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# Direct demonstration of $\beta_1$- and evidence against $\beta_2$- and $\beta_3$-adrenoceptors, in smooth muscle cells of rat small mesenteric arteries

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**1**   Recent evidence supports additional subtypes of vasodilator $\beta$-adrenoceptor ($\beta$-AR) besides the 'classical' $\beta_2$. The aim of this study was to investigate the distribution of $\beta$-ARs in the wall of rat mesenteric resistance artery (MRA), to establish the relative roles of $\beta$-ARs in smooth muscle and other cell types in mediating vasodilatation and to analyse this in relation to the functional pharmacology.

**2**   We first examined the vasodilator $\beta$-AR subtype using 'subtype-selective' agonists against the, commonly employed, phenylephrine-induced tone. Concentration-related relaxation was produced by isoprenaline (pEC$_{50}$: 7.70±0.1) ($\beta_1$ and $\beta_2$). Salbutamol ($\beta_2$), BRL 37344 ($\beta_3$) and CGP 12177 (atypical $\beta$) caused relaxation but were 144, 100 and 263 times less potent than isoprenaline; the '$\beta_3$-adrenoceptor agonist' CL 316243 was ineffective.

**3**   In arteries precontracted with 5-HT or U 46619, isoprenaline produced concentration-related relaxation but salbutamol, BRL 37344, CGP 12177 and CL 316243 did not. SR 59230A, CGP 12177 and BRL 37344 caused a parallel rightward shift in the concentration–response curve to phenylephrine indicating competitive $\alpha_1$-AR antagonism, explaining the false-positive 'vasodilator' action against phenylephrine-induced tone. Endothelial denudation but not L-NAME slightly attenuated isoprenaline-mediated vasodilatation in phenylephrine and U 46619 precontracted MRA.

**4**   The $\beta$-AR fluorescent ligand BODIPY TMR-CGP 12177 behaved as an irreversible $\beta_1$-AR antagonist in MRA and bound to the surface and inside vascular smooth muscle cells in intact vascular wall. $\beta$-ARs in smooth muscle cells were observed in a perinuclear location, consistent with the location of Golgi and endoplasmic reticulum.

**5**   Binding of BODIPY TMR-CGP 12177 was inhibited by BAAM (1 $\mu$M) in all three vascular tunics, confirming the presence of $\beta$-ARs in adventitia, media and intima. Binding in adventitia was observed in both neuronal and non-neuronal cell types. Lack of co-localisation with a fluorescent ligand for $\alpha$-ARs confirms the selectivity of BODIPY TMR-CGP 12177 for $\beta$-ARs over $\alpha$-ARs.

**6**   Our results support the presence of functional vasodilator $\beta_1$-ARs and show that they are mainly located in smooth muscle cells. Furthermore, we have demonstrated, for the first time, the usefulness of BODIPY TMR-CGP 12177 for identifying $\beta$-AR distribution in the 'living' vascular wall.
*British Journal of Pharmacology* (2005) **146,** 679–691. doi:10.1038/sj.bjp.0706369; published online 22 August 2005

## Introduction

In their groundbreaking study that divided $\beta$-adrenoceptors (ARs) into two subtypes, Lands *et al.* (1967) classified vasodilator $\beta$-ARs as $\beta_2$. Since then, several studies have shown that $\beta_1$-ARs can also participate in the relaxation of blood vessels (O'Donnell & Wanstall, 1984; Graves & Poston, 1993). The involvement of one or other $\beta$-AR subtype has been postulated to vary not only with the vascular bed but also with species (Guimaraes & Moura, 2001). Recently, Chruscinski *et al.* (2001) showed, by a combination of receptor knockouts and classical pharmacology, that vasodilator $\beta_1$-ARs were common and widespread amongst mouse blood vessels whereas $\beta_2$-ARs were confined to a narrower range of vessels. The participation of the $\beta_3$-AR subtype, originally described in adipose tissue and the gastrointestinal tract (Arch & Kaumann, 1993; Arch, 2002), has been implicated in rat and dog blood vessels (Shen *et al.*, 1996; MacDonald *et al.*, 1999;

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Tagaya *et al.*, 1999). A fourth atypical $\beta$-AR subtype that shares some properties with the $\beta_3$-AR subtype has been reported in cardiac and adipose tissue (Kaumann & Molenaar, 1996; Galitzky *et al.*, 1997; Malinowska & Schlicker, 1997) and also in several rat blood vessels (Oriowo, 1994; 1995; Dumas *et al.*, 1998; Brawley *et al.*, 2000a; Kozlowska *et al.*, 2003). Brahmadevara *et al.* (2003; 2004) provided evidence in rat thoracic aorta against the presence of $\beta_3$-ARs, suggesting that the relaxation induced by the 'atypical $\beta$-AR agonists' could be explained by an $\alpha$-AR-blocker action against the agonist (phenylephrine) commonly used to contract the vessels rather than to an interaction with $\beta_3$-ARs.

