solution maintained at 37°C and bubbled continuously with 95% O_2/5% CO_2 (pH 7.4) under 5 mN force. Ugo Basile isotonic transducers connected to a MacLab system with an Apple Macintosh IICi Computer were used to measure isotonic changes in the length of the tissues. Tissue strips were equilibrated for 30–45 min in the presence of desipramine (0.5 μM) to block neuronal uptake, hydrocortisone (30 μM) to block extraneuronal uptake of ISO and phentolamine (10 μM) to block β-ARs. Antagonists were added prior to the 30 min equilibration period.

Preliminary experiments

Carbachol concentration response (C-R) curves were performed to determine the concentration producing 70–80% maximum contraction. Carbachol (3 μM) was chosen to precontract longitudinal smooth muscle segments in subsequent experiments (data not shown). The tissue sensitivity to (−)-ISO changed when three successive (−)-ISO C-R curves were performed in individual segments. The first C-R curve ($pEC_{50} = 6.79 \pm 0.02$, $n = 4$) significantly increased the sensitivity ($P < 0.05$) of the tissue to the second C-R curve ($pEC_{50} = 7.14 \pm 0.02$, $n = 4$) and the third C-R curve had a significantly depressed maximum response ($P < 0.05$). By comparison (−)-ISO C-R curves in paired tissues were very consistent so single C-R curves were performed in the absence and presence of antagonists in paired ileum preparations obtained from the same animal.

Concentration response curves in rat ileal smooth muscle

Tissue strips were precontracted submaximally with carbachol (3 μM) for a 15–20 min equilibration period before relaxation by cumulative concentrations of agonists were performed. Agonist curves were constructed using 0.5 log unit increments until a stable state was observed. The time intervals between each concentration ranged from 4–5 min for isoprenaline and zinterol to 20–30 min for BRL 37344 and CL 316,243. At the end of the concentration response (C-R) curve, papaverine (50–100 μM) was added and responses were expressed as a percentage of the maximum papaverine relaxation.

Analysis

The activity of the agonists was expressed as an EC_{50} value obtained by analysis using the non-linear curve fitting program in PRISM (Intuitive Software for Science). When a range of antagonist concentrations was used a Schild plot was constructed (Arunlakshana & Schild, 1959) and the x-intercept of the Schild plot was taken as the pA_2 value. In circumstances where single concentrations of antagonist were used or if the Schild plot was clearly non-linear, pK_B values were calculated according to the method of Furchgott (1972). Statistical significance was determined using Student's *t*-test where $P < 0.05$ was considered to be significant. C-R curves were plotted as means ± s.e.mean of *n* individual experiments. The slope values of regression lines were expressed as slope ± 95% confidence limits (C.L.). All values are expressed as means ± s.e.mean.

Molecular methods

Adult male Sprague Dawley rats (250–300 g) were anaesthetized by 80% CO_2/20% O_2 and killed by cervical dislocation. Epididymal white adipose tissue, cerebral cortex, soleus muscle, ileal and colonic smooth muscle were removed, frozen in liquid N_2 and stored at −70°C. Ileum and colon were carefully dissected free of surrounding adipose tissue, cut open and pinned out, and mucosa removed by scraping with a scalpel. The resulting smooth muscle was washed in Krebs Henseleit solution and blotted dry prior to immersion in liquid N_2.

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, sodium acetate (pH 7.0) (100 mM), $MgSO_4$ (5 mM), dithiothreitol (5 mM), 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 using 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 prior to the addition of reaction mix containing 1 × RT buffer (supplied by Promega), dNTPs (1 mM), $MgCl_2$ (5 mM), 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 done on cDNA equivalent to 100 ng of starting RNA, using oligonucleotide primers specific for rat β_1-AR (forward, 5′-CCGCTGCTACAACGACCCCAAG-3′ and reverse, 5′-CGGATCGCCTCTTCGTCTTCTTCAA-3′), β_2-AR (forward, 5′-TGTGTCACAGCCAGCATCGAGA-CCCTGT-3′ and reverse 5′-TTGAAGGCAGAGTTGACA-TAGCCCAACCAGTT-3′), β_3-AR (set A: forward, 5′-AT-CATGAGCCAGTGGTGGCGTGTAG-3′ and reverse, 5′-GCGATGAAAACTCCGCTGGGAACTA-3′), β_3-AR (set B, intron spanning forward, 5′-TAGTCCTGGTGTG-GATCGTGTCCGC-3′ and reverse, 5′-CGCTCACCTTCA-TAGCCATCAAACC-3′), and β-actin (forward, 5′-ATCCTGCGTCTGGACCTGGCTG-3′ and reverse, 5′-CCTGCTTGCTGATCCACATCTGCTG-3′) synthesized at the Howard Florey Institute, Melbourne or by Life Technologies, Gaithersburg, MD, U.S.A. Reverse primers for actin were labelled prior to PCR in a reaction mix containing 120 pmol of oligonucleotide, 70 μCi [γ-^{33}P]-ATP, 1 × One-Phor-All Plus buffer (Pharmacia), and 20 U T4 polynucleotide kinase (Pharmacia) in a volume of 40 μl. Following incubation at 37°C for 30 min, reactions were diluted to 100 μl with H_2O and heated at 90°C for 2 min. The labelled primers were separated from unincorporated nucleotide by centrifugation through Chroma-spin 10 columns (Clontech), according to the manufacturer's instructions. PCR mixes contained 1 U of Taq polymerase (Life Technologies), the buffer supplied (Tris-HCl (pH 8.4) (20 mM) and KCl (50 mM)), dNTPs (200 mM), Mg-acetate (2 mM), 2.5 pmol of forward primer, 2.5 pmol of reverse primer and cDNA in a volume of 10 μl. For each set of tissues (e.g. all samples of ileum), a single reaction mix containing all components except the cDNA was prepared for the entire PCR experiment and aliquotted 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. Following amplification, PCR products were electrophoresed on 1.3% agarose gels and transferred onto Hybond N+ membrane by Southern blotting in 0.4 M NaOH/1 M NaCl. For detection of β-AR products, membranes were exposed to u.v. light for 2 min then pre-hybridized for 4 h at 42°C in a buffer containing 5 × Denharts solution (0.1% (w v^{-1}) ficoll type 400, 0.1% (w v^{-1}) poly-vinylpyrrolidone, 0.1% (w v^{-1}) BSA), 5 × SSC, 0.5% SDS, 100 μg ml^{-1} salmon sperm DNA and 0.1 mM ATP, prior to addition of labelled probe and hybridization for 16 h at 42°C. Probes (10 pmol) specific for β_1-AR (5′-AAGAAGATCGA-CAGCTGCGAGC-3′), β_2-AR (5′-AGCCAGGTGGAGCAG-GATGGG-3′) and β_3-AR (5′-TGCCAACTCCGCCTTC-AACCCGGTC-3′) were end-labelled with 15 μCi γ-^{33}P-ATP (2000 Ci $mmol^{-1}$; Bresatec) and T4 polynucleotide kinase (Pharmacia). Filters were washed in 2 × SSC/0.1% SDS, rinsed at room temperature then washed at 30°C for 20–30 min then at 37°C for 5 min. Radioactivity was detected using a Molecular Dynamics SI phosphorimager, and bands were quantitated using the 'Volume Report' function of Image-QuaNT software (Molecular Dynamics).

