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# $\beta_1$-Adrenoceptors compensate for $\beta_3$-adrenoceptors in ileum from $\beta_3$-adrenoceptor knock-out mice

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**1** This study examines $\beta_1$-, $\beta_2$- and $\beta_3$-adrenoceptor (AR)-mediated responses, mRNA levels and radioligand binding in ileum from $\beta_3$-AR knock-out (−/−) (KO) and wild type (+/+) (FVB) mice.

**2** In KO and FVB mice, SR59230A (100 nM) ($\beta_3$-AR antagonist) antagonized responses to (−)-isoprenaline in both KO and FVB mice. (−)-Isoprenaline mediated relaxation of ileum was antagonized weakly by ICI118551 (100 nM) ($\beta_2$-AR antagonist). Responses to (−)-isoprenaline were more strongly antagonized by CGP20712A (100 nM) ($\beta_1$-AR antagonist), propranolol (1 $\mu$M) ($\beta_1$-/$\beta_2$-AR antagonist), carvedilol (100 nM) (non-specific $\beta$-AR antagonist), and CGP12177A (100 nM) ($\beta_1$-/$\beta_2$-AR antagonist) in ileum from KO than in FVB mice.

**3** Responses to CL316243 ($\beta_3$-AR agonist) in ileum from FVB mice were antagonized by SR59230A (100 nM) but not by propranolol (1 $\mu$M) or carvedilol (100 nM). CL316243 was ineffective in relaxing ileum from KO mice.

**4** CGP12177A had no agonist actions in ileum from either KO or FVB mice.

**5** $\beta_1$-AR mRNA levels were increased 3 fold in ileum from KO compared to FVB mice. This was associated with an increased maximum number of $\beta_1$-/$\beta_2$-AR binding sites ($B_{max}$). $\beta_2$-AR mRNA levels were unaffected while no $\beta_3$-AR mRNA was detected in KO mice.

**6** In mouse ileum, $\beta_3$-ARs and to a lesser extent $\beta_1$-ARs are the predominant adrenoceptor subtypes mediating relaxation in ileum from FVB mice. In KO mice $\beta_1$-ARs functionally compensate for the lack of $\beta_3$-ARs, and this is associated with increased $\beta_1$-AR mRNA and levels of binding.

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**Abbreviations:** $\beta$-AR, $\beta$-adrenoceptor; $B_{max}$, maximum number of binding sites; CGP12177A, (±)-4-(3-t-butylamino-2-hydroxy-propoxy)benzimidazol-2-one; CGP20712A, 2-hydroxy-5(2-((2-hydroxy-3-(4-((1-methyl-4-trifluoromethyl)1H-imidazole-2-yl)-phenoxy)propyl)amino)ethoxy)-benzamide monomethane sulfonate, CL316243, (**R,R**)-5-[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]-amino]-propyl]1,3-benzodioxole-2,2-dicarboxylate; c-r, concentration-response; ICI118551, erythro-DL-1(7-methylindian-4-yloxy)-3-isopropylaminobutan-2-ol; ICYP, (−)-[$^{125}$I]-cyanopindolol; KO, knock-out; $R_{max}$, maximal relaxation; RT–PCR, reverse transcrition-polymerase chain reaction; SR59230A, 3-(2-ethylphenoxy)-1-[(1**S**)-1,2,3,4-tetrahydronapth-1-ylamino]-2**S**-2-propanol oxalate

## Introduction

$\beta_3$-ARs mediate relaxation in a wide variety of gastrointestinal tissues from various species including guinea-pig, rat, rabbit and man (for review see Manara *et al*., 1995). A functional role for $\beta_3$-ARs is supported by studies demonstrating $\beta_3$-AR mRNA in gastrointestinal tissues (Bensaid *et al*., 1993; Evans *et al*., 1996; 1998; Granneman *et al*., 1991; 1993; Hutchinson *et al*., 2000; Krief *et al*., 1993; Roberts *et al*., 1997; 1999) and radioligand binding studies using (−)-[$^{125}$I]-cyanopindolol (ICYP) show a site with characteristics of $\beta_3$-ARs (Hutchinson *et al*., 2000; Roberts *et al*., 1995; 1997).

While $\beta_3$-ARs predominate in gastrointestinal tissues, $\beta_1$- and $\beta_2$-ARs may also have roles. The $\beta_1$-AR agonist Ro363 relaxes rat ileum, and the relaxation is antagonized by CGP20712A (Roberts *et al*., 1999). Ro363 had a higher intrinsic activity than isoprenaline in rat colon and guinea-pig ileum (Molenaar *et al*., 1997a), and caused a relaxation in rat ileum which was 60–70% of the isoprenaline response (Hoey *et al*., 1996). Later studies showed responses that were less than 20% of maximum responses with $pEC_{50}$ values (6.2) (Roberts *et al*., 1999) intermediate between values at $\beta_1$- and $\beta_3$-ARs in rat colon (8.5 and 5.6 respectively) (Molenaar *et al*., 1997a).

However one of the problems in interpreting some studies is the use of $\beta_1$-/$\beta_2$-AR agonists that also act at $\beta_3$-ARs. Ro363 is a partial agonist at the cloned human $\beta_3$-AR and on intestinal $\beta_3$-ARs in the rat and guinea-pig (Molenaar *et al*., 1997a). Zinterol ($\beta_2$-AR agonist) causes relaxation in rat ileum, but the response was mediated through $\beta_3$-ARs since they were antagonized by SR58894A but not ICI118551 (Roberts *et al*., 1999). Ritodrine ($\beta_2$-AR agonist) causes relaxation in rat colon, and its responses are antagonized by alprenolol and propranolol but with lower potency than that expected for $\beta_2$-ARs and it probably also acts at $\beta_3$-ARs (Bianchetti & Manara, 1990).

The aim of the present study was to examine the importance of $\beta_3$-ARs in mouse ileum by comparing responses of ileum from $\beta_3$-AR KO and FVB mice.

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Relaxation of ileal smooth muscle to (−)-isoprenaline was performed to assess the relative roles of all three $\beta$-AR subtypes in mediating smooth muscle relaxation. This study demonstrates that $\beta_1$- and $\beta_3$-ARs mediate smooth muscle relaxation in mouse ileum and that $\beta_1$-ARs compensate for the lack of $\beta_3$-ARs in $\beta_3$-AR KO mice, suggesting that $\beta_3$-ARs are important in gastrointestinal function.

