British Journal of Pharmacology (1995) 116, 2549- 2556

Characterization and localization of atypical β-adrenoceptors in rat ileum

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1 Homogenate binding studies and receptor autoradiography have been used to examine the binding characteristics and localization of propranolol-resistant (−)-[^{125}I]-cyanopindolol (CYP) binding sites in rat ileum.

2 Saturation studies with (−)-[^{125}I]-CYP and homogenates of rat ileum identified a site with pK_D 8.89±0.08 and B_{max}=50.3±4.1 fmol mg^{-1} protein (n=6). Both β_1- and β_2-adrenoceptors (AR) were not detected in these preparations.

3 (−)-Isoprenaline infusion (400 μg kg^{-1} h^{-1}) for 14 days caused no significant change in the density of (−)-[^{125}I]-CYP binding which was 48.9±12.8 and 40.6±12.3 fmol mg^{-1} protein in control and isoprenaline-treated animals respectively (n=6) (P=0.97).

4 Competition for (−)-[^{125}I]-CYP binding in the presence of 0.1 μM (−)-propranolol gave affinity values for CYP, tertatolol, alprenolol, ICI 118551 and CGP 20712A that correspond to known affinities at atypical β-ARs. Stereoselectivity ratios for tertatolol and alprenolol were low.

5 Autoradiographic localization of propranolol resistant (−)-[^{125}I]-CYP binding showed sites associated with the mucosa and to a lesser extent to the muscularis. A small population of β_2-ARs were detected located predominantly in the longitudinal and circular smooth muscle layers.

6 This study identifies an (−)-[^{125}I]-CYP binding site in rat ileum that is resistant to blockade by propranolol (0.1 μM), is located predominantly in the mucosa, shows resistance to downregulation by isoprenaline and has binding characteristics of the atypical β-AR.

Keywords: β_3-Adrenoceptor; atypical β-adrenoceptor; (−)-[^{125}I]-cyanopindolol; regulation; localization; autoradiography; rat ileum

Introduction

There is evidence from functional studies for atypical β-adrenoceptors (β-AR) in gastrointestinal tissues where they mediate relaxation. The functional atypical β-AR is found in guinea-pig ileum (Bond & Clarke, 1988; Blue *et al.*, 1990), rat gastric fundus (McLauglin & MacDonald, 1991), ileum (Growcott *et al.*, 1990), jejunum (Van der Vliet *et al.*, 1990), colon (Croci *et al.*, 1988; McLaughlin & MacDonald, 1990) and oesophagus (Ford *et al.*, 1992; de Boer *et al.*, 1993). Atypical β-ARs in these tissues in some respects resemble the β_3-AR in adipocytes. Common features include: a catecholamine-mediated response that is resistant to blockade by concentrations of propranolol that effectively block β_1 and β_2-ARs; a low affinity of conventional β-AR antagonists with alprenolol being one of the more effective competitors of the response (Blue *et al.*, 1990); and a high potency of atypical β-AR agonists, particularly BRL 37344 (Bond & Clarke, 1988) and SR 58611A (Croci *et al.*, 1988) compared to isoprenaline. There is a high functional homology between the β_3-ARs mediating lipolysis in rat adipocytes and atypical β-ARs which inhibit colonic motility (Landi *et al.*, 1993). Atypical β-ARs also resemble the cloned β_3-AR from man (Emorine *et al.*, 1989), rat (Granneman *et al.*, 1991; Muzzin *et al.*, 1991) and mouse (Nahmias *et al.*, 1991). mRNA for the β_3-AR has been detected in human ileum and colon (Granneman *et al.*, 1993), rat ileum (Granneman *et al.*, 1991) and rat colon (Bensaid *et al.*, 1993) providing additional evidence for the presence of β_3-ARs in gastrointestinal tissue.

Attempts to detect atypical β-ARs with radioligand binding techniques in rat jejunum (Van der Vliet *et al.*, 1990) and rat colon (Landi *et al.*, 1992) were unsuccessful. In contrast, receptor binding assays for β_3-ARs were performed successfully in cell lines expressing the native receptor (Feve *et al.*, 1991) or transfected cells (Muzzin *et al.*, 1991) and have shown that (−)-[^{125}I]-CYP and [^{3}H]-CGP12177 have a lower affinity for the β_3- than for either β_1- or β_2-ARs. This may explain why many binding studies performed under conditions developed to study β_1- and β_2-ARs failed to identify the third subtype. Previously we have optimised conditions for (−)-[^{125}I]-CYP binding to rat soleus muscle homogenates and characterized a propranolol-resistant site with properties resembling the atypical β-AR (Roberts *et al.*, 1993). Similar sites have been characterized in rat skeletal muscle and brown adipose tissue (Sillence *et al.*, 1993). The aims of the present study were to use the atypical β-AR binding assay to examine the binding of (−)-[^{125}I]-CYP in rat small intestine and to localize the areas of binding.

Methods

Membrane preparation

Male Sprague-Dawley rats (250–350 g) were stunned by a blow to the back of the head and exsanguinated. A 10–15 cm segment of the small intestine was removed from the ileocaecal junction and the intraluminal contents flushed out with ice cold phosphate buffered saline (PBS) (composition, mM: NaCl 118.4, $NaH_2PO_42H_2O$ 10, KCl 4.7, $MgSO_47H_2O$ 1.2, $CaCl_2$ 1.3). The tissue was weighed and homogenized in 20–25 ml ice cold PBS containing phenylmethylsulphonylfluoride (PMSF) (10 μM) pH 7.4. Homogenates were filtered through a nylon filter (210 μm) and centrifuged at 4°C for 12 min at 50,000 g in a Beckman J2-MI centrifuge. The supernatant was discarded and the pellet resuspended in 20 ml of PBS and centrifuged a second time. The final pellet was resuspended in 5 volumes of

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PBS and stored on ice until use. Protein was determined by the method of Lowry *et al.* (1951) with BSA used as standard.

Implantation of osmotic mini pumps

Male Sprague Dawley rats (280–320 g) were anaesthetized with pentobarbitone sodium (Nembutal) 25 mg kg^{-1}, i.p., the back of the neck shaved and a local s.c. injection of lignocaine (2%) made. The skin was cut and a pocket under the skin formed by blunt dissection to allow implantation of osmotic mini pumps (Alzet, model 2002) to perfuse 0.5 μl h^{-1} of either (−)-isoprenaline (400 μg kg^{-1} h^{-1}) or vehicle (0.001 N HCl). The wound was closed with suture clips. After 14 days the animals were killed by a blow to the back of the head and exsanguinated; 10 cm (approx) of small intestine was removed from the ileocaecal junction, frozen in liquid nitrogen and stored at −80°C for use in receptor binding assays. Homogenates were prepared as described above for fresh tissue.

Binding assays

Radioligand binding assays were performed in PBS as previously described (Roberts *et al.*, 1993). Preliminary experiments in rat ileum revealed that high concentrations of 5-hydroxytryptamine (5-HT) (10 μM and 100 μM) only inhibited specific (−)-[^{125}I]-CYP (500 pM) binding by 12±5% and 10±3% (n=3) respectively indicating that (−)-[^{125}I]-CYP binding was not to 5-HT receptors (Hoyer *et al.*, 1985). It was therefore not necessary to include 5-HT in the radioligand assay buffer.