Drugs and reagents

The authors thank the following companies and individuals for gifts of: ICI D7114 ((S)-4-[2-hydroxy-3-phenoxypropylamino ethoxy] - N - (2 - methoxyethyl) phenoxyacetamide), (Imperial Chemical Industries, Wilmslow, Cheshire, England); (+)-alprenolol (Professor B. Jarrott, Monash University, Victoria, Australia); (±)-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) (Dr G. Anderson, Ciba-Geigy AG Australia); (−)-CYP, (±)-CYP (Sandoz, Basel, Switzerland); BRL 37344 (sodium-4-[-2[-2-hydroxy-2-(-3-chloro-phenyl) ethylamino] propyl] phenoxyacetate) (Dr M.A. Cawthorne, Smith Kline Beecham, Great Burgh, Epsom, U.K.); (−) tertatolol, (+)-tertatolol (Servier, Paris, France); SR 58611A (RS-N-(7-carbethoxymethoxyl 1,2,3,4-tetrahydronophth-2-yl)-2 hydroxy 2-(3-chlorophenyl)-ethanamine), SR58894 (3-(2-allyl-phenoxy)-1-[(1S)-1,2,3,4-tetrahydronaphth - 1 - ylamino] - (2S) - 2 -propanol hydrochloride) (Dr Luciano Manara, SANOFI-MIDY S.p.A. Research Centre, Milan, Italy); zinterol hydrochloride (Bristol-Myers Squibb, Noble Park, Australia); CL 316 243 (disodium (R,R)-5- [2- [[2-3-Chlorophenyl)-2-hydroxyethyl] -amino] propyl]-1,3-benzodioxole-2,2-dicarboxylate) (Dr Tim Nash, Wyeth Pharmaceuticals, Sydney, Australia).

Other chemicals were from commercial sources as indicated: (−)-propranolol, (±)-ICI 118551 (erythro-DL-1(7-methylindian-4-yloxy)-3-isopropylaminobutan-2-ol) (Imperial Chemical Industries, Wilmslow, Cheshire, England); (−)-alprenolol, (−)-isoprenaline bitartrate, hydrocortisone, desipramine HCl, carbachol (carbamylcholine chloride), papaverine, forskolin (Sigma Chemical Company, St Louis, MO, U.S.A.); phentolamine HCl (Regitine; Ciba-Geigy AG Australia); (±)-CGP 12177 hydrochloride ((−)-4-)3-t-butylamino-2-hydroxypropoxy) benzimidazol-2-one) (Research Biochemicals Inc., MA, U.S.A.); RO 363 (±)-1-(3,4-dimethoxy-phenethylamino)-3-(3,4-dihydroxyphenoxy)-2-propanol)-oxalate) (Institute of Drug Technology, Boronia, Australia); L(+)-ascorbic acid (Merck, Frankfurt, Germany); EDTA (ethylenediaminetetraacetic acid di-sodium salt (AJAX Chemicals, Melbourne, Australia).

Results

(−)-Isoprenaline mediated relaxation of rat ileal longitudinal smooth muscle

(−)-ISO produced concentration-dependent relaxation of carbachol-precontracted rat ileum longitudinal smooth muscle with a pEC_{50} value of 6.80 ± 0.02 (n = 4). The β_1/β_2-AR antagonist (−)-propranolol (0.1, 1, 10 μM) caused concentration-dependent rightward shifts of the (−)-ISO C-R curve. The corresponding Schild plot was biphasic and the slope of the plot was significantly less than unity (Table 1, Figure 1). Calculation of pK_B values for each propranolol concentration

showed that the higher concentrations (1 and 10 μM) of the drug caused a reduced rightward shift compared to the lower concentration (0.1 μM) indicating the possible involvement of two sites with different affinities for propranolol (Table 1).

Both the selective β_1-AR antagonist CGP 20712A (30, 100, 300 nM) and the selective β_2-AR antagonist ICI 118551 (30, 100, 300 nM) failed to produce significant shifts of the (−)-ISO CR curve (Figure 1). ICI 118551, at a concentration of 30 μM caused a small shift with a pK_B value of 5.06±0.13 ($n=4$). CGP 20712A did not cause a significant shift in the (−)-ISO C-R curves (pEC_{50} control 6.69±0.04, $n=5$; pEC_{50} CGP 20712A (300 nM) 6.55±0.06, $n=4$; $P>0.05$). It was not possible to use CGP 20712A at a concentration equivalent to its affinity at a β_3-AR (1 mM) in the organ bath due to solubility difficulties.

The β_1/β_2-AR antagonist (±)-CYP (30 nM, 0.1, 0.3, 1 μM) caused concentration dependent rightward shifts of the (−)-ISO C-R curve. A Schild plot produced a line with a slope of unity (0.97) and a corresponding pA_2 value of 7.98 (Figure 2). The stereoselectivity of the isomers of alprenolol and tertatolol was examined against (−)-ISO C-R curves (Figure 3). Schild plots for (−)-alprenolol and (−)-tertatolol had slope values which did not differ significantly from unity with pA_2 values of 7.12 and 7.29 respectively (Table 1). The Schild plots for the (+)-stereoisomers of both alprenolol and tertatolol had slope values significantly less than unity and pA_2 values of 5.83 and 5.65 respectively. The stereoselectivity displayed for alprenolol (19.5 fold) and tertatolol (43.7 fold) were somewhat less than expected at β_1- or β_2-AR but in the range expected for a β_3-AR. (Table 1).

Effects of selective β_1-, β_2- and β_3-AR agonists on longitudinal smooth muscle

β_1-AR agonist The selective β_1-AR agonist RO 363 produced a small relaxation of carbachol precontracted rat longitudinal smooth muscle (<20% of papaverine maximum) at a high concentration of 10 μM. The pEC_{50} for this response was 6.18±0.34 ($n=4$) and this relaxation was abolished by CGP 20712A (100 nM) (Figure 4).

β_2-AR agonist The β_2-AR antagonist, zinterol, caused a concentration dependent relaxation with a pEC_{50} of 5.71±0.16 ($n=6$) and this relaxation was not affected by ICI 118551 (100 nM; $pEC_{50}=5.93\pm0.16$, $n=8$) or CGP20712A (100 nM; $pEC_{50}=5.94$, $n=2$) but was shifted to the right by increasing concentrations of the β_3-AR antagonist SR58894A in the presence of ICI 118551 (100 nM). pK_B values of 7.23±0.02 (0.1 μM, $n=4$) and 6.95±0.09 (1 μM, $n=5$) were calculated (Figure 4).

β_3-AR agonists BRL 37344 was a slower acting agonist (each concentration required approximately 15–20 min to achieve equilibrium) and BRL 37344 C-R curves were biphasic. The first phase (pEC_{50} (1) = 7.31±0.04, $n=4$) occurred at lower concentrations than (−)-ISO and caused a response that was approximately 50% of the maximum produced by (−)-ISO (10 μM). This component of the response was shifted to the right by (−)-propranolol (1 μM) (pK_B 6.51). The second phase had an estimated pEC_{50} (2) value of 4.39±0.09, $n=4$ but failed to reach a maximum within the concentration range used and was not shifted by 1 μM propranolol (Figure 5).

pK_B values for the high affinity component of the BRL 37344 CR curve were determined for single concentrations of antagonists (Table 2). The affinities of the antagonists against BRL 37344 responses were compared with the pK_B values obtained against (−)-ISO responses. This data has been represented as a correlation plot (Figure 5). The regression line has an r value of 0.973 ($P=0.0011$) and a slope of 0.863 (95% C.L. 0.58–1.14, $n=6$).

SR 58611A SR 58611A C-R curves were performed using a concentration range of 1×10^{-10} to 3×10^{-5} M. A maximum response was not achieved but the C-R curve appeared monophasic and was not affected by the presence of 1 μM propranolol (Figure 5).