# Methods

## *Animals and genotyping*

FVB mice (female, ~4 months old) were obtained from the Animal Resources Centre, Canning Vale, Western Australia. $\beta_3$-AR KO mice (male, ~1 year old) were the offspring of a previously described strain (Susulic *et al*., 1995). Genomic DNA analysis was conducted on mouse tails to determine the genotype of all mice used for breeding and experimental studies. Genomic DNA was isolated by proteinase K digestion overnight followed by phenol-chloroform extraction (Miller *et al*., 1988). PCR was performed on 0.5 $\mu$g DNA (62°C, 32 cycles) using primers designed to indicate the presence/absence of neomycin disruption to the $\beta_3$-AR allele (see Table 1). Products were run on a 1.3% agarose gel and the bands photographed. In all experiments conducted, 8–14 week old male mice were used.

## *Analysis of $\beta$-AR mRNA levels in ileum*

Tissue was obtained from mice anaesthetized with 80% $CO_2$/20% $O_2$ and decapitated. Tissue was prepared as previously described (Roberts *et al*., 1999). Total RNA was extracted by homogenization in Trizol reagent (Gibco BRL, Life Technologies) using a PRO200 homogenizer. The yield and quality of RNA was assessed by measuring absorbance at 260 and 280 nm and by electrophoresis on 1.3% agarose gels. cDNAs were synthesized by reverse transcription of 1 $\mu$g of each total RNA using oligo $(dT)_{15}$ (Gibco BRL, Life Technologies) as a primer (Roberts *et al*., 1999). PCR amplification was carried out on cDNA equivalent to 100 ng of starting RNA using primers specific for $\beta_1$-, $\beta_2$- or $\beta_3$-AR and the internal standard actin (Gibco BRL, Life Technologies; see Table 1). For $\beta_1$-AR PCR, PCR mixes contained 0.5 U Platinum® Pfx DNA polymerase (Life Technologies), 1× AMP buffer and 1× Enhancer solution (as supplied by Life Technologies), dNTPs (130 $\mu$M) (Amersham Pharmacia Biotech), $MgSO_4$ (1.5 mM), 5.8 pmol forward primer and 5.8 pmol reverse primer. $\beta_2$-, $\beta_3$-AR and actin PCR has been described elsewhere (Roberts *et al*., 1999). The actin reverse primer was labelled prior to PCR with [$\gamma$-$^{33}$P]-ATP (Evans *et al*., 1998). The annealing temperature for all PCR reactions was 64°C except for $\beta_1$-AR PCR where the annealing temperature was 60°C. For each set of primers the log (PCR product) *versus* cycle number was plotted and a cycle number chosen within the linear portion of the graph (data not shown). Cycle numbers were 16 for actin, 26 for $\beta_1$- and $\beta_2$-AR, and 30 for $\beta_3$-AR. Following amplification, PCR products were electrophoresed on 1.3% agarose gels and transferred onto Hybond $N^+$ membranes by Southern blotting in 0.4 M NaOH/1 M NaCl. For detection of $\beta_1$-, $\beta_2$-, and $\beta_3$-AR products, membranes were hybridized with a probe specific for each product expected (Table 1). Probes were end labelled with 50 $\mu$Ci [$\gamma$-$^{33}$P]-ATP and T4 polynucleotide kinase (Amersham Pharmacia Biotech) (Roberts *et al*., 1999). Radioactivity was detected with a Molecular Dynamics SI phosphorimager, and bands quantitiated using ImageQuantNT Software (Molecular Dynamics). $\beta$-AR levels were normalized for actin levels ($\beta$-AR/actin) and expressed as a percentage of the mean ± s.e.mean value of *n* animals from FVB tissue. Student's unpaired *t*-test (2-tailed) was used to determine significance of differences. Probability values less than or equal to 0.05 were considered significant.

## *Receptor binding assay*

Membranes were prepared as previously described (Hutchinson *et al*., 2000). Saturation experiments were performed at room temperature in a total volume of 100 $\mu$l in microcentrifuge tubes. Homogenate (approximately 20–40 $\mu$g protein) was incubated with (−)-[$^{125}$I]-cyanopindolol (ICYP) (5–100 pM) for 60 min at room temperature in the absence or presence of (−)-propranolol (1 $\mu$M) to define non-specific

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

| | | *Strand length* | *Sequence* (5′->3′) | $T_m$ (°C)[a] |
|---|---|---|---|---|
| *Primers* | | | | |
| $\beta_1$-AR | for | 22 | CCG CTG CTA CCA CGA CCC CAA G | 71 |
| $\beta_1$-AR | rev | 26 | AGC CAG TTG AAG AAG AGC AAG AGG CG | 71 |
| $\beta_2$-AR | for | 26 | GGT TAT CGT CCT GGC CAT CGT GTT TG | 71 |
| $\beta_2$-AR | rev | 29 | TGG TTC GTG AAG AAG TCA CAG CAA GTC TC | 70 |
| $\beta_3$-AR | for | 26 | TCT AGT TCC CAG CGG AGT TTT CAT CG | 76 |
| $\beta_3$-AR | rev | 25 | CGC GCA CCT TCA TAG CCA TCA AAC C | 77 |
| $\beta$-actin | for | 22 | ATC CTG CGT CTG GAC CTG GCT G | 71 |
| $\beta$-actin | rev | 25 | CCT GCT TGC TGA TCC ACA TCT GCT G | 71 |
| neo | for | 25 | CGC ATC GCC TTC TAT CGC CTT CTT G | 77 |
| wt | for | 24 | GTT GCG AAC TGT GGA CGT CAG TGG | 77 |
| neo/wt | rev | 22 | AAT GCC GTT GGC GCT TAG CCA C | 75 |
| *Probes* | | | | |
| $\beta_1$-AR | | 22 | AAG AAG ATC GAC AGC TGC GAG C | 68 |
| $\beta_2$-AR | | 20 | GCC AGC ATC GAG ACC CTG TG | 70 |
| $\beta_3$-AR | | 25 | TGC CAA CTC CGC CTT CAA CCC CCT C | 81 |

[a]$T_m$ determined by Primer (version 0.5, Whitehead Institute).

binding. Tubes were centrifuged briefly after incubation, supernatants discarded and the pellet resuspended in 100 $\mu$l binding buffer (50 mM Tris pH 7.4 room temperature, 5 mM $MgCl_2$, 1 mM EDTA, 10 mg $ml^{-1}$ bacitracin, 10 mg $ml^{-1}$ leupeptin, 10 mg $ml^{-1}$ pepstatin A, 0.5 mg $ml^{-1}$ aprotinin) for 30 min to minimize any low affinity binding. Reactions were terminated by rapid filtration through GF/B filters using a Packard Cell Harvester. Filters were washed four times with buffer (50 mM Tris pH 7,4 room temperature), dried, 30 $\mu$l Microscint-O (Packard) added and radioactivity measured using a Packard Top Count. Experiments were performed in duplicate with tissues from $n$ animals. Results are expressed as mean $\pm$ s.e.mean of $n$. Data was analysed using non-linear curve fitting (GraphPad PRISM version 2.0) using a one-site fit to obtain $K_D$ and $B_{max}$ values. Two-way ANOVA tests were used to determine significance of variations between curves. Probability values less than or equal to 0.05 were considered significant.