Kinetics Homogenate (50 μl) was incubated with (−)-[^{125}I]-CYP (500 pM) and (−)-propranolol (0.1 μM) at 37°C for up to 120 min in association experiments. The dissociation of (−)-[^{125}I]-CYP was determined after a 60 min incubation at 37°C for up to 90 min after the addition of (±)-alprenolol (500 μM).

Saturation Homogenate (50 μl) was incubated with (−)-[^{125}I]-CYP (5–1000 pM) in the absence or presence of (±)-alprenolol (500 μM) to define non-specific binding. In studies examining β_1- and β_2-ARs, homogenate was incubated with (−)-[^{125}I]-CYP (5–200 pM) in the absence or presence of (−)-propranolol (1 μM) to define non-specific binding.

Competition Homogenate (50 μl) was incubated with (−)-[^{125}I]-CYP (500 pM) with or without (−)-propranolol (0.1 μM) and a range of concentrations of competitor. Non-specific binding was defined by (±)-alprenolol (500 μM). In experiments using (−)-isoprenaline and (−)-noradrenaline the incubation mixtures contained 0.1 mM ascorbic acid to prevent catecholamine oxidation.

All tubes were prepared in duplicate or triplicate and incubated for 60 min at 37°C. Incubations were terminated by the addition of 4 ml ice cold PBS followed by rapid vacuum filtration (Brandel M-30R cell Harvester). Membranes were washed onto Whatman GF/B filters presoaked in 2% polyethylenimine and washed with 3 × 4 ml buffer. Radioactivity retained on the filters was measured in a Packard gamma counter (Model B5424) at an efficiency of 79%.

Analysis All experiments were performed in duplicate or triplicate with tissues from n animals. All results are expressed as means±s.e.mean of n. Kinetic experiments were analysed with KINFIT (Williams & Summers, 1990). Saturation binding data and competition experiments were analysed using EBDA (McPherson, 1983) to obtain preliminary binding estimates and the non-linear curve fitting analysis programme PRISM (Intuitive Software for Science) to obtain final pK_D and n_H values. All curves were analysed for both a one site and two site fit. Significant line shifts for stereoselectivity were determined with ANCOVA which is part of the REAP package (Gamma Research Systems, Knoxfield Australia).

Autoradiography

Sprague-Dawley rats (male or female, 250–350 g) were stunned and exsanguinated, the chest cavity opened and the left ventricle punctured with a cannula which was inserted into the aorta. The animal was perfused via the aorta with PBS containing PMSF 10 μM/sucrose 0.32 M (1 : 1; 40 ml, 37°C), and then lightly fixed by adding 0.05% formaldehyde and 0.05% glutaraldehyde to the perfusate (20 ml, 37°C). A distal segment of ileum was removed and the intraluminal contents flushed out with PBS/sucrose. The tissue was frozen in liquid nitrogen-cooled isopentane, mounted in O.C.T. Compound and sections (10 μm) cut with a Reichert-Jung Cryocut 1800 cyrostat and collected on microscope slides coated with a gelatin (0.5%) and chromic potassium sulphate (0.05%) solution. Every 10th slide was stained with haematoxylin and eosin to show tissue morphology. Other sections were incubated at room temperature (30 min) with guanosine triphosphate (GTP, 0.1 mM) containing ascorbic acid (1 mM) and EDTA (0.1 mM). Slides were shaken vigorously to remove excess GTP solution and placed in humidified chambers on a slide warming tray (Ratek Instruments, Australia). The sections were incubated with (−)-[^{125}I]-CYP (540 pM) in PBS containing (−)-propranolol (0.1 μM) to block β_1- and β_2-ARs (60 min, 37°C). (±)-Alprenolol (500 μM) was used to determine non-specific binding. Slides were drained, dipped and then washed (2 × 5 min, 37°C) in PBS to remove unbound radioligand.

The distribution of β_1- and β_2-ARs was determined using (−)-[^{125}I]-CYP (43 pM) prepared in PBS containing 5-HT (10 μM) in the absence or presence of the β_2-AR-selective antagonist, ICI 118,551 (70 nM), or the β_1-AR-selective antagonist, CGP 20712A (100 nM). Non-specific binding was determined with (−)-propranolol (0.1 μM). These slides were drained, dipped and then washed (2 × 15 min, 37°C) in PBS to remove unbound radioligand. All slides were dipped in distilled water (room temperature) and dried under a stream of medical air. X-ray film was apposed to the sections and exposed for 3–5 weeks. The X-ray film was developed in Kodak D19 activator (5 min, 18°C), rinsed in distilled water and fixed with Kodak Rapid Fixer (5 min, 18°C). Haematoxylin and eosin stained sections were photographed under a Wild Heebrugg Photomacroscope M400 with Wild MPS45 Photoautomat control meter attachment and the autoradiographs printed directly from X-ray film using a Leitz Focomat 1C enlarger. For quantitative autoradiographic analysis the X-ray film images were illuminated with a Northern Light (Model B90) light source and viewed with a Minitron (MTV-1801 CB) camera. The density of propranolol-resistant binding sites in the muscularis (4-7 sections for each of total and non-specific binding) was determined with a Microcomputer Imaging Device (MCID, Imaging Research Inc.) system. Regions of the muscularis were traced using the outlining tool. The density of binding sites was determined by comparing the grey scale from the X-ray film with the standard curve produced using ^{125}I standards prepared in guinea-pig heart paste.

Materials

The materials used were: (−)-[^{125}I]-CYP (Amersham International, Buckinghamshire, U.K.), (±)-ICI 118551 (erythro-DL-1(7-methylindian-4-yloxy)-3-iso-propylaminobutan-2-ol), ICI D7114 ((S)-4-[2-hydroxy-3-phenoxy-propylaminoethoxyl]-*N*-(2-methoxyethyl)phenoxyacetamide) (Imperial Chemical Industries, Wilmslow, Cheshire, England); (±)-CGP 20712A (2-hydroxy-5(2-((2-hydroxy-3(4-((1-methylo-4-trifluoromethyl) 1H-imidazole-2-yl)-phenoxy)propyl)amino)ethoxy)-benzamide monomethane sulphonate) (Ciba-Geigy Ag, Basel, Switzerland); (−)-tertatolol, (+)-tertatolol (Servier, Paris, France), (+)-alprenolol (Professor B. Jarrott, Monash University), (±)-alprenolol, (−)-alprenolol, (−)-isoprenaline, (−)-noradrenaline, (−)-propranolol, 5-hydroxytryptamine, phenylmethylsuphonyl fluoride, haematoxylin (Sigma Chemical Company, St. Louis, MO, U.S.A.); SR 58611A (RS-N-(7-

carbethoxymethoxyl 1,2,3,4-tetrahydronaphth-2-yl)-2 hydroxy 2-(3-cholorphenyl)ethanamine) (SANOFI-MIDY S.p.A. Research Centre, Milan, Italy), BRL 37344 (sodium-4-[-2[-2-hydroxy-2-(-3-chloro-phenyl) ethylamino] propyl] phenoxyacetate) (Smith Kline Beecham, Great Burgh, Epsom, Surrey, U.K.), (±)-CGP 12177 hydrochloride ((−)-4-)3-*t*-butylamino-2-hydroxypropoxy)benzimidazol-2-one) (Research Biochemicals Inc. Massachusetts, U.S.A.), (±)-CYP (Sandoz, Basel, Switzerland), guanosine triphosphate (Boehringer Mannheim, Melbourne, Australia); Rapid Fixer, D19 (Kodak, Melbourne, Australia); eosin (Medos); O.C.T. Compound (Miles, Elkhart, IN, U.S.A.). Stock solutions of CGP 20712A and ICI 118,551 were prepared in 10 mM HCl, PMSF in absolute ethanol, isoprenaline and noradrenaline in 0.1 mM ascorbic acid and the remaining drugs in distilled water.