CL 316243 Like BRL 37344 the response to CL 316243 was slow and required 25–30 min to reach a plateau at each concentration. To determine whether this slow response was due to the thickness of the ileal segment, relaxation responses were also examined by inverting the segment, gently scraping off the mucosa with a glass slide, and reinverting the segment to its original orientation. Mucosal removal did not alter the time course of the response or significantly change the potency

Table 1 Affinity values (pA_2) for antagonists against (–)-isoprenaline relaxation of rat ileum. Values are given as pK_B value when single concentrations of antagonists were used or if the Schild plot was clearly non-linear

Antagonist		*pA_2±s.e.mean* (n)	*slope±s.e.mean*	*95% CL*
(–)-tertatolol		7.29±0.17 (28)	1.03±0.09	0.84–1.22
(+)-tertatolol		5.65±0.05 (19)	0.59±0.17	0.24–0.95*
(–)-alprenolol		7.12±0.16 (12)	1.19±0.18	0.78–1.60
(+)-alprenolol		5.83±0.31 (11)	0.73±0.11	0.47–0.98*
CGP 12177		7.65±0.08 (14)	0.77±0.05	0.65–0.89*
(±)-cyanopindolol		7.98±0.07 (14)	0.97±0.06	0.83–1.11
		pK_B±s.e.mean		
Propranolol	0.1μM	7.50±0.10 (4)		
	1μM	6.81±0.20 (4)		
	10μM	6.69±0.07 (4)		
ICI 118551	30μM	5.06±0.13 (4)		
CGP 20712A	300nM	no shift (4)		
CGP 12177	0.1μM	7.55±0.07 (5)		
	1μM	7.15±0.08 (5)		
	10μM	7.09±0.06 (4)		
ICI D7114	0.1μM	7.41±0.03 (4)		
	1μM	7.62±0.13 (4)		

*Regression line significantly different from unity.

of CL 316243 (pEC_{50} 8.05±0.10 (intact mucosa) 7.85±0.11 (mucosa removed), $P>0.05$, $n=4$), although the maximum relaxation was significantly enhanced in preparations after the mucosa was removed ($P=0.005$) (Figure 6). The CL 316243 CR curve was monophasic and was not shifted by the β_1-AR antagonist CGP 20712A or the β_2-AR antagonist ICI118551 at 100 nM but was shifted to the right by the selective β_3-AR antagonist SR 58894A ($pA_2=7.80\pm0.06$, $n=21$; Figure 6).

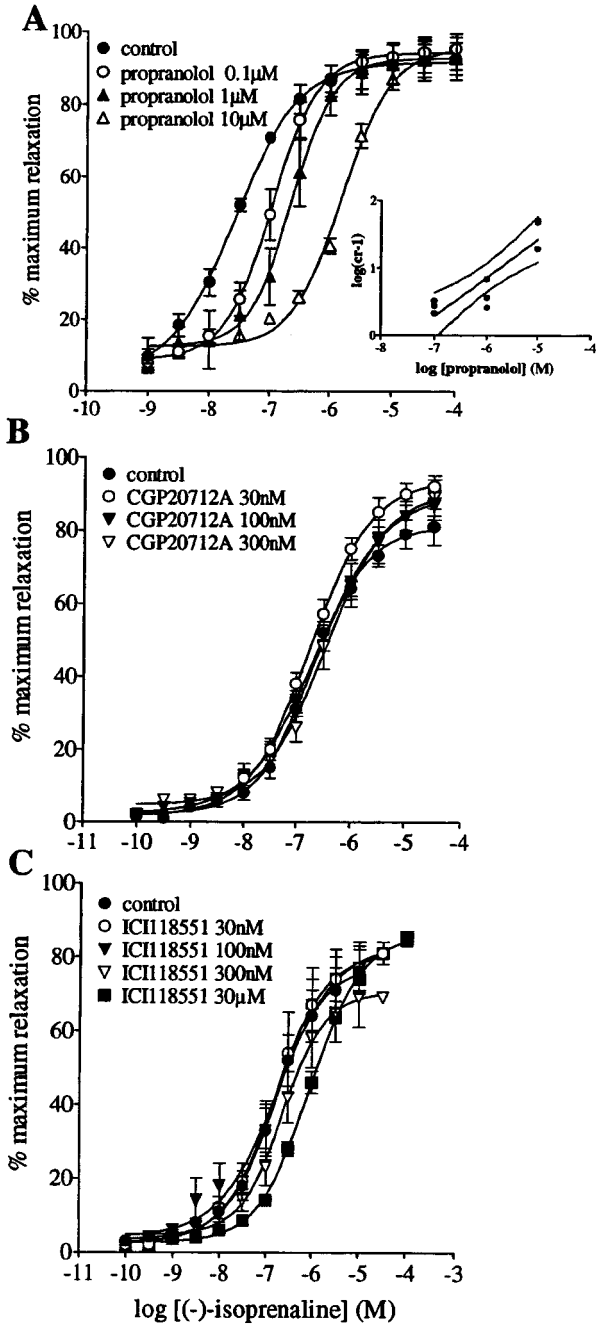

Figure 1 The effect of β_1/β_2 antagonists on the relaxation responses to (−)-isoprenaline in the rat isolated ileum precontracted with carbachol. Graph shows (−)-isoprenaline concentration response curves in the absence and presence of (A) propranolol (B) the β_1-AR antagonist CGP 20712A and (C) the β_2-AR antagonist ICI 118551. Inset (A) shows a Schild plot with a slope significantly less than unity. Note the lack of effect of CGP 20712A and the small rightward shift caused only by a high concentration of ICI 118551. Points show mean±s.e.mean ($n=4$) and are expressed as a percentage of maximum relaxation by papaverine (100 μM).

Effects of putative β_3-adrenoceptor agonists

In the rat ileum neither ICI D7114 or CGP 12177 showed any agonist activity at concentrations up to 10 μM. The Schild plot for CGP 12177 against ISO responses had a slope of 0.77 (95% C.L. 0.65–0.89, $n=12$) and pK_B values are shown in Table 1. ICI D7114 at 0.1 and 1 μM produced rightward shifts of the (−)-ISO C-R curve with pK_B values of 7.41 and 7.62 respectively (Table 1).

Effects of forskolin and pertussis toxin on relaxation of rat ileal longitudinal smooth muscle

Forskolin (0.1 and 1 μM) was added 30 min prior to the commencement of the CR curve. Both concentrations produced a slight relaxation in the basal tone of the tissue–this effect being more pronounced at the higher concentration. Carbachol precontractions were maintained in a similar manner to that observed in the absence of forskolin. A forskolin concentration of 0.1 μM had no effect on ISO and CL 316243 CR curves. At 1 μM, forskolin caused a significant enhancement of the maximum relaxation of both agonists. The ISO response increased by 31% and the CL 316243 response increased by 38% of the maximum relaxation to papaverine. There was no significant change in the pEC_{50} values for CL 316243 or ISO in the presence of forskolin 1 μM (CL 316243 pEC_{50} 7.72±0.20, with forskolin 7.94±0.18 and for ISO pEC_{50} 6.53±0.18, with forskolin 6.93±0.15, $n=4$) (Figure 7). Preincubation of rat ileum for 2 h with 0.5 μg ml^{-1} pertussis toxin also produced a significant increase of 21% in the maximum relaxation of the smooth muscle to CL 316243 (Figure 7).