### *Organ bath studies*

Mice were anaesthetized with 80% $CO_2$/20% $O_2$ and decapitated. Approximately 10 cm of small intestine was removed 2 cm above the ileocaecal junction. Segments of approximately 2 cm were mounted on tissue hooks and suspended in jacketed organ baths containing 6 ml Krebs-Henseleit solution (composition (mM): NaCl 118.4, KCl 4.7, $MgSO_4.7H_2O$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 25, glucose 11, $CaCl_2$ 2.5) containing ascorbic acid (0.1 mM) and EDTA (0.04 mM) maintained at 37°C and bubbled with 95% $O_2$/5% $CO_2$ (pH 7.4) under 4 mN force. Responses were measured with a UgoBasile isotonic transducer connected to a MacLab system and an Apple IIci computer. Tissues were allowed to equilibrate with or without antagonists (as specified) for 30 min. Following equilibration, tissues were contracted with carbachol (1 $\mu$M) (approximately 80% maximal response; data not shown) until responses maintained plateau (10–15 min). Control experiments were conducted and showed that the carbachol-evoked contraction was stable for the whole period of the experiment (data not shown). Cumulative concentration-response (c-r) curves to specified agonists were constructed with increments of 0.5 log units until a stable state was observed. At the end of each c-r curve, tissues were maximally relaxed with papaverine (10 $\mu$M) and all responses were expressed as a percentage of this papaverine response. Non-linear regression was used to fit sigmoid c-r curves to the data (GraphPad PRISM version 2.0) and to determine $pEC_{50}$ values. Values are expressed as mean $\pm$ s.e.mean of $n$ individual experiments, with each $n$ value referring to the number of individual animals used. In experiments where antagonists were used, $pK_B$ values were calculated according to the method of Furchgott (1972). Student's $t$-test was used to determine statistical significance where $P<0.05$ was considered to be significant.

### *Drugs and reagents*

CL316243 (Dr T. Nash), SR59230A (Dr L. Manara) and carvedilol (Dr R.R. Ruffolo III) were gifts from Wyeth-Ayerst, Sanofi-Midi and SB Pharmaceuticals respectively. The drugs and reagents used were as follows: [$\gamma$-$^{33}$P]-ATP (2000 Ci $mmol^{-1}$, Geneworks, Adelaide, SA, Australia); (−)-[$^{125}$I]-CYP (2200 Ci $mmol^{-1}$, NEN Life Science Products, Boston, MA, U.S.A.); ICI118551 (Imperial Chemical Industries, Wilmslow, Cheshire, U.K.); CGP12177A (Research Biochemicals Inc., MA, U.S.A.); CGP20712A (Ciba-Geigy AG, Australia); aprotinin, bacitracin, carbachol (carbamylcholine chloride), (−)-isoprenaline bitartate, papaverine hydrochloride, (−)-propranolol (Sigma Chemical Company, St. Louis, MO, U.S.A.); leupeptin, pepstatin A, (Gibco BRL, Life Technologies, Gaithersburg, MD, U.S.A.); L(+)-ascorbic acid (Merck, Frankfurt, Germany); EDTA (AJAX Chemicals, Melbourne, VIC, Australia). All other chemicals were of analytical grade.

Stock solutions of SR59230A were prepared in 50% distilled water, 25% ethanol and 25% DMSO, pepstatin A in DMSO, (−)-isoprenaline bitartate, CGP20712A, ICI118551 and CGP12177A in 10 mM HCl, carvedilol in 0.1 mM ascorbic acid, and the remaining drugs in distilled water.

## Results

### *Detection of $\beta_1$-, $\beta_2$- and $\beta_3$-AR mRNA in FVB and $\beta_3$-AR KO ileum*

RT–PCR detected all three $\beta$-AR subtypes and actin mRNA in ileal smooth muscle from FVB mice. Direct comparison of the levels of $\beta$-AR mRNA in KO compared to FVB samples showed that $\beta_1$-AR mRNA levels were increased 3 fold in the $\beta_3$-AR KO ileum (FVB 100 $\pm$ 37.8%, KO 350.9 $\pm$ 92.5%; $n=6$; Student's $t$-test $^{*}P<0.05$), $\beta_2$-AR mRNA levels were not significantly altered (FVB 100 $\pm$ 12.2%, KO 95.2 $\pm$ 9.0%, $n=6$), and as expected no $\beta_3$-AR mRNA was detected in KO samples (FVB 100 $\pm$ 15.5%, KO 2.4 $\pm$ 1.1%, $n=6$; Student's $t$-test $^{***}P<0.001$) (Figure 1).

### *[$^{125}$I]-Cyanopindolol binding in membranes from FVB and $\beta_3$-AR KO ileum*

ICYP binding occurred in a saturable manner (Figure 2) to a single population of sites in FVB ileum ($K_D$ 44.6 $\pm$ 30.4 pM; $B_{max}$ 16.7 $\pm$ 4.9 fmol $mg^{-1}$ protein; $n=5$). ICYP binding levels were increased in KO ($K_D$ 57.2 $\pm$ 25.5 pM; $B_{max}$ 30.6 $\pm$ 6.5 fmol $mg^{-1}$ protein; $n=6$) compared to FVB samples (2-way

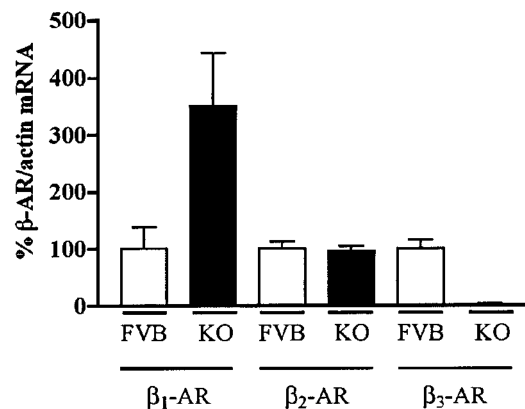


**Figure 1** $\beta_1$-, $\beta_2$-, and $\beta_3$-AR mRNA levels in KO and FVB mice. $\beta_1$-AR mRNA levels were increased 3 fold in ileum from KO as compared to FVB mice. $\beta_2$-AR mRNA levels were unaltered and no detectable levels of $\beta_3$-AR mRNA was observed in KO mice.

ANOVA ***$P=0.0001$). At the highest concentration of ICYP used (100 pM), approximately 66% of the $\beta$-AR binding sites in FVB and KO ileum would be labelled.