Results

Binding characterization

Kinetics Association of (−)-[^{125}I]-CYP (500 pM) to the propranolol (0.1 μM)-resistant site in rat ileum homogenates reached equilibrium in 30 min at 37°C and remained at this level for 90 min (Figure 1). Association occurred in a single phase with an association constant (K_1) of $4.38 \pm 0.66 \times 10^8$ min^{-1} ($n=6$). Dissociation kinetics determined at 37°C showed that binding was reversible on the addition of (±)-alprenolol (500 μM) (Figure 1). Dissociation was monophasic with a K_1 of 0.19 ± 0.03 min^{-1} ($n=4$). The kinetic K_D (K_{-1}/K_1) was 424 pM.

Saturation (−)-[^{125}I]-CYP binding occurred in a concentration-dependent manner (Figure 2). Specific binding defined by (±)-alprenolol (500 μM) ranged from 50–69%. (−)-[^{125}I]-CYP bound to a single population of sites with a pK_D value of 8.89 ± 0.08 (1.29 nM) and B_{max} of 50.3 ± 4.1 fmol mg^{-1} protein ($n=6$).

Competition Competition by (−)-propranolol (non-selective β-AR antagonist), CGP20712A (selective-β_1-AR antagonist) or ICI 118551 (selective β_2-AR antagonist) were monophasic (Figure 3). The affinity of each compound for the binding site was much lower than expected for β_1- or β_2-ARs (Table 1). It should be noted that the slope values of these curves were low particularly for (−)-propranolol (n_H 0.63) and ICI 118551 (n_H 0.67) indicating that two sites may be present but cannot be differentiated by computer analysis.

Subsequent competition experiments were performed in the presence of 0.1 μM (−)-propranolol which will block only 3% of the propranolol-resistant binding sites while blocking 97% and 98% of β_1- and β_2-ARs (Gille *et al.*, 1985). Competition by (±)-cyanopindolol (CYP) (pK_D 6.02) revealed an affinity lower than that of (−)-[^{125}I]-CYP (pK_D 8.89). The stereoisomers of alprenolol and tertatolol show small but significant differences between the isomeric forms ($P<0.05$). In each case the (−)-isomer was more potent than (+)-isomer with stereoselectivity indices of 3.0 fold for alprenolol and 1.2 fold for tertatolol. The rank order of affinities was (−)-tertatolol (5.89) > (+)-tertatolol (5.80) > (−)-alprenolol (5.38) > (+)-alprenolol (4.91) (Table 1). The pK_D values for atypical β-AR agonists were determined from single site competition binding analysis and the rank order of affinity was ICI D7114 (4.44) > SR 58611A (4.09) > (±)-CGP 12177A (3.94) > BRL 37344 (3.64) as were affinities of catecholamines isoprenaline (4.56) > noradrenaline (<3) (Table 1). Competition curves for CGP 12177A and (−)-isoprenaline both showed low slope values of 0.53 and 0.41 respectively and biphasic competition curves were significantly better fits than monophasic curves ($P<0.05$). High (pK_{D1}) and low (pK_{D2}) affinity components for biphasic competition analysis were pK_{D1} 5.70 ± 0.53 and pK_{D2} 3.67 ± 0.16 (CGP 12177A) and pK_{D1} 5.29 ± 0.21 and pK_{D2} 2.68 ± 0.46 ((−)-isoprenaline).

The values obtained in this study were compared to those

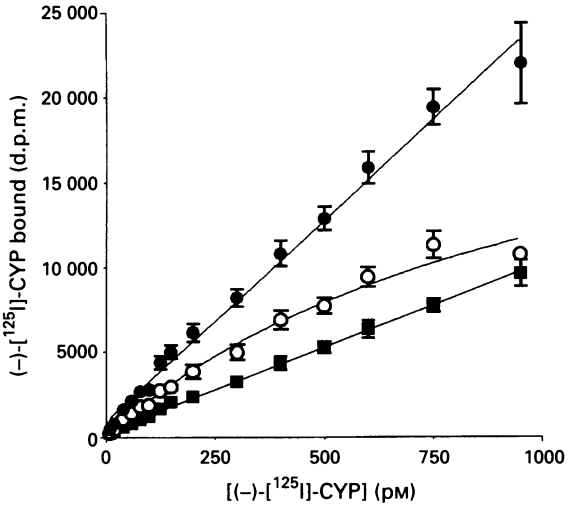

Figure 2 Saturation binding curve of (−)-[^{125}I]-CYP to rat ileal membrane preparations. Graph shows total binding (●), specific binding (○) and non-specific binding (■) defined by (±)-alprenolol (500 μM). PRISM analysis of data showed a single binding site with pK_D 8.89 ± 0.08 (B_{max} 50.3 ± 4.1 fmol mg^{-1} protein) ($n=6$).

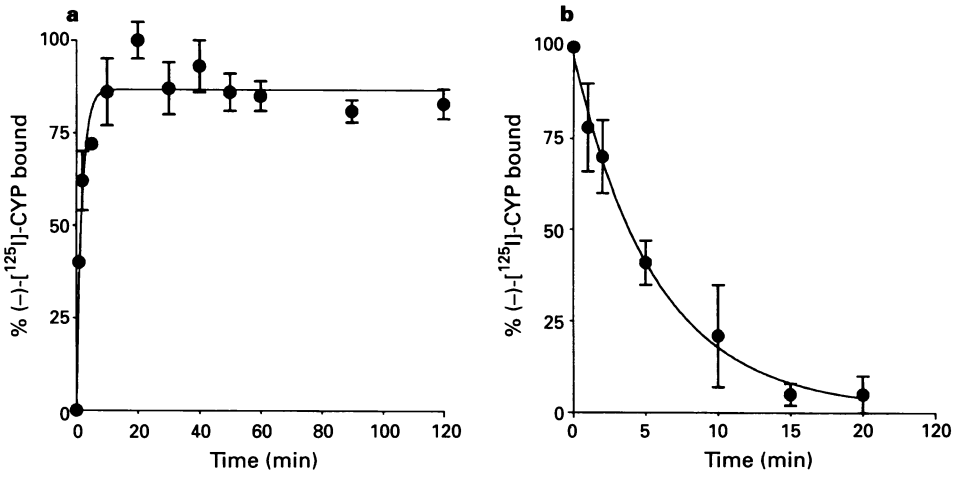

Figure 1 Association and dissociation of (−)-[^{125}I]-CYP propranolol-resistant binding sites in rat ileal membranes at 37°C. (a) Shows the rapid association of (−)-[^{125}I]-CYP while (b) shows the dissociation at various time points after the addition of 500 μM alprenolol to membranes incubated with 500 pM (−)-[^{125}I]-CYP for 60 min. Points show mean ± s.e.mean ($n=5$).