Detection of β-adrenoceptor mRNA in rat colon and ileum

RT–PCR was used to detect β_1-AR, β_2-AR, β_3-AR and actin mRNA in circular/longitudinal smooth muscle from samples of rat ileum and colon. Determinations were also made with samples of cortex, epididymal white adipose tissue (WAT) and soleus muscle which are known to contain varying levels of the

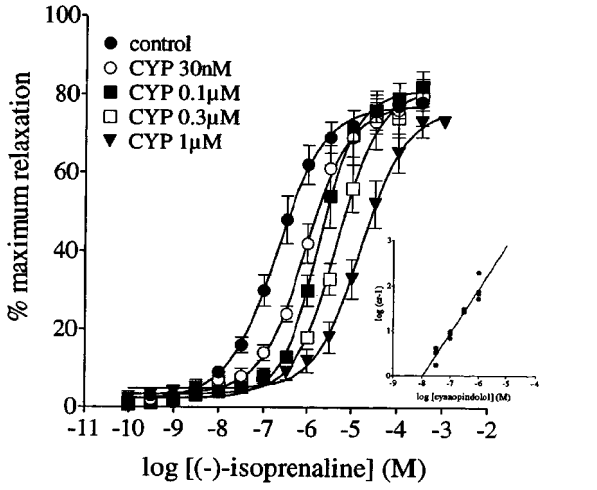

Figure 2 The effect of (±)-cyanopindolol (CYP) on the relaxation responses to (−)-isoprenaline in the rat isolated ileum precontracted with carbachol. Points show mean±s.e.mean and are expressed as a percentage of maximum relaxation by papaverine (100 μM) ($n=4$). Inset shows the Schild plot for (±)-CYP with slope 0.97 and pA_2 value of 7.98.

β-AR subtypes (Figure 8). The PCR products demonstrated the expected sizes of 435 (β_1-AR), 620 (β_2-AR), 473 (β_3-AR), and 559 bp (actin). To ensure that the products were derived exclusively from mRNA and not from contaminating genomic DNA, all RNA samples were treated with DNase, and intron-spanning primers were used to detect actin mRNA. The

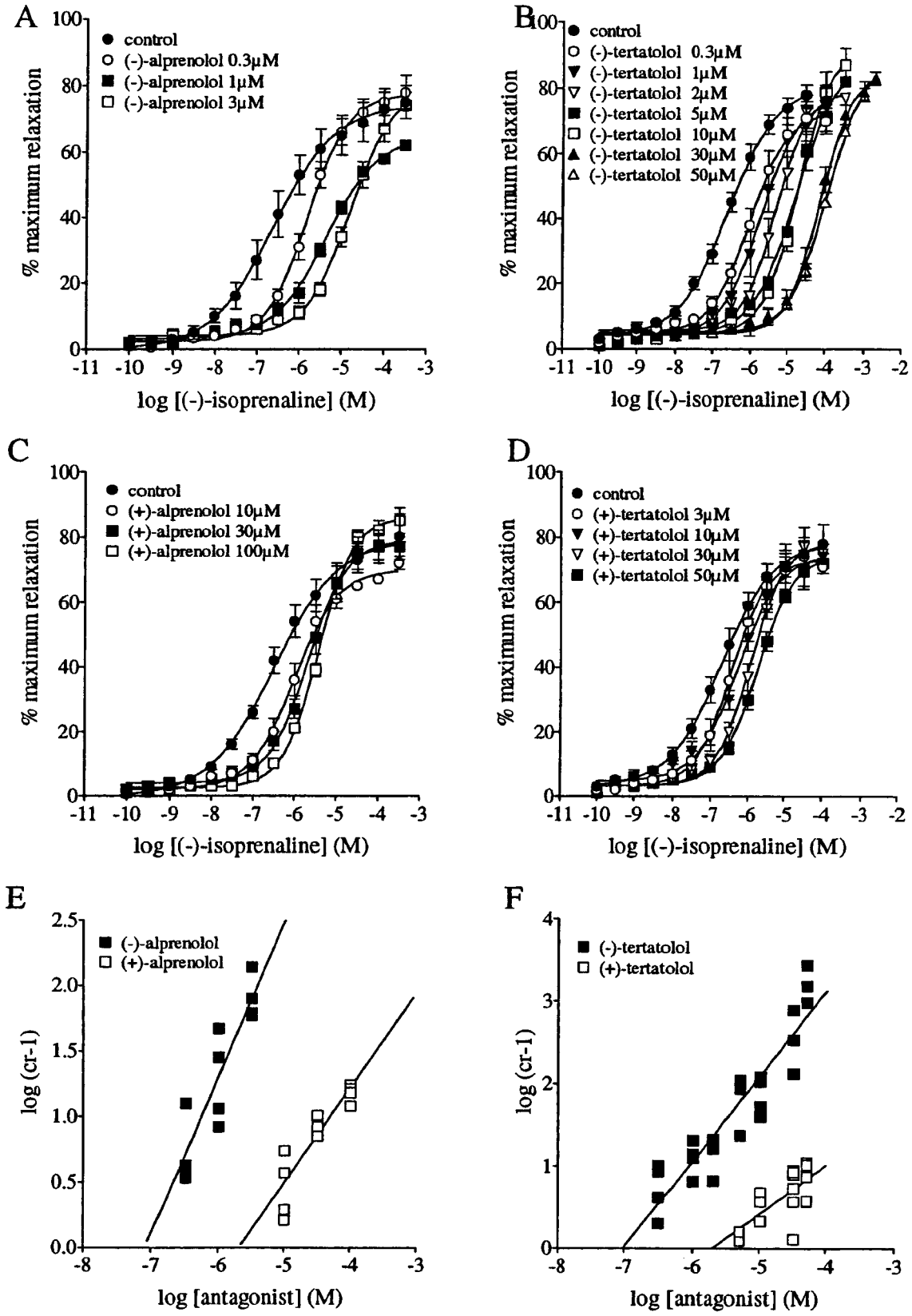

Figure 3 The effect of the stereoisomers of alprenolol (A, C and E) and tertatolol (B, D and F) on the relaxation responses to (−)-isoprenaline in the rat isolated ileum precontracted with carbachol. Shown are (−)-isoprenaline concentration response curves in the absence and presence of (A) (−)-alprenolol (B) (−)-tertatolol (C) (+)-alprenolol and (D) (+)-tertatolol. Points show mean ± s.e.mean ($n = 4-6$) and are expressed as a percentage of maximum relaxation by papaverine (100 μM). Slope values calculated from Schild plots for alprenolol (E) and tertatolol (F) show that the (−)-isomer in each case has a slope value not significantly different from unity while both (+)-isomers had slope values significantly less than unity. Slope values and pA_2 values were calculated and are shown in Table 1.

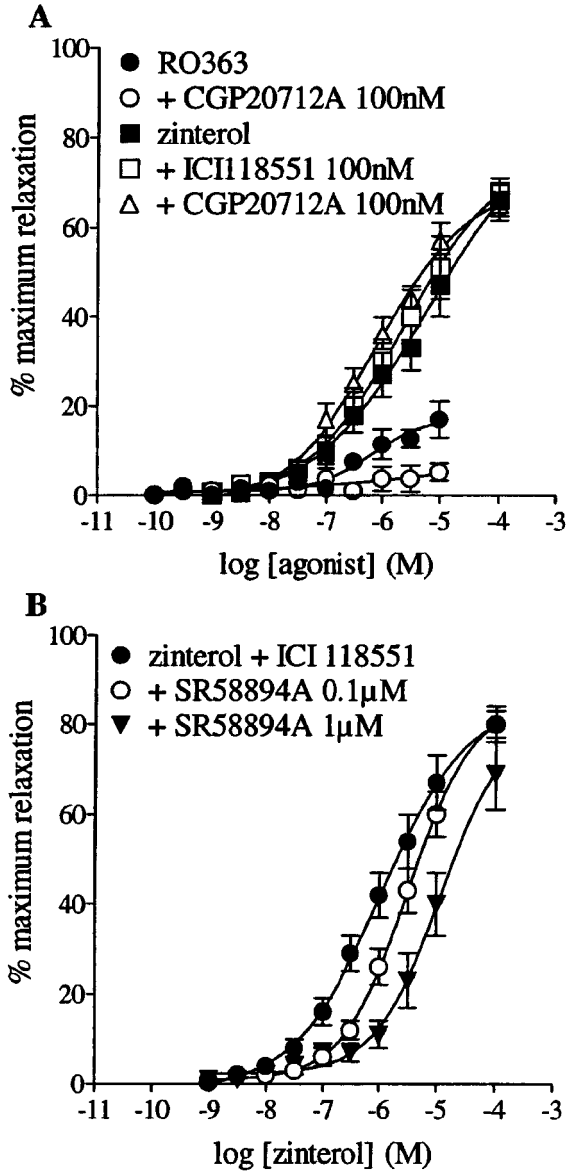

Figure 4 The effect of β_1- and β_2-AR selective agonists on relaxation in rat ileum precontracted with carbachol. Graph (A) shows relaxation with the β_2-AR agonist zinterol in the absence and presence of ICI 118551 and CGP 20712A. Also shown in (A) are the relaxation responses by the β_1-AR agonist RO363 in the absence and presence of CGP 20712A ($n=4$). Graph (B) shows the inhibition of zinterol relaxation by the β_3-AR antagonist SR 58894A in the presence of ICI 118551.

observed actin PCR product corresponded to the expected 559 bp, whereas contaminating DNA would have given a PCR product of 771 bp. Larger bands were not observed in any samples of cDNA used for the subsequent measurement of β-AR mRNA.