### *Organ bath studies of ileum from $\beta_3$-AR KO and FVB mice*

*Effect of CL316243* The $\beta_3$-AR agonist CL316243 relaxed smooth muscle in carbachol precontracted FVB ileum in a dose-dependent manner (pEC$_{50}$ 8.5$\pm$0.3, $n=7$) whereas ileum from KO animals ($n=7$) was unresponsive to CL316243 (Figure 3a). Responses to CL316243 in FVB ileum were antagonized by SR59230A (100 nM) (control: pEC$_{50}$ 8.8$\pm$0.5, $n=6$; SR59230A (100 nM): pEC$_{50}$ 7.6$\pm$0.2, $n=6$) with a pK$_B$ value of 8.3$\pm$0.2 ($n=6$) (Figure 3b). Antagonism of CL316243 responses by both propranolol (100 nM) (control: pEC$_{50}$ 8.8$\pm$0.6, $n=6$; propranolol (100 nM): pEC$_{50}$ 8.3$\pm$0.2, $n=6$) and carvedilol (100 nM) (control: pEC$_{50}$ 8.3$\pm$0.3, $n=3$; carvedilol (100 nM): pEC$_{50}$ 8.1$\pm$0.1, $n=3$) was relatively weak (Figure 3c,d).

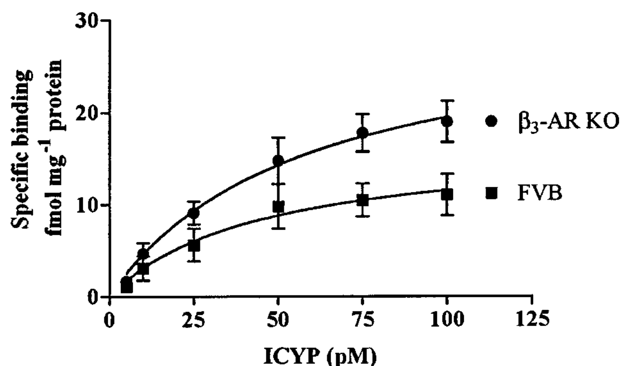


**Figure 2** Saturation binding curve of ICYP to KO and FVB ileal membrane preparations. Graph shows specific binding, showing a significant increase in ICYP binding in ileum from KO as compared to FVB mice (2-way ANOVA ***$P<0.0001$). Points show mean$\pm$s.e.mean ($n=5-6$).

*Effect of (−)-isoprenaline* The non-subtype selective $\beta$-AR agonist (−)-isoprenaline caused concentration-dependent relaxation of carbachol-precontracted ileum from both FVB and KO animals (Figure 4).

The $\beta_1$-AR selective antagonist CGP20712A (100 nM) (Figure 4a) caused a rightward shift in the (−)-isoprenaline c-r curve in both KO (control: pEC$_{50}$ 8.2$\pm$0.1, $n=9$; CGP20712A (100 nM): pEC$_{50}$ 5.7$\pm$0.3, $n=9$) and FVB ileum (control: pEC$_{50}$ 7.8$\pm$0.1, $n=7$; CGP20712A (100 nM): pEC$_{50}$ 6.6$\pm$0.1, $n=7$), with the larger shift observed in the KO compared to FVB ileum (pK$_B$ values 9.4$\pm$0.3 ($n=9$) and 8.1$\pm$0.3 ($n=7$) respectively; Student's $t$-test ***$P<0.001$).

The $\beta_2$-AR antagonist ICI118551 (100 nM) (Figure 4b) was a very weak antagonist in both KO (control: pEC$_{50}$ 8.0$\pm$0.2, $n=8$; ICI118551 (100 nM): pEC$_{50}$ 7.5$\pm$0.1, $n=8$) and FVB (control: pEC$_{50}$ 7.7$\pm$0.1, $n=6$; ICI118551 (100 nM): pEC$_{50}$ 7.4$\pm$0.1, $n=6$) ileum and failed to significantly shift c-r curves to (−)-isoprenaline.