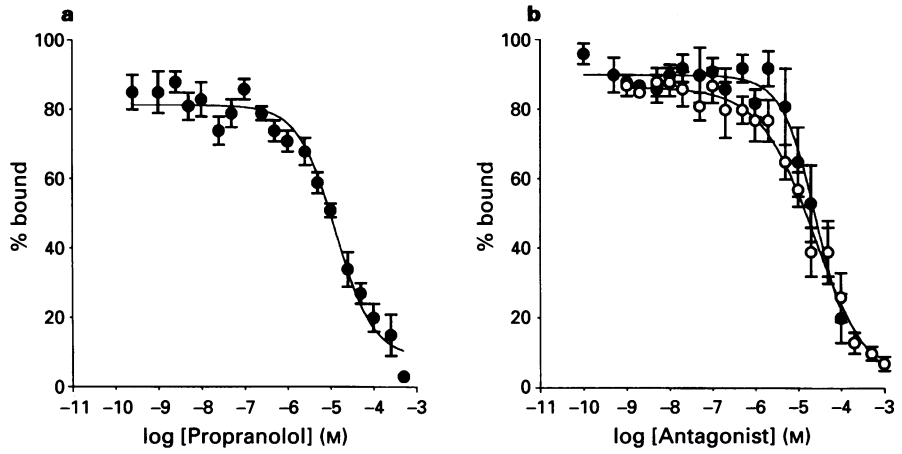

Figure 3 Competition for (−)-[^{125}I]-CYP binding (500 pM) by (a) (−)-propranolol and (b) ICI 118551 (○) and CGP 20712A (●) in rat ileal membranes. Incubations were for 1 h at 37°C and nonspecific binding was defined by (±)-alprenolol (500 μM). pK_D and n_H values are shown in Table 1. Points show mean ± s.e.mean (n = 4–6).

Table 1 Affinity (pK_D) and Hill coefficient (n_H) values of compounds competing for (−)-[^{125}I]-CYP binding in rat ileum

Competitor	n	pK_D ± *s.e.mean*	n_H ± *s.e.mean*
Absence of propranolol			
(−)-Propranolol	6	5.04 ± 0.08	0.63 ± 0.09
(±)-ICI 118551	4	4.90 ± 0.07	0.67 ± 0.09
(±)-CGP 20712A	4	4.65 ± 0.11	1.08 ± 0.27
(−)-[^{125}I]-CYP	6	8.89 ± 0.08	0.96 ± 0.01
Presence of 0.1 μM propranolol			
(±)-Cyanopindolol	4	6.02 ± 0.09	0.81 ± 0.14
(−)-Tertatolol	6	5.89 ± 0.05	0.86 ± 0.09
(+)-Tertatolol	6	5.80 ± 0.07	0.91 ± 0.15
(−)-Alprenolol	5	5.38 ± 0.07	0.88 ± 0.16
(+)-Alprenolol	5	4.91 ± 0.09	0.60 ± 0.09
SR 58611A	5	4.09 ± 0.07	1.30 ± 0.23
ICI D7114	4	4.44 ± 0.13	0.89 ± 0.30
BRL 37344	4	3.31 ± 0.18	1.40 ± 0.55
CGP 12177A	5	5.70 ± 0.53 3.67 ± 0.16	0.53 ± 0.17
(−)-Isoprenaline	4	5.29 ± 0.21 2.68 ± 0.46	0.41 ± 0.04
(−)-Noradrenaline	4	< 3.0	ND
Atropine	4	< 3.0	ND

ND, Non linear regression analysis was not able to determine these values

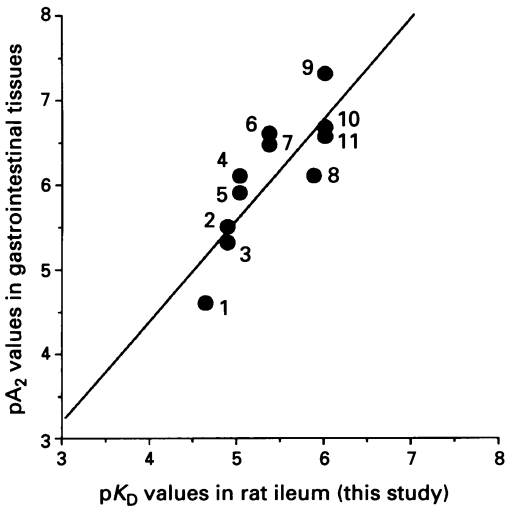

Figure 4 Correlation plot of pK_D values from binding studies in rat ileum (this study) with affinities of the same compounds used as antagonists in functional studies. The regression line has a correlation coefficient of r = 0.83, P = 0.0015 and a slope (1.20) not significantly different from one (95% confidence limits, (CL) = 0.60–1.80) (n = 11). Key: **1**[a], CGP 20712A; **2**[b], **3**[a] ICI 118551; **4**[c], **5**[b] propranolol; **6**[d], **7**[c] alprenolol; **8**[c] tertatolol; **9**[e], **10**[f], **11**[c] cyanopindolol. [a]de Boer *et al.* (1993); [b]Van der Vliet *et al.* (1990); [c]Ford *et al.* (1992); [d]Blue *et al.* (1990); [e]McLaughlin & McDonald (1991); [f]McLaughlin & McDonald (1990).

obtained for the same compounds used in functional studies in a variety of gastrointestinal tissues and show a high degree of similarity (r = 0.83, P = 0.0015, n = 11) (Figure 4). We also compared the affinities of several compounds from the present study with those obtained in binding studies at the cloned rat β_3-AR expressed in CHO cells (Muzzin *et al.*, 1991; Liggett, 1992). A correlation with r = 0.71 (P = 0.048, n = 8) was found when comparing the affinities for ICI 118551, propranolol, alprenolol, noradrenaline and the high affinity binding values for (−)-isoprenaline and CGP 12177A. The regression line for this analysis is shown in Figure 5. However when points representing the atypical β-AR agonist BRL 37344 and the low affinity values for CGP 12177 and (−)-isoprenaline were included the correlation using linear regression was no longer significant (r = 0.26, P = 0.38) (Figure 5).

Regulation

Using binding conditions designed for typical β-ARs, β_1- and β_2-ARs were not detected in saturation binding experiments in either vehicle- or isoprenaline-treated animals. Atypical β-AR binding did not change significantly with isoprenaline treatment (400 μg kg^{-1} h^{-1} for 14 days). Binding parameters were pK_D 8.78 ± 0.11 and B_{max} 48.9 ± 12.8 fmol mg^{-1} protein in control and pK_D 8.81 ± 0.12 and B_{max} 40.6 ± 12.3 fmol mg^{-1} protein in isoprenaline treated animals (n = 6) (P = 0.97).