It is generally assumed that vasodilatation caused *via* $\beta$-ARs involves vascular smooth muscle cells rather than any other cell type in the vascular wall, although the role of other cell types cannot be discounted. Nevertheless, using autoradiography, $\beta$-ARs have been located in the media, adventitia and/or endothelium in canine (Molenaar *et al.*, 1988a) and human (Nakai *et al.*, 1986; Molenaar *et al.*, 1988b; Amenta *et al.*, 1991). Radioligand binding studies using cultured bovine endothelial cells have also reported the presence of $\beta$-ARs (Steinberg *et al.*, 1984). Thus, although no evidence exists for the function of adventitial $\beta$-AR, a role for endothelial $\beta$-ARs in vasodilatation mediated by $\beta$-AR agonists remains controversial (Guimaraes & Moura, 2001; Vanhoutte, 2001). Some studies in blood vessels of rat (Moncada *et al.*, 1991; Oriowo, 1994) and human (Molenaar *et al.*, 1988b) support the concept that $\beta$-AR agonist-induced vasodilatation is endothelium independent. In contrast, other studies suggest that the relaxation mediated by $\beta$-ARs in rat (Gray & Marshall, 1992; Trochu *et al.*, 1999; Brawley *et al.*, 2000b; Georgescu *et al.*, 2005) and mouse (Akimoto *et al.*, 2002) blood vessels relies on the presence of the endothelium. The studies indicating that the $\beta_2$-AR should no longer be thought of as the principal vascular subtype also prompts reanalysis of the function and distribution of the vascular $\beta$-AR subtypes.

We have undertaken visualisation and functional analysis of $\beta$-ARs in a standard resistance artery model to compare receptor location and vasodilator function.

BODIPY-TMR-CGP 12177 is a fluorescent ligand for $\beta$-ARs that has been described as a long-lasting $\beta_2$-AR partial agonist in cells expressing the recombinant human $\beta_2$-AR (Baker *et al.*, 2003). Its affinity for $\beta_1$-ARs is not known, although there is no *prima facie* case to assume that it would be selective between $\beta$-AR subtypes. In addition, its pharmacology has not been reported in the rat resistance vasculature so it was necessary to first validate the receptor subtype present in the small mesenteric arteries and then to test it as an antagonist of these receptors.

The main objective of this study was to employ the fluoligand, with confocal microscopy, to show the cellular distribution of $\beta$-ARs in the wall of a 'live' resistance artery and to resolve whether smooth muscle, adventitia and/or endothelial $\beta$-ARs exist and to relate this to vasodilation. To attain this objective, it was necessary to (i) characterise the $\beta$-AR subtype that mediates vasodilatation in the preconstricted mesenteric resistance artery (MRA), (ii) consider the participation of endothelium in $\beta$-AR mediated vasodilatation. (iii) evaluate the $\beta$-AR agonist and/or antagonist capacity of BODIPY-TMR-CGP 12177 since the class of $\beta$-blocker from which it is derived can have diverse actions and (iv) examine the binding of the fluorescent ligand in MRA.

## Methods

### Tissue preparation

Male Wistar Kyoto rats, 6-month old, (Autonomous University of Madrid, Spain) were decapitated and the mesenteric arcade was removed and placed in physiological salt solution (PSS) of the following composition (in mM): NaCl 112.0, KCl 4.7, $CaCl_2$ 2.5, $KH_2PO_4$ 1.1, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 11.1, maintained at $4^\circ C$ and continuously gassed with 95% $O_2$ and 5% $CO_2$. Segments (1.8–2 mm) of third-order branches of the mesenteric arteries were dissected free of fat and connective tissue. In some vessels, the endothelial layer was removed by passing a human hair through the lumen. Vessels were mounted on wires on an isometric myograph (mod 410 A, J.P. Trading, Denmark) filled with PSS kept at $37^\circ C$. The vessels were then allowed to equilibrate for 30 min and set up with a 'normalised' internal circumference 0.9 $L_{100}$ estimated to be 0.9 times the passive circumference at 100 mmHg transmural pressure. This was calculated for each individual vessel based on the passive length-tension characteristics of the artery and the Laplace relationship (Mulvany & Halpern, 1977). This procedure optimised active force generation by these vessels and the internal diameters referred to were derived from this calculation. After the normalization procedure, the vessels were allowed to equilibrate for a further 30 min. The tissue was then contracted three times with KCl 100 mM every 5 min to ensure that the size of the contractile response was constant. After a 30 min period, each ring was contracted with phenylephrine (2–3 $\mu$M) and relaxed with acetylcholine (ACh, 1–10 $\mu$M) to verify the functionality of the endothelium. Only preparations that relaxed more than 80% were classified as endothelium intact and those that failed to relax (0%) were classified as endothelium removed. After washing, the tissues were left to equilibrate for a further 30 min before starting the experiments.