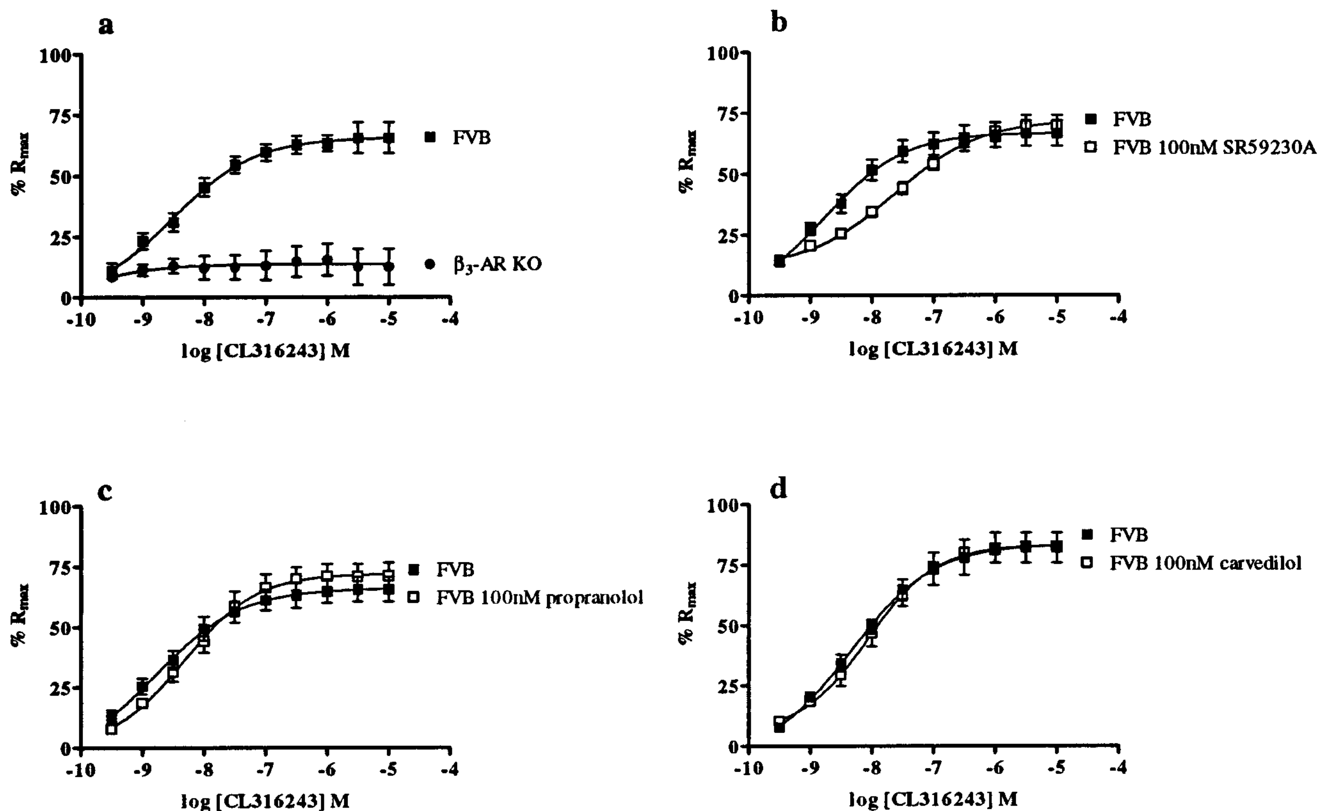


**Figure 3** The effect of CL316243 on ileal responses in KO and FVB mice. Graph shows: (a) responses to CL316243 are abolished in ileum from KO as compared to FVB mice ($n=7$); and responses to CL316243 in FVB mice in the absence and presence of (b) SR59230A ($n=6$), (c) propranolol ($n=6$) and (d) carvedilol ($n=3$). Points show mean and vertical lines indicate s.e.mean.

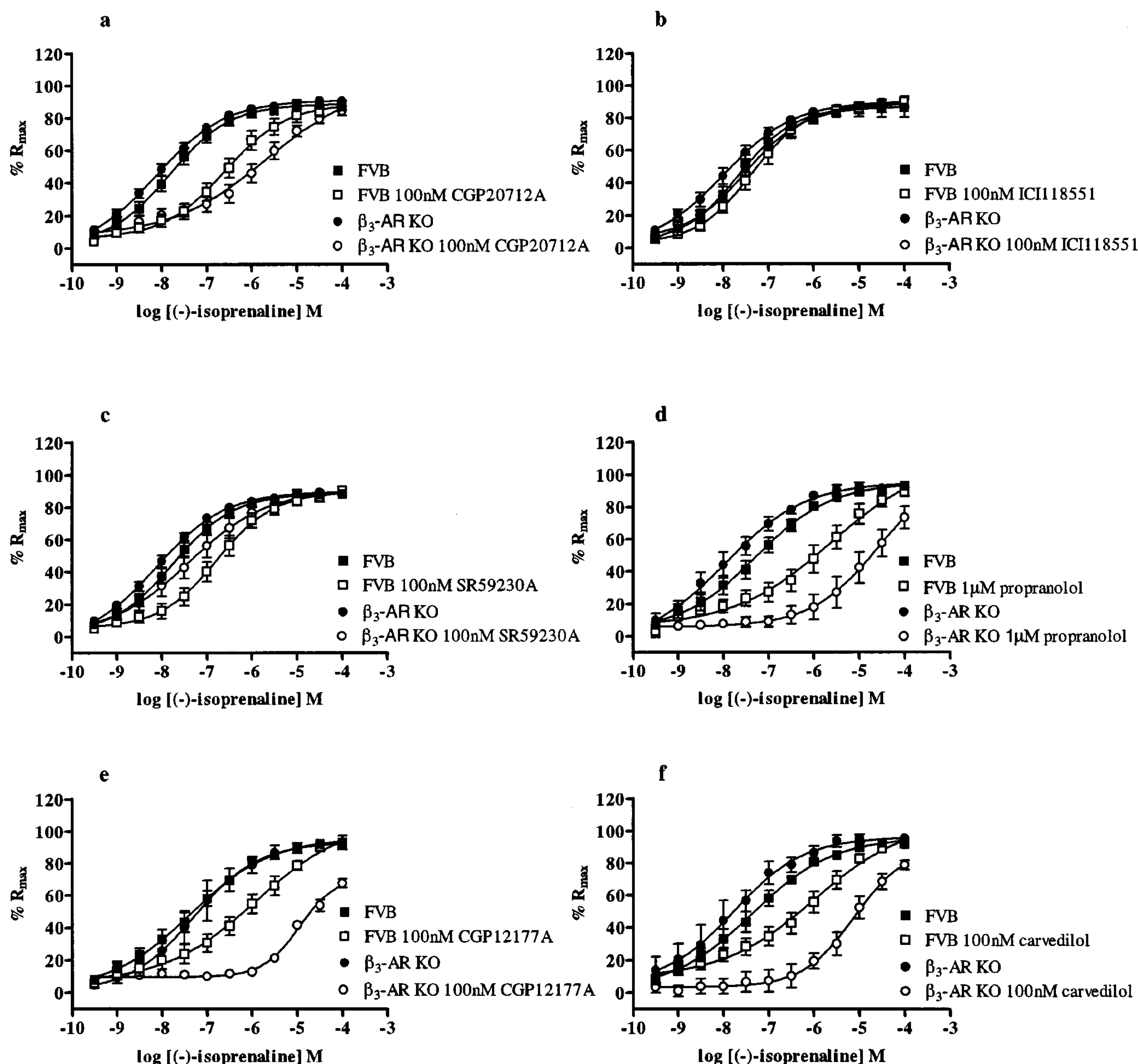


**Figure 4** The effect of (−)-isoprenaline on ileal responses in KO and FVB mice. Graph shows responses to (−)-isoprenaline are more strongly antagonized by (a) CGP20712A ($n=7-9$), (d) propranolol ($n=6-7$), (e) CGP12177A ($n=3-5$), and (f) carvedilol ($n=3-5$) in KO as compared to FVB mice. Responses to (−)-isoprenaline are weakly antagonized by (b) ICI118551 in both KO and FVB mice ($n=6-8$) while (c) SR59230A antagonized (−)-isoprenaline responses in FVB mice but was still effective in KO mice but to a lesser degree ($n=8-9$). Points show mean and vertical lines indicate s.e.mean.

The $\beta_3$-AR antagonist SR59230A (100 nM) (Figure 4c) significantly shifted the (−)-isoprenaline c-r curve to the right in FVB ileal samples (control: $pEC_{50}$ 7.8±0.1, $n=8$; SR59230A (100 nM): $pEC_{50}$ 6.8±0.1, $n=8$; $pK_B$ 8.0±0.2 ($n=8$)). (−)-Isoprenaline responses in KO ileum was antagonised to a weaker degree by SR59230A (100 nM) (control: $pEC_{50}$ 8.1±0.1, $n=9$; SR59230A (100 nM): $pEC_{50}$ 7.4±0.2, $n=9$; $pK_B$ 7.4±0.3 ($n=9$)). There was no statistical difference between the $pK_B$ values obtained for FVB or KO mice (Student's $t$-test NS).