Autoradiographic localization of propranolol-resistant binding sites and β_1- and β_2-ARs in rat ileum

Haematoxylin and eosin stained sections were used to identify various anatomical structures in the rat ileum (Figure 6a). The mucosa contains a muscularis mucosa which forms a border between villi at the luminal surface and plica circulares. The submucosa lies adjacent to the muscularis, is highly vascularised and extends upwards to form the plica circulares. The muscularis consists of an outer longitudinal and an inner circular muscle layer.

A differential distribution of propranolol-resistant binding sites and typical β-ARs was observed in the rat ileum. A high density of propranolol-resistant (−)-[^{125}I]-CYP binding was

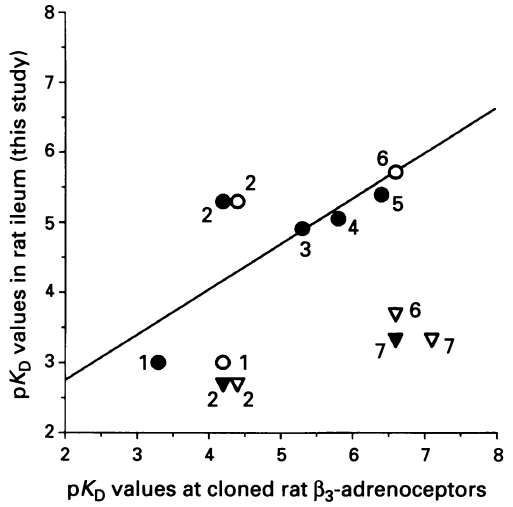

Figure 5 Correlation plot for pK_D values of compounds competing for (−)-[^{125}I]-CYP binding in rat ileum and affinities of the same compounds in competition with (−)-[^{125}I]-CYP binding at the cloned β_3-AR in cell systems. (Liggett, 1992 (open symbols); Muzzin *et al.*, 1991 (filled symbols)). The regression line is shown for compounds represented by circles only (correlation coefficient 0.71, $P=0.048$, $n=8$). The points shown as triangles represent the low affinity binding sites for CGP 12177A and (−)-isoprenaline and also the single binding affinity for BRL 37344. When these points were included in the analysis a significant linear regression could not be obtained. Key: **1** (−)-noradrenaline, **2** (−)-isoprenaline, **3** ICI 118551, **4** propranolol, **5** alprenolol, **6** CGP 12177A, **7** BRL 37344.

associated with most luminal portions of the mucosa and was inhibited by (±)-alprenolol (500 μM). Binding to the muscularis and to discrete regions at the base of the mucosa was resistant to blockade by a combination of (−)-propranolol (0.1 μM) and (±)-alprenolol (500 μM) in the labelling medium. Propranolol resistant binding in the muscularis (155.4±3.8 d.p.m. mm^{-2}) was significantly higher than non-specific binding (143±5.5 d.p.m. mm^{-2}, $n=5$, $P=0.014$; paired t test), indicating that a low density of propranolol-resistant binding sites are located in the muscularis of the rat ileum.

Typical β-ARs were evenly distributed in the longitudinal and circular muscle layers (Figure 6). Labelling was inhibited by the β_2-AR selective antagonist, ICI 118,551 (70 mM), but not by the β_1-AR-selective antagonist, CGP 20712A (100 nM), indicating that β_2-ARs are located in the muscularis of the rat ileum. Non-specific binding, determined in the presence of (−)-propranolol (0.1 μM), was low.

Discussion

In the present study, receptor binding assays with high concentrations of (−)-[^{125}I]-CYP (500 pM) showed a low affinity site in rat small intestine membrane preparations that was not blocked by 5-HT (10 μM) or (−)-propranolol (0.1 μM). (−)-[^{125}I]-CYP bound to this site rapidly, reaching equilibrium in approximately 20 min. Dissociation after 60 min incubation was also rapid with complete dissociation after 20 min. These kinetics differ markedly from those of β_1- and β_2-ARs from which (−)-[^{125}I]-CYP associates and dissociates at a slower rate (Molenaar *et al.*, 1987). The more rapid dissociation of ligand from the site was also reflected in the shorter wash periods used in the autoradiography experiments (2 × 5 min cf. 2 × 15 min at 37°C for β_1, β_2-ARs) (Molenaar *et al.*, 1987).

Saturation studies revealed a single site for (−)-[^{125}I]-CYP binding to rat ileal membranes and the results clearly showed that the affinity of (−)-[^{125}I]-CYP was significantly lower than expected at β_1 and β_2-ARs (24 pM and 12 pM respectively, Neve *et al.*, 1986). The estimated affinity of (−)-[^{125}I]-CYP in this study (1.29 nM) correlates closely to the K_D values quoted for this ligand at cloned β_3-ARs in mouse (880 pM) (Nahmias *et al.*, 1991) and man (500 pM) (Emorine *et al.*, 1989) and the β_3-AR in 3T3F442A adipocytes (1.9 nM) (Feve *et al.*, 1991), (500 pM) (Thomas *et al.*, 1992). Ideally the concentration-range of (−)-[^{125}I]-CYP in saturation studies would span 10-times the K_D value, however the present assay conditions were designed to examine both typical and atypical β-ARs and the concentration-range was chosen to identify both sites using a limited amount of radioligand. Other constraints involved in using high concentrations of (−)-[^{125}I]-CYP include the high proportion of non-specific binding that occurred as concentrations approached 1 nM and the associated problems of high radioactivity retained on other components of the binding system such as the tubing which increased the variability between filtered groups with increased ligand concentrations.

Competition by (−)-propranolol, the selective β_1-AR antagonist, CGP 20712A and the selective β_2-AR antagonist, ICI 118551 in homogenates failed to reveal a population of β_1- and β_2-ARs in these preparations. β_1- and β_2-ARs were also not detected in control tissues using binding conditions that were optimal for typical β-ARs. This would indicate that although typical β_1- and β_2-ARs are present in the muscle layers as shown by autoradiography they form only a minor component of the total tissue binding.

Competition studies performed in the presence of 0.1 μM (−)-propranolol showed that β-AR antagonists with affinity for atypical β-ARs competed for binding with affinities comparable to those displayed in functional studies. A high correlation is seen between the affinities of compounds such as CGP 20712A, ICI 118551, propranolol, alprenolol, tertatolol and cyanopindolol in functional studies in gastrointestinal tissues and their affinities at the binding site identified in the current study (Figure 4).

The stereoisomers of alprenolol and tertatolol displayed low stereoselectivity for the binding site compared to stereoselectivity shown at β_1- and β_2-ARs (Harms *et al.*, 1977). It has been this relative lack of stereoselectivity that has led other workers to ignore possible atypical β-AR binding sites and label it as non-specific binding (Van der Vliet *et al.*, 1990; Landi *et al.*, 1992). Yet low stereoselectivity by antagonists is a distinguishing feature of the atypical β-AR. Studies of lipolysis in rat adipocytes observed that stereoselectivity indices for isomers of propranolol and alprenolol were lower in adipocytes compared to rat atria (Harms *et al.*, 1977). Low stereoselectivity was also 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 in agonists in functional studies at atypical β-ARs, particularly the isomers of isoprenaline which display 25 fold separation in rat small intestine (Van der Vliet *et al.*, 1990) and 31 fold separation at the human β_3-AR (Emorine *et al.*, 1989).