To compare the relative levels of β_1-AR, β_2-AR and β_3-AR in ileum, colon, cortex, soleus muscle and WAT the relationship between the log (PCR product) and cycle number was determined for individual samples. Although RT–PCR methods without control templates cannot be used to make direct comparisons between expression of different genes, the amplification of β_1-AR, β_2-AR and β_3-AR cDNA occurred with similar efficiency both in comparison to each other and between tissues as judged by the similar slopes of the product/cycle relationships. All plots were linear up to 28 cycles, and 27 cycles of PCR were used to determine the relative levels of β_1-AR, β_2-AR and β_3-AR mRNA from multiple samples of colon and ileum versus cortex, WAT and soleus muscle. In this experiment intron-spanning primers were used for both actin and β_3-AR, again with no evidence of larger genomic DNA bands. In accord with previous data (Summers *et al*., 1995; Evans *et al*., 1996), the greatest expression of β_3-AR mRNA was in WAT > colon = ileum > cerebral cortex > soleus. Levels of β_3-AR mRNA in ileum and colon smooth muscle were not significantly different from each other ($P=0.24$), and amounted to approximately 20% of that in WAT. In contrast β_1-AR mRNA was most abundant in cerebral cortex > WAT > ileum = colon > soleus with the levels in ileum and colon being 7–8% of that in cortex. β_2-AR mRNA was expressed in soleus > WAT > ileum = colon > cerebral cortex. β_2-AR mRNA levels in ileum and colon were approximately 25% of that in soleus muscle.

Discussion

In the present study, the β-AR subtypes mediating smooth muscle relaxation in rat ileum have been examined. The initial characterization of this receptor was performed using the non-selective β-AR agonist (−)-ISO. The effects of (−)-propranolol on (−)-ISO C-R curves suggested the presence of both typical and atypical β-ARs given the reduced shift with progressively higher concentrations. However this was not supported by experiments with the β_1- and β_2-AR selective antagonists CGP 20712A and ICI 118551 which both failed to cause any effect at concentrations up to 300 nM. It would therefore appear that the major component of smooth muscle relaxation was due to activation of a β-AR subtype that was not effectively blocked by propranolol, ICI 118551 or CGP 20712A. The affinity of propranolol ($pK_B=6.69$ at 10 μM) was somewhat higher than previously reported in rat ileum (5.5–5.7) (Growcott *et al*., 1993) but close to values quoted in rat distal colon (6.57, 6.39) (McLaughlin & MacDonald, 1990). The pK_B values of both propranolol and ICI 118551 (6.69 and 5.06 respectively) in rat ileum corresponded to the affinities of these compounds in rat jejunum (6.1 and 5.5; Van der Vliet *et al*., 1990), rat colon (6.0, Kaumann & Molenaar, 1996) and also at the rat cloned β_3-AR (5.8 and 5.3; Muzzin *et al*., 1991).

Evidence for a β_3-AR in rat ileal smooth muscle was strongly supported by concentration-dependent relaxation of the smooth muscle by the β_3-AR agonists CL 316243, BRL 37344 and SR 58611A. The biphasic responses caused by BRL 37344 were consistent with observations made by Growcott *et al*. (1993), as was the slower onset of action by BRL 37344 (also seen for CL 316243 and SR 58611A in the present study) compared to the rapid (−)-ISO response. The pEC_{50} value of 7.31 for the first component of the BRL 37344 C-R curve corresponded well with that at the cloned rat β_3-AR ($pEC_{50}=7.1$, Granneman *et al*., 1991). This component of the curve was sensitive to high concentrations of β-AR antagonists and a pK_B value of 6.51 for propranolol (1 μM) indicated activity at a β_3-AR. By comparison, the second phase of the BRL 37344 C-R curve was completely resistant to propranolol, cyanopindolol and the stereoisomers of tertatolol and alprenolol and may indicate another mechanism activated by high concentrations of BRL 37344.

The phenylethanolaminotetraline SR 58611A is a potent β_3-AR agonist that was originally described as a colon specific atypical β-AR agonist (see Manara *et al*., 1995, for review). However SR 58611A was relatively ineffective in the present