The $\beta_1$-/$\beta_2$-AR antagonist (−)-propranolol (1 $\mu$M) (Figure 4d) caused a rightward shift of the (−)-isoprenaline c-r curve with $pK_B$ values of 7.7±0.2 ($n=7$) and 8.9±0.3 ($n=6$) (Student's $t$-test $^{***}P<0.001$) in FVB (control: $pEC_{50}$ 7.3±0.1, $n=7$; propranolol (1 $\mu$M): $pEC_{50}$ 5.6±0.4, $n=7$) and KO (control: $pEC_{50}$ 8.0±0.3, $n=6$; propranolol (1 $\mu$M): $pEC_{50}$ 4.6±0.8, $n=6$) ileum respectively.

CGP12177A ($\beta_1$-/$\beta_2$-AR antagonist) (Figure 4e) antagonized responses to (−)-isoprenaline in both FVB and KO ileum, with responses more strongly antagonized in ileum from KO (control: $pEC_{50}$ 7.3±0.2, $n=3$; CGP12177A (100 nM): $pEC_{50}$ 5.0±0.1, $n=3$) as compared to FVB mice (control: $pEC_{50}$ 7.4±0.1, $n=5$; CGP12177A (100 nM): $pEC_{50}$ 5.9±0.3, $n=5$) with $pK_B$ values of 9.6±0.6 ($n=3$) and 8.6±0.4 ($n=5$) respectively (Student's $t$-test $^{***}P<0.001$).

Responses to (−)-isoprenaline were antagonized by carvedilol (100 nM) (non-specific $\beta$-AR antagonist) (Figure 4f) more strongly in ileum from KO (control: $pEC_{50}$ 7.8±0.3, $n=3$; carvedilol (100 nM): $pEC_{50}$ 5.1±0.2, $n=3$) compared to FVB mice (control: $pEC_{50}$ 7.4±0.1, $n=5$; carvedilol (100 nM): $pEC_{50}$ 6.0±0.3, $n=5$) with $pK_B$ values of 10.1±0.4 ($n=3$) and 8.4±0.4 ($n=5$) respectively (Student's *t*-test $***P<0.001$).

*Effect of CGP12177A* CGP12177A failed to exert any significant agonistic actions in both KO and FVB ileum, even at concentrations of 10 $\mu$M (Figure 5). Subsequent addition of (−)-isoprenaline (10 $\mu$M) failed to produce any relaxation of the ileum, suggesting that CGP12177A acted purely as an antagonist in this preparation. Subsequent additions of CL316243 (1 $\mu$M) in FVB mice caused relaxation in the same tissue, suggesting an intact $\beta_3$-AR response, whereas in KO mice CL316243 had no effect (Figure 5).

## Discussion

### *$\beta_1$-, $\beta_2$- and $\beta_3$-AR mediated relaxation of mouse ileum*

Both $\beta_1$- and $\beta_3$-ARs mediate relaxation of mouse ileal smooth muscle in FVB mice. The relaxant effects of (−)-isoprenaline are antagonized strongly by the selective $\beta_1$- (CGP20712A) and $\beta_3$-AR (SR59230A) antagonists but

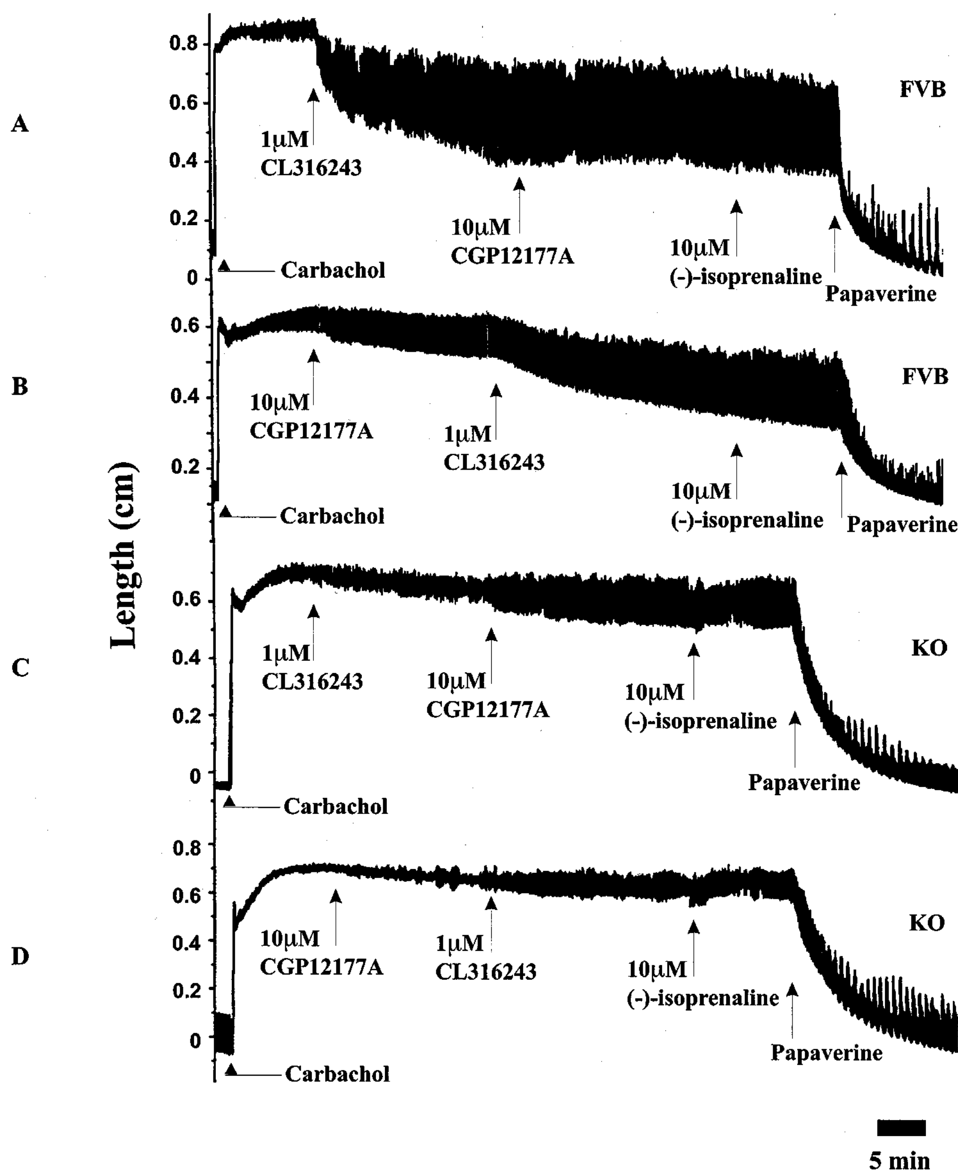


**Figure 5** Original traces obtained from (A,B) FVB and (C,D) KO ileum tissues. Tissues were contracted with carbachol (1 $\mu$M) and then exposed to CL316243 (1 $\mu$M) followed by CGP12177A (10 $\mu$M) or, CGP12177A (10 $\mu$M) followed by CL316243 (1 $\mu$M) as indicated. (−)-Isoprenaline (10 $\mu$M) and finally papaverine (10 $\mu$M) were added. Arrows indicate administration of drug. CL316243 was effective only in FVB ileum while CGP12177A was ineffective in causing ileal relaxation in both FVB and KO mice. Note the lack of effect of (−)-isoprenaline in FVB and KO ileum following CGP12177A administration. Traces are representative of $n=4$ experiments.