Compounds previously classified as agonists or antagonists at β_1- and β_2-ARs have different profiles of activity at the β_3-AR. Compounds such as (±)-cyanopindolol and CGP 12177 are described as antagonists at β_1- and β_2-ARs but as partial agonists at some atypical β-ARs while CGP 12177 is a full agonist at the human cloned β_3-AR when the receptor is expressed at high levels in cells (Granneman *et al.*, 1993). Both (±)-cyanopindolol and CGP 12177 showed affinities at the propranolol-resistant binding site in rat ileum that were similar to their known affinities at the β_3-AR. The biphasic nature of the CGP 12177A competition curve indicates that this compound may recognise different affinity states of the receptor.

Selective atypical β-AR agonists such as ICI D7114, SR 58611A and BRL 37344 competed with low affinity for propranolol-resistant (−)-[^{125}I]-CYP binding. The rank order of affinity of these compounds in rat ileum (ICI D7114>SR 58611A>BRL 37344) differed slightly from that at a similar propranolol-resistant (−)-[^{125}I]-CYP binding site in rat soleus muscle preparations (SR58611A>ICI D7114>BRL 37344) (Roberts *et al.*, 1993). Variations of responses to the selective atypical β-AR compounds have been observed in gastro-

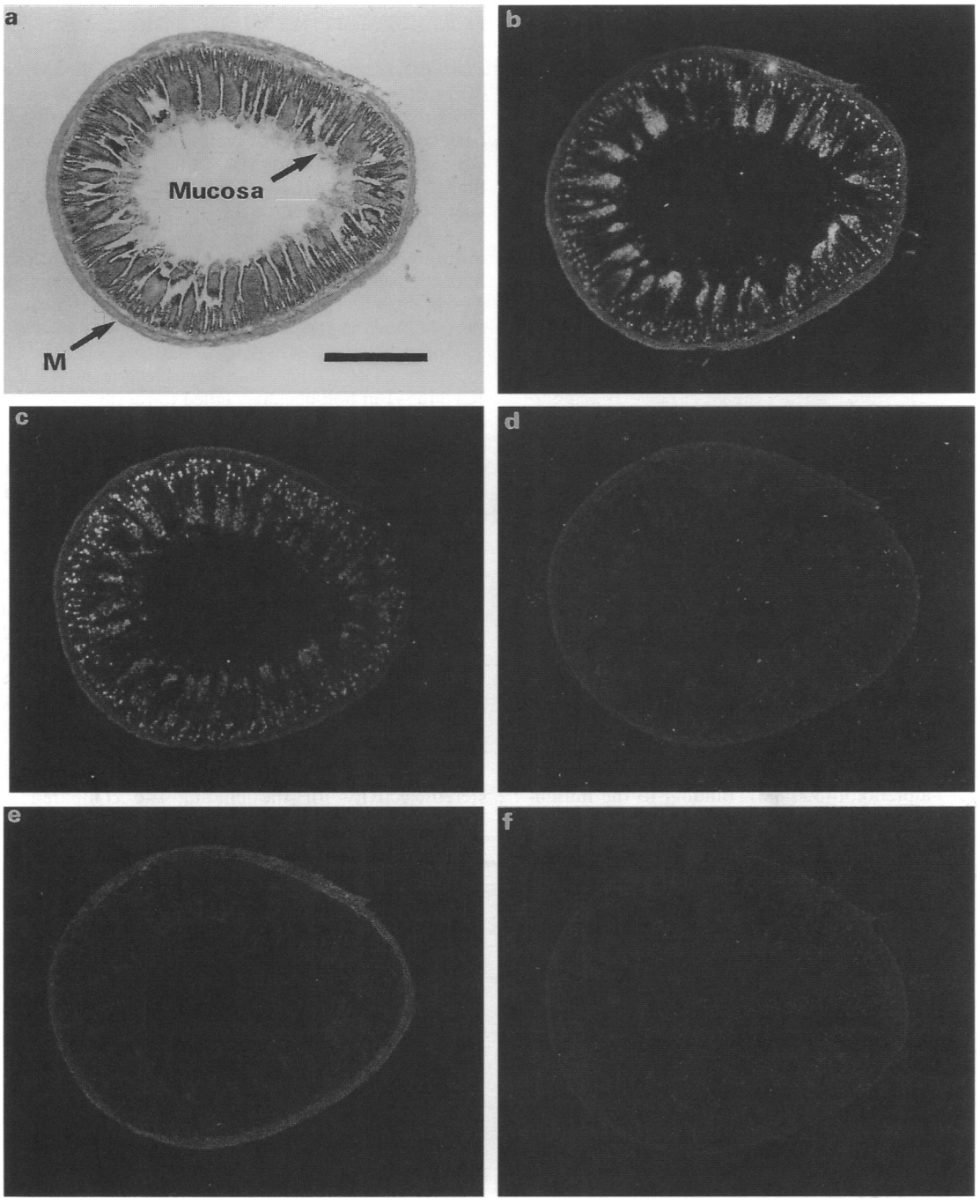

Figure 6 Localization of atypical β-AR binding in rat ileum. Photomicrographs show haematoxylin and eosin stained sections of rat ileum (a), and corresponding autoradiographs showing total (b) and non-specific (c) propranolol-resistant (−)-[^{125}I]-CYP binding. β_1- (d) and β_2- (e) AR distribution and corresponding non-specific binding (f). Note the high density of propranolol-resistant binding in the inner regions of the mucosa with high levels of non-specific binding associated with the muscularis (M) and discrete regions at the base of the mucosa (arrows). β_2-ARs were located in the circular and longitudinal smooth muscle layers of the rat ileum. Typical β-ARs were absent from the mucosa. Scale bar = 1 mm, autoradiography.

intestinal tissues, for example ZD7114 (formerly known as ICI D7114) is described as an antagonist in rat ileum (Growcott *et al.*, 1993) but as an agonist in guinea-pig ileum (Growcott *et al.*, 1991). These differences have been attributed to different tissues having varying numbers of receptors. The catecholamines, (−)-isoprenaline and (−)-noradrenaline, also competed for (−)-[^{125}I]-CYP at the ileal binding site with low affinities which corresponded to their affinities at the cloned β_3-AR in binding studies (Liggett, 1992). In a similar study comparing [^{125}I]-CYP binding in rat skeletal muscle and adipose tissue it has been proposed that the low affinity of agonists in both tissues could be explained if the radioligand did not occupy the active agonist site of the receptor under the chosen binding conditions (Sillence *et al.*, 1993).