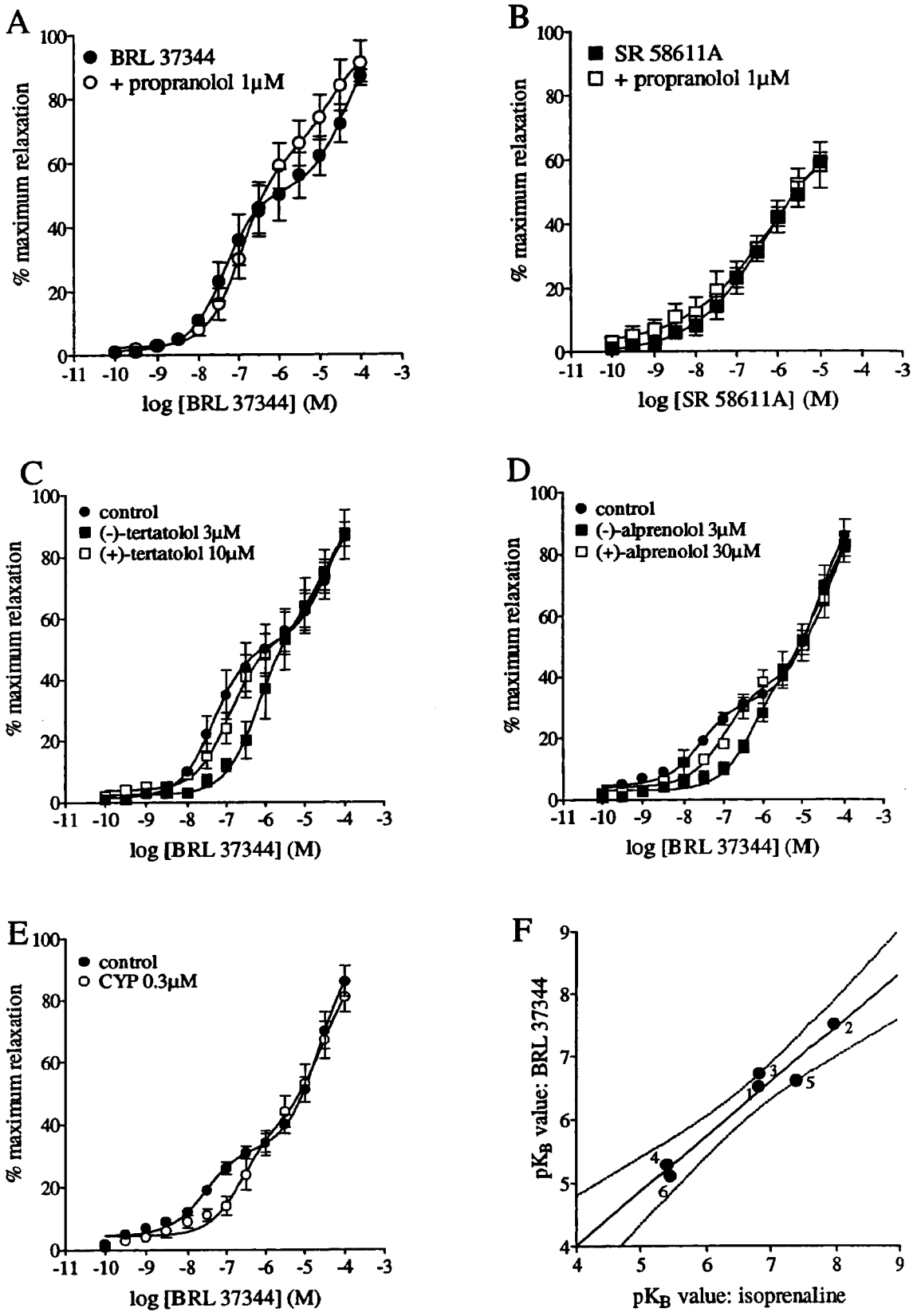

Figure 5 Mean concentration response curves for the relaxation of carbachol precontracted rat ileum by the atypical β-AR agonists (A) BRL 37344 and (B) SR 58611A. Both graphs show control concentration response curves and in the presence of (−)-propranolol (1 μM). Also shown are BRL 37344 concentration response curves in the presence of (C) (−)-tertatolol 3 μM and (+) tertatolol 10 μM; (D) (−)-alprenolol 3 μM and (+)-alprenolol 30 μM and (E) (±)-cyanopindolol 0.3 μM. Points are expressed as a percentage of maximum relaxation by papaverine (100 μM). Points show mean±s.e.mean ($n=4-6$). The pK_B values for all compounds were calculated and are shown in Table 2. Graph (F) shows a correlation plot of pK_B values for a range of compounds obtained against either (−)-isoprenaline concentration-response or BRL 37344 concentration-response curves. The regression line has a correlation coefficient of $r=0.973$, $P=0.0011$ and a slope of 0.863 (95% C.L. 0.58–1.14, $n=6$).

rat ileum preparation and maximum responses could not be obtained within the concentration range available. The reduced activity of SR 58611A in rat ileum compared to rat colon (EC_{50} 3.5 nM, Bianchetti & Manara, 1990) may be due to the hydrolysis of SR 58611A to a more potent acid metabolite by esterases specifically located in the colon giving this compound its preferential action in the colon preparation (Bianchetti & Manara, 1990). Alternatively, SR 58611A may be a partial agonist which is more effective in colon as this tissue has a greater receptor reserve.

Table 2 The affinities of a variety of antagonists against isoprenaline relaxation compared with affinities against relaxation to BRL 37344

Compound	*(−)-isoprenaline* $pK_B \pm$ *s.e.mean* (n)	*BRL 37344* $pK_B \pm$ *s.e.mean* (n)
(−)-propranolol (1 μM)	6.81 ± 0.20 (4)	6.51 ± 0.16 (3)
ICI 188551 (30 μM)	5.06 ± 0.13 (4)	n.d.
CGP 20712A (300 nM)	no shift (4)	n.d.
(−)-tertatolol (3 μM)	6.82 ± 0.15 (4)	6.71 ± 0.10 (5)
(+)-tertatolol (10 μM)	5.39 ± 0.15 (4)	5.28 ± 0.10 (5)
(−)-alprenolol (3 μM)	7.40 ± 0.08 (4)	6.61 ± 0.26 (5)
(+)-alprenolol (30 μM)	5.44 ± 0.04 (4)	5.10 ± 0.07 (5)
(±)-CYP (0.3 μM)	7.96 ± 0.02 (4)	7.50 ± 0.25 (4)

n.d., affinities of these compounds were not determined.

In studies of glycerol release from adipocytes (Bloom *et al.*, 1992) and colonic relaxation (Dolan *et al.*, 1994), CL 316243 was described as selective for β_3-AR compared to β_1-(atrial) and β_2-ARs (soleus muscle) and more β_3-AR selective than BRL 37344. In rat ileum, CL 316243 was the most potent β_3-AR agonist and produced a monophasic relaxation of the ileal smooth muscle. This response was not blocked by β_1- and β_2-AR antagonists but was blocked by the β_3-AR antagonist SR58894A with a pA_2 value of 7.8 which corresponds to its published affinity at rat colonic β_3-ARs ($pA_2 = 8.06$, Manara *et al.*, 1996).