weakly by the $\beta_2$-AR antagonist (ICI118551), suggesting both $\beta_1$- and $\beta_3$-AR involvement in relaxation to (−)-isoprenaline. Responses to (−)-isoprenaline were also antagonized by the $\beta_1$-/$\beta_2$-AR antagonist propranolol, the non-selective $\beta$-AR antagonist carvedilol and the $\beta_1$-/$\beta_2$-AR antagonist (and putative $\beta_4$-AR agonist) CGP12177A. The degree of blockade caused by CGP20712A and SR59230A was similar, indicating that $\beta_1$- and $\beta_3$-ARs contribute to a similar extent to (−)-isoprenaline relaxation.

The $pK_B$ values observed in ileum from FVB mice with propranolol (7.7) was much higher than the values found in studies in rat distal colon (6.6; McLaughlin & MacDonald, 1990), ileum (6.8; Roberts *et al*., 1999) and gastric fundus (6.3; McLaughlin & MacDonald, 1991), but not other studies where higher $pK_B$ values have been reported (8.4, guinea-pig taenia caecum (Koike *et al*., 1995); 8.2 and 8.5, rat colon (Bianchetti & Manara, 1990; Croci *et al*., 1988)). In rat colon, there is a mixture of $\beta$-ARs present since in studies where higher $pA_2$ values for propranolol are reported, Schild plot slopes well below unity were found, suggesting the presence of more than one $\beta$-AR subtype (Bianchetti & Manara, 1990; Croci *et al*., 1988; McLaughlin & MacDonald, 1990). Other studies of antagonism of (−)-isoprenaline by propranolol show higher $pK_B$ values in guinea-pig atria ($\beta_1$-AR) ($pA_2$ 8.7–8.9), trachea ($\beta_2$-AR) ($pA_2$ 8.3) and diaphragm ($\beta_2$-AR) ($pA_2$ 9.2) (Harms *et al*., 1977), all tissues with predominantly $\beta_1$- or $\beta_2$-AR responses. Antagonism with CGP20712A also revealed a $pK_B$ value of 8.1 which was lower than that at cardiac $\beta_1$-ARs ($pA_2$ 9.0; Molenaar & Summers, 1987) but higher than that at atypical $\beta$-ARs ($pA_2$ 4.1–4.6; Hollenga & Zaagsma, 1989; Van Liefde *et al*., 1993). Studies carried out in rat ileum, distal colon and guinea-pig colon (De Ponti *et al*., 1995; MacDonald & Lamont, 1993; Roberts *et al*., 1999) demonstrated no significant shift with CGP20712A. Carvedilol antagonized (−)-isoprenaline responses in FVB ileum with a $pK_B$ (8.4) similar to that observed in ferret myocardium ($pK_B$ 8.1; Lowe *et al*., 1999). This data taken together suggests that a significant $\beta_1$-AR component exists in mouse ileum, as evidenced by the higher than expected $pK_B$ values obtained from antagonism of (−)-isoprenaline by propranolol and CGP20712A at $\beta_3$-ARs, and the similarity of the $pK_B$ values to those reported in tissues rich in $\beta_1$-ARs.

The functional role of the $\beta_3$-AR in mouse ileum was also confirmed in this study, as in FVB mice responses were produced to the $\beta_3$-AR agonist CL316243 and were antagonized by SR59230A but not by propranolol or carvedilol. Affinity values for antagonism by SR59230A were consistent with previous reports (this study $pK_B$ 8.3, in rat colon $pA_2$ 8.1 (Manara *et al*., 1996)). Carvedilol was investigated since it was reported to have high affinity at the cloned human $\beta_3$-AR ($K_i$ 0.4 nM (Candelore *et al*., 1999)). However in the present study it had no significant antagonist action against CL316243 in mouse ileum. Propranolol resistance of $\beta_3$-AR mediated responses has been used in many studies of gastrointestinal smooth muscle to demonstrate the presence of atypical $\beta$-ARs (Bianchetti & Manara, 1990; De Ponti *et al*., 1995; McLaughlin & MacDonald, 1991), and this was confirmed in this study where propranolol failed to antagonize responses to CL316243. In the present study no responses to CL316243 were observed in ileum from KO animals. This is supported by other studies using $\beta_3$-AR KO animals where responses to CL316243 were abolished in stomach fundus (Cohen *et al*., 2000), colon (Oostendorp *et al*., 2000), adipose tissue (Grujic *et al*., 1997; Preitner *et al*., 1998; Susulic *et al*., 1995) and there was also decreased gastrointestinal motility (Fletcher *et al*., 1998).

A role for $\beta_1$- and $\beta_3$-ARs is supported by the presence of mRNA for both receptors in ileum. In contrast $\beta_2$-AR mRNA was present yet responses to (−)-isoprenaline were only weakly antagonized by the $\beta_2$-AR antagonist ICI118551 in FVB mice. Despite other *in vitro* studies showing a minor $\beta_2$-AR mediated relaxation in mouse colon (Oostendorp *et al*., 2000), rat distal colon, and jejunum (MacDonald & Lamont, 1993; van der Vliet *et al*., 1990), and *in vivo* studies in rat duodenum and jejunum showing (−)-isoprenaline and ritodrine can disrupt migrating myoelectric complexes which are blocked by ICI118551 (Thollander *et al*., 1996), other studies in rat ileum show that despite $\beta_2$-AR mRNA being present, no $\beta_2$-AR mediated relaxation responses could be found (Roberts *et al*., 1999). It is likely therefore that $\beta_2$-ARs play roles other than relaxation in mouse ileum. $\beta$-AR agonists such as isoprenaline, adrenaline and terbutaline are known to increase glucagon-like peptide-1 and peptide YY secretions in rat ileum and this effect is antagonized by propranolol and likely to be mediated by $\beta_2$-AR activation (Claustre *et al*., 1999; Dumoulin *et al*., 1995).