The possible existence of two affinity states of the receptor may contribute to the poor correlation seen between binding affinities obtained in rat ileum with those at the cloned β_3-AR, particularly for compounds known to be β_3-AR agonists (Muzzin *et al.*, 1991; Liggett, 1992) (Figure 5). Whether the biphasic competition observed for CGP 12177 and (−)-isoprenaline indicated two affinity states of the receptor or competition at two distinct sites was not determined in this study however we did observe that the high affinity binding values for these compounds correlated more closely to values obtained at the cloned β_3-AR. The tendency to overexpress cloned receptors increases the apparent affinity of agonists in binding studies making agonists poor tools for comparing binding sites between tissue and expression systems. It was

unfortunate that only a few antagonists were common to these studies but it was encouraging to note that the affinites of propranolol, alprenolol and ICI 118551 were similar in the rat ileum and at the rat cloned β_3-AR. This correlation could prove to be more valuable when a larger number of common compounds, preferably antagonists, are available for comparison.

Atypical β-AR binding was localized to the mucosa and regionally distinct from β_2-AR binding found in the longitudinal and circular smooth muscle layers. Although atypical β_2-AR binding was present in the muscle layers as shown by densitometric analysis they were predominantly located in the mucosa. This would also suggest that the atypical β-AR labelled by (−)-[^{125}I]-CYP in homogenates resides predominantly on the mucosa with the smooth muscle providing a small contribution. The predominance of propranolol-resistant binding in the mucosa may indicate that this site has a metabolic or secretory role. Studies of gastric acid secretion in rat stomach (Canfield & Paraskevas, 1992) and of bicarbonate secretion in rat caecum (Canfield & Abdul-Ghaffar, 1992) have suggested that atypical β-ARs play a role in these processes.

Resistance of the atypical binding site to downregulation by chronic agonist treatment clearly shows this site to be distinct from typical β_1- and β_2-ARs. β_2-ARs are desensitized by phosphorylation of serine and threonine residues in the C-carboxyl terminus by β-AR kinase (β-ARK) and receptor uncoupling from the G protein. Based on the lack of phosphorylation sites in the carboxy terminus of the β_3-AR it has been suggested that this receptor may be more resistant to phosphorylation than β_2-ARs (Emorine *et al.*, 1991). β_3-ARs and mRNA levels in 3T3 F442A cells have been shown to increase in response to chronic isoprenaline exposure (Thomas al., 1992). Human β_3-ARs expressed in cells are resistant to desensitization following short term isoprenaline exposure (Nantel *et al.*, 1993) and with chronic noradrenaline infusion in hamsters the lipolytic response of adipocytes is not decreased (Carpene *et al.*, 1993). Acute noradrenaline treatment in rats however has been shown to reduce greatly β_3-AR mRNA in both white and brown adipose tissue (Granneman & Lahners, 1992). *n*-Butyric acid, present in the gastrointestinal tract after ingestion of polysaccharides, has been shown to downregulate selectively β_3-ARs in 3T3 F442A fibroblasts while β_1- and β_2-AR levels are increased (Krief *et al.*, 1994). Such regulation may be important at receptors exposed to the luminal surface such as the atypical β-AR.

In conclusion, this study characterizes an (−)-[^{125}I]-CYP binding site in rat ileum that resembles the atypical β-AR described in functional studies in a variety of gastrointestinal tissues. This site clearly differs pharmacologically and in location from β_1- and β_2-ARs, is localized predominantly in the mucosa of the ileum and is resistant to downregulation by chronic agonist treatment.

This work was supported by the National Health and Medical research Council. P.M. is an NH and MRC research fellow, S.J.R. is an Australian Postgraduate Research Scholar. The authors are grateful to SmithKline Beecham, Sanosi, ICI, Ciba-Geigy, Servier and Sandoz for gifts of compounds used in this study.

References

BENSAID, M., KAGHAD, M., RODRIGUEZ, M., LEFUR, G. & CAPUT, D. (1993). The rat β_3-adrenergic receptor gene contains an intron. *FEBS Letts.*, **318**, 223–226.

BLUE, D.R., BOND, R.A., ADHAM, N., DELMENDO, R., MICHEL, A.D., EGLEN, R.M., WHITING, R.L. & CLARKE, D.E. (1990). Antagonist characterisation of atypical beta adrenoceptors in guinea pig ileum: blockade by alprenolol and dihydroalprenolol. *J. Pharmacol. Exp. Ther.*, **252**, 1034–1042.

BOND, R.A. & CLARKE, D.E. (1988). Agonist and antagonist characterization of a putative adrenoceptor with distinct pharmacological properties from the α- and β-subtypes. *Br. J. Pharmacol.*, **95**, 723–734.

CANFIELD, P. & ABDUL-GHAFFAR, T. (1992). Stimulation of bicarbonate secretion by atypical β-receptor agonists in rat cecum in vitro. *Eur. J. Pharmacol.*, **216**, 293–297.

CANFIELD, P. & PARASKEVA, P. (1992). β-Adrenoceptor agonist stimulation of acid secretion by rat stomach *in vitro* is mediated by 'atypical' β-adrenoceptors. *Br. J. Pharmacol.*, **106**, 583–586.

CARPENE, C., GALITZKY, J., COLLON, P., ESCALPEZ, F., DAUZATS, M. & LAFONTAN, M. (1993). Desensitisation of beta-1 and beta-2 but not beta-3, adrenoceptor mediated lioplytic responses of adipocytes after long term norepinephrine infusion. *J. Pharmacol Exp. Ther.*, **265**, 237–247.

CROCI, T., CECCHI, R., TARANTINO, A., AUREGGI, G., BIANCHETTI, A., BOIGEGRAIN, R. & MANARA, L. (1988). Inhibition of rat colon motility by stimulation of atypical beta-adrenoceptors with new gut-specific agents. *Pharmacol. Res. Commun.*, **20**, 147–151.

DE BOER, R.E.P., BROUWER, F. & ZAAGSMA, J. (1993). The β-adrenoceptors mediating relaxation of rat oesophageal muscularis mucosae are predominantly of the β_3-, but also of the β_2-subtype. *Br. J. Pharmacol.*, **110**, 442–446.

EMORINE, L.J., FEVE, B., PAIRAULT, J., BRIEND-SUTREN, M., MARULLO, S., DELAVIER-KLUTCHKO, C. & STROSBERG, D.A. (1991). Structural basis for functional diversity of β_1-, β_2- and β_3-adrenergic receptors. *Biochem. Pharmacol.*, **41**, 853–859.

EMORINE, L.J., MARULLO, S., BRIEND-SUTREN, M., PATEY, G., TATE, K., DELAVIER-KLUTCHKO, C. & STROSBERG, D.A. (1989). Molecular characterisation of the human β_3-adrenergic receptor. *Science*, **245**, 1118–1121.

FEVE, B., EMORINE, L.J., LASNIER, F., BLIN, N., BAUDE, B., NAHMIAS, C., STROSBERG, A.D. & PAIRAULT, J. (1991). Atypical β-adrenergic receptor in 3T3-F442A adipocytes. *J. Biol. Chem.*, **266**, 20329–20336.

FORD, A.P.D.W., TANEJA, D.T. & CLARKE, D.E (1992). Atypical β-adrenoceptor-mediated relaxation and cyclic AMP formation in tunica muscularis mucosae (TMM) of the rat oesophagus. *Br. J. Pharmacol.*, **105**, 235P.