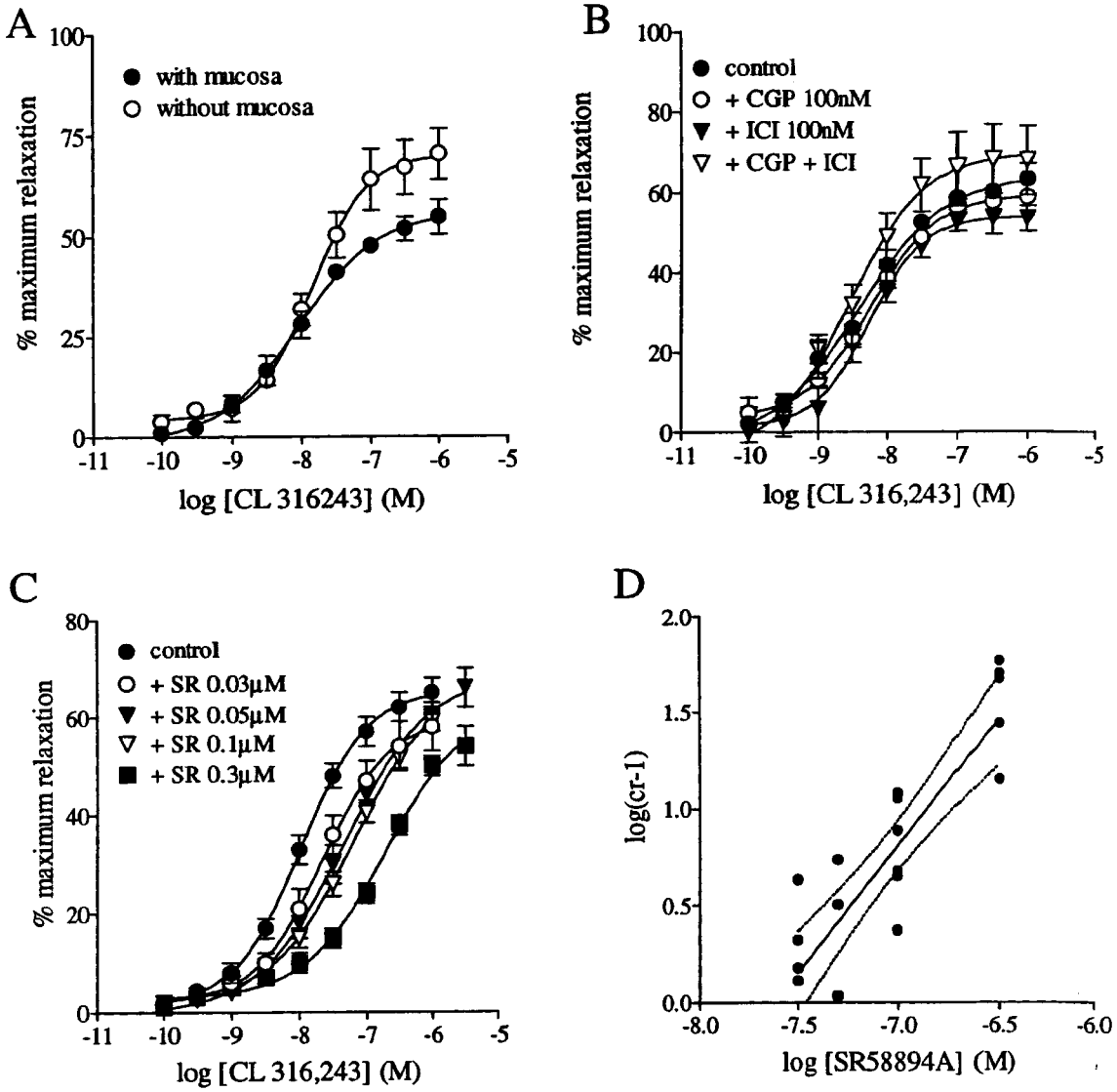

Figure 6 Mean concentration response curves for the relaxation of carbachol precontracted rat ileum by the β_3-AR agonist CL 316243. Graph (A) shows monophasic CL 316243 concentration-response curves in intact preparations and preparations with the mucosa removed. Graph (B) shows a control CL 316243 C-R curve and in the presence of the β_1-AR antagonist CGP 20712A 100 nM, the β_2-AR antagonist ICI 118551 100 nM and both antagonists in combination. Graph (C) shows the concentration dependent rightward shifts of the CL 316243 C-R curve with the β_3-AR antagonist SR 58894A. Graph (D) shows the Schild Plot (slope 95% C.I. 0.94–1.68) for SR 58894A. Points show mean ± s.e.mean ($n = 4–6$).

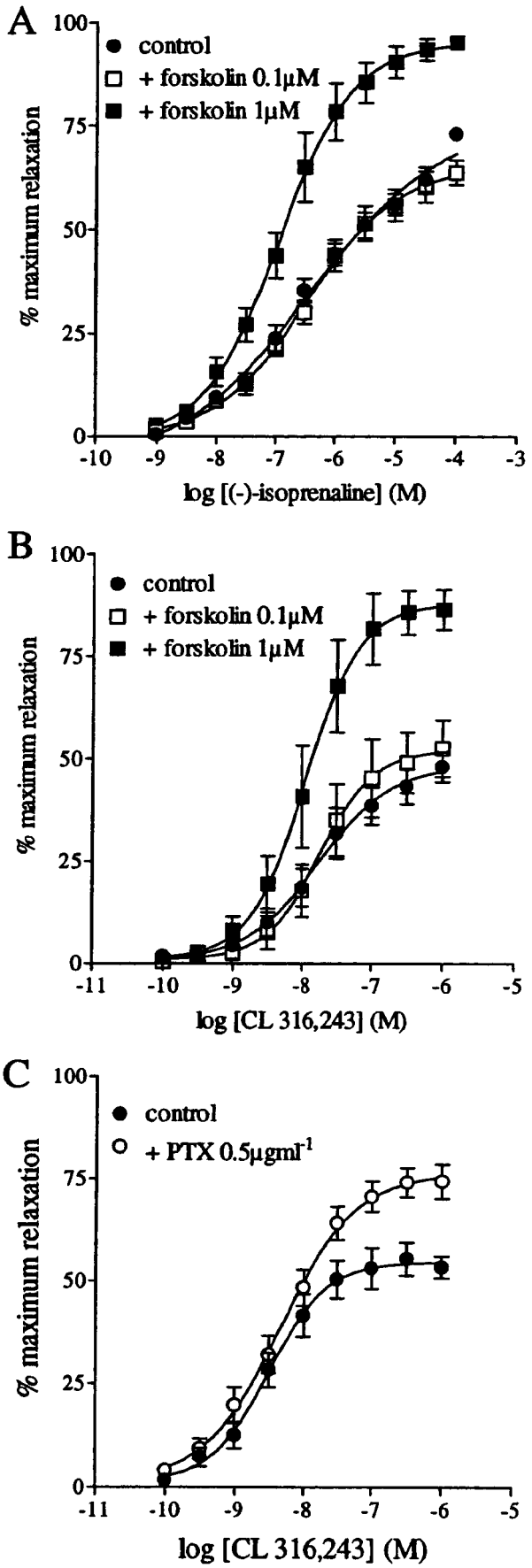

Figure 7 Effects of preincubation of rat ileal strips with forskolin for 45 min at 37°C on concentration-response curves to (A) isoprenaline and (B) CL 316243. Also shown (C) is the effect of preincubation with pertussis toxin (2 h, 37°C, $n=4$) on CL 316243 concentration-response curves.

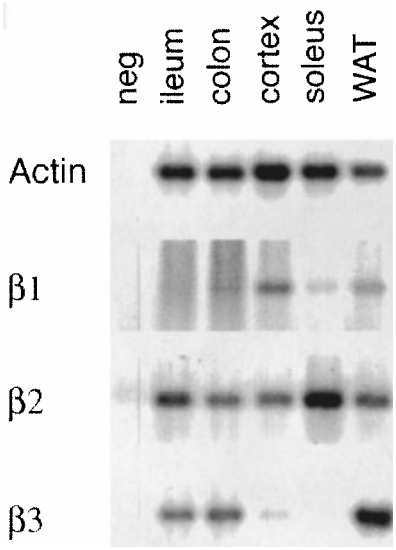

Figure 8 Detection of β_1-AR, β_2-AR, β_3-AR and actin mRNA by RT/PCR. Cycle numbers were 27 for β-ARs and 16 for actin. Sizes of the PCR products were determined from ethidium bromide stained gels by comparison with 100 bp DNA ladder (Pharmacia). The blots were apposed to phosphorimager plates for 7 h prior to scanning.

The affinity of (−)-alprenolol (3 μM) against ISO C-R curves in the present study (pK_B 7.12) was higher than when BRL 37344 was used as the agonist (pK_B 6.61). Other functional studies of β-ARs in gastrointestinal tissues have found affinities of alprenolol ranging from 7.2 in rat jejunum (Van der Vliet *et al*., 1990) to 6.5 in rat ileum (Growcott *et al*., 1993) and 6.47 in guinea-pig ileum (Blue *et al*., 1990). The latter study also demonstrated partial agonist activity of alprenolol in guinea-pig ileum (Blue *et al*., 1990). Tertatolol has also been described as an effective antagonist at β_3-ARs with a pA_2 value of 6.8 in guinea-pig ileum (Bond & Vanhoutte, 1992) compared to values of 7.29 and 6.71 (pK_B) measured in rat ileum (present study). It is interesting to note that the affinities of both alprenolol and tertatolol in the present study fall closer to values described for atypical β-ARs in other studies when BRL 37344 rather than ISO is used as the agonist. Another interesting observation was a tendency for Schild plots generated for the (+)-isomers to have slope values of less than unity, a feature not shared by the (−)-isomers. The reason for this difference is not known. Low stereoselectivity was observed for isomers of alprenolol competing for (−)-[^{125}I]-CYP binding in rat skeletal muscle (Molenaar *et al*., 1991; Roberts *et al*., 1993; Sillence *et al*., 1993) and brown adipose tissue membranes (Sillence *et al*., 1993). It is interesting to note that much higher stereoselectivity has been observed for agonists at atypical β-ARs, particularly the isomers of ISO which display 25 fold separation in rat small intestine (Van der Vliet *et al*., 1990) and 31 fold separation at the cloned human β_3-AR expressed in CHO cells (Emorine *et al*., 1989).