$\beta_1$-ARs appear to compensate for the lack of $\beta_3$-ARs in ileum from $\beta_3$-AR KO mice. This was supported functionally by the increased antagonism of (−)-isoprenaline mediated relaxation by CGP20712A, propranolol and carvedilol in ileum from KO as compared to FVB mice. The increase in $pK_B$ values for these agents suggests an enhanced $\beta_1$-AR response. There may also be a slight increase in $\beta_2$-AR function in KO mice since while responses to (−)-isoprenaline were very weakly antagonized by ICI118551 in FVB mice, there was a somewhat greater shift in KO mice, despite the absence of a change in $\beta_2$-AR mRNA levels. Compensation by $\beta_1$-ARs was associated with an increase in ICYP binding and $\beta_1$-AR mRNA levels. Interestingly, this parallels the increase in $\beta_1$-AR mRNA levels seen in adipose tissue in this strain of KO mice (Grujic *et al*., 1997; Susulic *et al*., 1995) where these animals compensate for the lack of $\beta_3$-ARs in adipose tissue by upregulation of $\beta_1$-AR gene expression. More recent studies with another strain of KO mice on a 129Sv+C57BL/6 mouse background (Revelli *et al*., 1997) show that in mouse colon, while $\beta_1$-ARs also compensate for the lack of $\beta_3$-ARs functionally, no differences in $\beta_1$-AR mRNA levels were observed in 129Sv+C57BL/6 and KO ileum (Oostendorp *et al*., 2000). These authors suggested that in the KO animals, existing $\beta_1$-ARs may become coupled more strongly to signalling pathways governing gastrointestinal relaxation. Two groups have successfully targeted the inactivation of the mouse $\beta_3$-AR gene. Both groups used different approaches (for review see Rohrer, 1998) and this may be a factor in the differing results obtained. Susulic *et al*. (1995) showed that the lack of $\beta_3$-ARs resulted in upregulation of $\beta_1$-AR mRNA in adipose tissue, whereas the model established by Revelli *et al*. (1997) showed downregulation of $\beta_1$-ARs in adipose tissue. This difference may be a result of the different strain backgrounds of the mice used, the knock-out strategy used or other unknown variables. Nevertheless, both studies show several similar findings, including KO animals being susceptible to a slowly developing mild increase in body fat content, with no

associated increase in food intake, and no changes in circulating levels of insulin, glucose or free fatty acids.

Another interesting feature of (−)-isoprenaline mediated relaxation in KO ileum was that antagonism by SR59230A was still observed, although much weaker than that in FVB mice. This would suggest that SR59230A may not be as specific for the $\beta_3$-AR as previously suggested (Manara *et al*., 1996). In this study, responses to the $\beta_3$-AR agonist SR58611A were antagonized by SR59230A with a $pA_2$ value of 8.76 in rat proximal colon, similar to that observed here with either CL316243 or (−)-isoprenaline as the agonist ($pK_B$ 8.3 and 8.0 respectively in FVB mice). In KO mice, a $pK_B$ value against (−)-isoprenaline of 7.4 was observed, in comparison with a $pA_2$ value of 7.3 observed in guinea-pig atria against (−)-isoprenaline (Manara *et al*., 1996). A recent study has indicated that at cloned human $\beta$-ARs, SR59230A displays little selectivity in binding for the $\beta_3$-AR compared to $\beta_1$- or $\beta_2$-ARs (Candelore *et al*., 1999). This may suggest SR59230A is not as selective as previously reported and may also interact with $\beta_1$-ARs but this needs further investigation.

## *Action of CGP12177A*

CGP12177A is a high affinity $\beta_1$-/$\beta_2$-AR antagonist (Staehelin & Hertel, 1983) which also shows partial agonist activity at $\beta_3$-ARs in adipose tissues, and has been suggested to have partial agonist actions at another site termed the putative $\beta_4$-AR. It has been used to describe a putative $\beta_4$-AR in the heart of several species including man and mouse (Kaumann, 1996; Kaumann & Molenaar, 1997; Molenaar *et al*., 1997b), as well as in mouse and human adipocytes (Galitzky *et al*., 1997; Preitner *et al*., 1998). In cardiac tissues CGP12177A and other non-conventional partial agonists cause antagonism of responses mediated by $\beta_1$- and $\beta_2$-ARs but at higher concentrations have agonist effects that are resistant to blockade by propranolol but not by bupranolol. These effects cannot be mediated by $\beta_3$-ARs since the heart does not express this subtype (Evans *et al*., 1996) and CGP12177A still produces these effects in the heart from $\beta_3$-AR KO mice (Kaumann *et al*., 1998). In tissues that do express $\beta_3$-ARs such as rat colon or guinea-pig taenia caecum, CGP12177A acts as an agonist (Kaumann & Molenaar, 1996; Molenaar *et al*., 1997a; Sennitt *et al*., 1998; Koike *et al*., 1995; 1996). Support for the $\beta_4$-AR concept came from studies showing that CGP12177A activated brown adipose tissue and cardiac responses in $\beta_3$-AR KO mice (Kaumann *et al*., 1998; Preitner *et al*., 1998). In $\beta_3$-AR KO mice, CGP12177A has agonist actions in atria (Cohen *et al*., 2000), adipose tissue (Preitner *et al*., 1998) and oesophageal and colonic smooth muscle (Oostendorp *et al*., 2000). However it is clear that CGP12177A effects at the putative $\beta_4$-AR only occur in tissues (heart and adipose) that express high levels of $\beta_1$-AR, and also in cultured cells overexpressing the $\beta_1$-AR (Pak & Fishman, 1996). It is now clear from a recent study in $\beta_1$- or $\beta_3$-AR KO mice, that $\beta_1$-ARs mediate most, if not all, of the $\beta_3$-AR independent effects of CGP12177A on brown adipocyte adenylate cyclase activity (Konkar *et al*., 2000). In the present study in mouse ileum, no agonist actions of CGP12177A were observed in either KO or FVB ileum, even with concentrations up to 10 $\mu$M, indicating no agonist actions at either $\beta_3$- or $\beta_1$-ARs. Instead, CGP12177A acted as a potent antagonist of (−)-isoprenaline mediated relaxations with $pK_B$ values of 9.6 in KO and 8.6 in FVB ileum. These values are much higher than those reported in rat ileum (7.6; Roberts *et al*., 1999) and consistent with those in guinea-pig taenia caecum (9.3; Koike *et al*., 1996). These findings are in accord with an action of CGP12177A to block $\beta_1$-ARs which are expressed at higher levels in KO ileum.

In conclusion, in mouse ileum, $\beta_3$-ARs and to a lesser extent $\beta_1$-ARs mediate smooth muscle relaxation, with no $\beta_2$-AR involvement. In KO mice, $\beta_1$-ARs functionally compensate for the lack of $\beta_3$-ARs, and these animals have increased $\beta_1$-AR mRNA and levels of binding. CGP12177A acts as an antagonist in this preparation with no agonist actions and in mice SR59230A appears not to be as selective for the $\beta_3$-AR as previously reported.

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