GILLE, E., LEMOINE, H., EHLE, B. & KAUMANN, A.J. (1985). The affinity of (–)-propranolol for B_1- and B_2-adrenoceptors of human heart. *Naunyn-Schmied. Arch. Pharmacol.*, **331**, 60–70.

GRANNEMAN, J.G. & LAHNERS, K.N. (1992). Differential adrenergic regulation of β_1- and β_3-adrenergic receptor messenger ribonucleic acids in adipose tissue. *Endocrinology*, **130**, 109–114.

GRANNEMAN, J.G., LAHNERS, K.N. & CHAUDHRY, A. (1991). Molecular cloning and expression of the rat β_3-adrenergic receptor. *Mol. Pharmacol.*, **40**, 895–899.

GRANNEMAN, J.G., LAHNERS, K. & CHAUDRY, A. (1993). Characterisation of the human β_3-adrenergic receptor gene. *Mol. Pharmacol.*, **44**, 264–270.

GROWCOTT, J.W., HOLLOWAY, B., GREEN, M. & WILSON, C. (1993). Zeneca ZD7114 acts as an antagonist at atypical β_3-adrenoceptors in rat isolated ileum. *Br. J. Pharmacol.*, **110**, 1375–1380.

GROWCOTT, J.W., WILSON, C., HOLLOWAY, B. & MAINWARING, S. (1991). Evaluation of ICI D7114, a putative stimulant of brown adipocytes, on histamine precontracted guinea-pig ileum. *Br. J. Pharmacol.*, **104**, 155P.

HARMS, H.H., ZAAGSMA, J. & DE VENTE, J. (1977). Differentiation of β-adrenoceptors in right atrium, diaphragm and adipose tissue of the rat, using stereoisomers of propranolol, alprenolol, nifenalol and practolol. *Life Sci.*, **21**, 123–128.

HOYER, D., ENGEL, G. & KALKMAN, H.O. (1985). Characterisation of the 5-HT_{1B} recognition site in rat brain: Binding studies with (−)-[^{125}I]-iodocyanopindolol. *Eur. J. Pharmacol.*, **118**, 1–12.

KRIEF, S., FEVE, B., BAUDE, B., ZILBERFARB, V., STROSBERG, A.D., PAIRAULT, J. & EMORINE, L.J. (1994). Transcriptional modulation by n-butyric acid of β_1- β_2- and β_3-adrenergic receptor balance in 3T3-F442A adipocytes. *J. Biol. Chem.*, **269**, 6664–6670.

LANDI, M., BIANCHETTI, A., CROCI, T. & MANARA, L. (1992). Phenylethanolaminotetralines compete with [^{3}H]dihydroalprenolol binding to rat colon membranes without evidencing atypical β-adrenoceptor sites. *Biochem. Pharmacol.*, **44**, 665–672.

LANDI, M., CROCI, T. & MANARA, L. (1993). Similar atypical β-adrenergic receptors mediate in vitro rat adipocyte lipolysis and colonic motility inhibition. *Life Sci.*, **53**, 297–302.

LIGGETT, S.B. (1992). Functional properties of the rat and human β_3-adrenergic receptors: differential agonist activation of recombinant receptors in chinese hamster ovary cells. *Mol. Pharmacol.*, **42,** 634–637.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193,** 265–275.

MCLAUGHLIN, D.P. & MACDONALD, A. (1990). Evidence for the existence of 'atypical' β-adrenoceptors (β_3-adrenoceptors) mediating relaxation in the rat distal colon *in vitro*. *Br. J. Pharmacol.*, **101,** 569–574.

MCLAUGHLIN, D.P. & MACDONALD, A. (1991). Characterisation of catecholamine-mediated relaxations in rat isolated gastric fundus: evidence for an atypical β-adrenoceptor. *Br. J. Pharmacol.*, **103,** 1351–1356.

MCPHERSON, G.A. (1983). A practical computer-based approach to the analysis of radioligand binding experiments. *Comput. Programs Biomed.*, **17,** 107–114.

MOLENAAR, P., CANALE, E. & SUMMERS, R.J. (1987). Autoradiographic localization of beta-1 and beta-2 adrenoceptors in guinea-pig atrium and regions of the conducting system. *J. Pharmacol. Exp. Ther.*, **241,** 1048–1064.

MOLENAAR, P., ROBERTS, S.J., KIM, Y.S., PAK, H.S., SAINZ, R.D. & SUMMERS, R.J. (1991). Localisation and characterisation of two propranolol resistant (−)-[^{125}I]-cyanopindolol binding sites in rat skeletal muscle. *Eur. J. Pharmacol.*, **209,** 257–262.

MUZZIN, P., REVELLI, J.-P., KUHNE, F., GOGAHNE, J.D., MCCOMBIE, W.R., VENTER, J.C., GIACOBINO, J.-P. & FRASER, C.M. (1991). An adipose tissue-specific β-adrenergic receptor. *J. Biol. Chem.*, **266,** 24053–24058.

NAHMIAS, C., BLIN, N., ELALOUF, J.M., MATTEI, M.G., STROSBERG, A.D. & EMORINE, L.J. (1991). Molecular characterisation of the mouse β_3-adrenergic receptor: relationship with the atypical receptor of adipocytes. *EMBO J.*, **10,** 3721–3727.

NANTEL, F., BONIN, H., EMORINE, L.J., ZILBERFARB, V., STROSBERG, A.D., BOUVIER, M. & MARULLO, S. (1993). The human β_3-adrenergic receptor is resistant to short term agonist promoted desensitisation. *Mol. Pharmacol.*, **43,** 548–555.

NEVE, K.A., MCGONIGLE, P. & MOLINOFF, P.B. (1986). Quantitative analyses of the selectivity of radioligands for subtypes of beta adrenergic receptors. *J. Pharmacol. Exp. Ther.*, **238,** 46–53.

ROBERTS, S.J., MOLENAAR, P. & SUMMERS, R.J. (1993). Characterisation of propranolol-resistant (−)-[^{125}I]-cyanopindolol binding sites in rat soleus muscle. *Br. J. Pharmacol.*, **109,** 344–352.

SILLENCE, M.N., MOORE, N.G., PEGG, G.G. & LINDSAY, D.B. (1993). Ligand binding properties of putative β_3-adrenoceptors compared in brown adipose tissue and in skeletal muscle membranes. *Br. J. Pharmacol.*, **109,** 1157–1163.

THOMAS, R.F., HOLT, B.D., SCHWINN, D.A. & LIGGETT, S.B. (1992). Long-term agonist exposure induces upregulation of the β_3-adrenergic receptor expression via multiple cAMP response elements. *Proc. Natl. Acad. Sci. U.S.A.*, **89,** 4490–4494.

VAN DER VLIET, A., RADEMAKER, B. & BAST, A. (1990). A beta adrenoceptor with atypical characteristics is involved in the relaxation of the rat small intestine. *J. Pharmacol. Exp. Ther.*, **255,** 218–226.

WILLIAMS, D.W. & SUMMERS, R.J. (1990). A program for the estimation of kinetic parameters using two stage regression analysis. *Clin. Exp. Pharmacol. Physiol.*, **17,** 86.

(Received February 20, 1995
Revised July 4, 1995
Accepted July 14, 1995)