CYP and CGP 12177 are both β_1/β_2-AR antagonists that have also been described as partial agonists at atypical β-ARs (Mohell & Dicker, 1989; McLaughlin & MacDonald, 1991; McKean & MacDonald, 1994). ICI D7114 was originally described in guinea-pig ileum as having both agonist and antagonist activity, but behaved as an antagonist in rat ileum (Growcott *et al*., 1993) and colon (MacDonald & Lamont, 1993). All three compounds behaved as antagonists in the present study. (±)-CYP and CGP 12177 produced concentra-

tion dependent rightward shifts of (−)-ISO C-R curves. A single concentration of (±)-CYP (0.3 μM) gave similar pK_B values against both (−)-ISO (7.96) and BRL 37344 (7.50) induced relaxation. This differs from that seen by McLaughlin & MacDonald (1991) in rat gastric fundus where the pK_B value of (±)-CYP was significantly lower against BRL 37344 (6.56) than against (−)-ISO (7.44) induced relaxation. Several other studies have used CYP to identify atypical β-AR populations in gastrointestinal tissues and affinity values quoted include 6.67, 7.12 and 7.07 in rat distal colon (McLaughlin & MacDonald, 1990; McKean & MacDonald, 1994), 6.56 and 7.44 in rat gastric fundus (McLaughlin & MacDonald, 1991) and 7.01 in rat jejunum (MacDonald *et al*., 1994). One study has reported partial agonist activity of CYP ($pD_2 = 5.3$) and IodoCYP ($pD_2 = 7.0$) in rat ileum preparations (Hoey *et al*., 1996). The present study confirms reports that ICI D7114 (also known as ZD7114), a reversible competitive antagonist in rat distal colon with a pA_2 value of 7.29 (MacDonald & Lamont, 1993), is also an antagonist in rat ileum (pK_B values 7.41, 7.62). These values are higher than those previously reported in rat ileum (6.3–6.7; Growcott *et al*., 1993) making ZD7114 a higher affinity β_3-AR antagonist than either alprenolol or tertatolol in our rat ileum preparation. These results are again in contrast to those of Hoey *et al*. (1996) who reported that ICI D7114 was a partial agonist in rat ileum with a pseudo pD_2 value of 6.92.

In previous studies we have characterized (−)-[^{125}I]-CYP binding to β-ARs in rat ileum and atypical (β_3)-AR binding sites were clearly the predominant subtype while β_1- and β_2-ARs were undetectable even using classical binding conditions that favour binding to β_1- and β_2-ARs (Roberts *et al*., 1995). The marked relaxation observed to the β_2-AR agonist zinterol was therefore unexpected. However this response was not altered by the β_2-AR antagonist, ICI 118551, but was inhibited by the β_3-AR antagonist, SR 58894A. This suggests that zinterol causes relaxation of rat ileal smooth muscle through activation of β_3-ARs. This observation is of particular note with regard to the interpretation of results obtained in diabetic rat models which showed decreased gastrointestinal responses in rat duodenum to the β_2-AR agonist salbutamol (Altan *et al*., 1987; Yildizoglu-Ari *et al*., 1988). The low potency of salbutamol in these studies (pD_2 5.9–6.6) compared to its known affinity at β_2-ARs (7.6, from Arch & Kaumann, 1993 review) strongly suggests that these reduced responses may reflect decreases in either β_1- or β_3-AR rather than β_2-AR responsiveness in duodenum in diabetes. It is interesting to note that β_2-AR mRNA was detected in rat ileal smooth muscle at about 25% of the level in soleus muscle a tissue where β_2-AR mediated responses can be readily identified. This may indicate that amplification of the β_2-AR PCR product is particularly efficient, that the mRNA is not translated into functional receptors or that the β_2-AR subserve functions other than smooth muscle relaxation. *In vivo* electromyographic recordings of migrating myoelectric complexes (MMC) in rat duodenum and jejunum have shown that isoprenaline and the β_2-AR agonist ritodrine can disrupt the regular MMC pattern and the irregular spiking can be blocked with ICI 118551 (Thollander *et al*., 1996). It is therefore possible that although no relaxation response was detected there may be another role for a small population of β_2-ARs which are also undetected in binding studies (Roberts *et al*., 1995).

The β_1-AR agonist RO363 has a high intrinsic activity compared to ISO in rat colon and guinea-pig ileum (Molenaar *et al*., 1997) and a study by Hoey *et al*. (1996) found that RO363 caused a relaxation of rat ileum that was 60–70% of the isoprenaline response. In the present study however RO363 caused a relaxation that was less than 20% of the maximum and had a potency ($pEC_{50} = 6.2$) between its potency at β_1- and β_3-ARs in rat colon ($pEC_{50} = 8.5$ and 5.6 respectively, Molenaar *et al*., 1997). We found that the RO363 relaxation in rat ileum was almost completely blocked by the β_1-AR antagonist CGP 20712A. Although it was not possible to directly compare expression of the β-AR subtypes we found that the level of β_1-AR mRNA in ileum was significantly lower than levels of the same receptor detected in either cortex or white adipose tissue, which both contain functional β_1-ARs (Morin *et al*., 1992; Arch & Kaumann, 1993). The levels of expression in ileum were comparable to levels found in soleus muscle, a tissue where there is little functional evidence for the presence of β_1-ARs (Roberts *et al*., 1993).

Finally, we examined the effects of alteration of components of the adenylate cyclase signalling pathway to determine whether β_3-AR mediated relaxation responses were altered. Low concentrations of forskolin, which produced no change in tissue responses by themselves, are known to enhance the responses to compounds acting through adenylate cyclase. In rat ileum, the maximum relaxation of the smooth muscle was significantly enhanced after pretreatment *in vitro* with the adenylate cyclase activator, forskolin. Pertussis toxin pretreatment to ADP ribosylate G_i also enhanced ideal smooth muscle relaxation suggesting that G_i signalling contributes to either the basal or receptor stimulated tone in rat ileum. Granneman and coworkers showed that the adipocyte β_3-AR can couple through both the stimulatory (G_s) protein and the inhibitory (G_i) protein and that abolition of the interaction with G_i using pertussis toxin caused a marked increase in cyclic AMP accumulation in these cells (Chaudry *et al*., 1994). Whether or not the gastrointestinal β_3-AR also activates both signalling pathways however clearly requires further investigation.

Several anomalies are apparent from these findings. The biphasic nature of the propranolol Schild Plot and the shallow slopes of several antagonists suggests that multiple subtypes are involved in relaxation yet the selective β_1- and β_2-AR antagonists CGP 20712A and ICI 118551 did not shift the ISO C-R curve. However, as the selective β_1-AR agonist RO 363 appears to act through a small population of β_1-ARs it is possible that β_1-ARs play a role in relaxation that is functionally of little significance when β_3-ARs are also being stimulated. The strong signal for β_2-AR mRNA in ileum, colon and white adipose tissue was also interesting as these tissues have been characterized functionally as having predominantly β_1- and/or β_3-ARs (Arch & Kaumann, 1993). Possible explanations may be that in these tissues the β_2-AR mRNA is in blood vessels or nerves or that β_2-AR are mediating a different function from the β_1- and β_3-ARs. Given the absence of β_2-AR binding in rat ileum however (Roberts *et al*., 1995) it is also possible that the β_2-AR mRNA may not be translated into a functional protein.

The current criteria for classification of the β_3-AR include: (1) stimulation by selective β_3-AR agonists; (2) partial agonist activity of non-conventional β-AR antagonists; (3) low affinity of typical β-AR antagonists; (4) atypically low stereoselectivity indices and (5) blockade by selective β_3-AR antagonists (Strosberg & Pietri-Rouxel, 1996; Kaumann & Molenaar, 1996). The studies presented in this paper show that the responses elicited by ISO and by various β_3-AR agonists in rat ileum smooth muscle are blocked with low affinity by conventional β-AR antagonists, that low stereoselectivity was demonstrated for alprenolol and tertatolol, and that the β_3-AR antagonist, SR 58894A, has high affinity for blocking relaxation to β_3-AR agonists. We also demonstrated the presence of β_3-AR mRNA in rat ileal smooth muscle. The

present characterization of smooth muscle relaxation to β-AR agonists confirms that β_3-ARs are the predominant subtype mediating these responses with little to no evidence for a relaxation role for β_1- or β_2-ARs in rat ileum.

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