### Functional experiments

In the first series of experiments, MRA with or without endothelium were incubated (30 min) with $\beta$-AR antagonists, or vehicle, before being exposed to a concentration of either phenylephrine ($\alpha_1$-AR agonist), [9,11,-dideoxy-9$\alpha$-11$\alpha$-epoxymenthane prostaglandin-F$_{2\alpha}$] (U 46619, thromboxane $A_2$ mimetic) or serotonin (5-HT) that produces a contractile response between 70 and 80% of 100 mM KCl-induced contraction. The contractile response was allowed to stabilize and a concentration–response curve (CRC) was obtained to test for vasodilator $\beta$-AR agonism of each compound. When the vessels were precontracted with either U 46619 or 5-HT, phentolamine (10 $\mu$M) was also present.

In a second series of experiments, CRCs to phenylephrine were performed in the presence and absence of the test drugs (putative $\beta_3$-AR agonists and antagonists) to establish their $\alpha$-AR antagonist capacity.

In addition, the fluo-ligand BODIPY-TMR-CGP 12177 was assessed for potential $\beta$-AR agonism (*versus* phenylephrine-induced tone) and antagonism (*versus* isoprenaline) following protocols similar to those in the first series. In addition, to determine if this compound binds irreversibly to $\beta$-ARs, in a separate series of experiments, tissues were incubated (30 min) with BODIPY-TMR-CGP 12177 (1 $\mu$M) or DMSO and then

washed successively every 5 min for 30 min after which the vessels were contracted with phenylephrine and a CRC to isoprenaline was constructed.

### $\beta$-AR localization by confocal microscopy

MRA segments were incubated (1 h, 37°C) with the irreversible $\beta$-AR antagonist bromoacetylalprenolol menthane (BAAM; 1 $\mu$M) or vehicle (0.0001% ethanol) and washed every 10 min over 30 min followed by incubation (15 min) with BODIPY-TMR-CGP 12177 (1 $\mu$M). Some vessels were coincubated (1 h) at room temperature with 1 $\mu$M quinazolinyl piperazine borate-dipyrromethene (QAPB, Miquel *et al.*, 2005), an $\alpha$-AR fluorescent ligand (Shafaroudi *et al.*, 2005) and BODIPY-TMR-CGP (1 $\mu$M). Vessels were then mounted in PSS on slides with a small well (400 $\mu$M depth) made of silicon spacers to avoid vessel compression. The vessels were visualised with either a Leica TCS SP2 or BIORAD Radiance 2100 confocal system fitted to an inverted microscope comprising argon and helium–neon laser sources. Serial optical sections (stacks of images) from the adventitia to the lumen ($z$ step $= 0.5\,\mu$m) were captured with either $\times$63 or $\times$40 oil objectives (NA $= 1.3$) using 488 or 543 nm excitation of the helium–neon laser as appropriate. A minimum of two stacks of different regions was captured in each arterial segment. For comparative studies, images were taken under identical conditions of laser intensity, brightness and contrast.

In a separate set of experiments (to visualise endothelial cells), MRA were pressure-fixed (70 mmHg) with 4% phosphate-buffered paraformaldehyde (pH $= 7.6$) for 1 h and washed in three changes of phosphate-buffered saline solution (pH $= 7.4$). Transverse sections (100 $\mu$m) were cut on to gelatine-coated slides and incubated with BODIPY-TMR-CGP 12177 (1 $\mu$M, 3 h). Some sections were preincubated with BAAM (1 $\mu$M, 3 h, 37°C) or vehicle (0.0001% ethanol) prior to incubation with BODIPY-TMR-CGP 12177.

### Imaging methods

Serial confocal sections were stored as a series of TIFF images for offline processing and analysis. Metamorph (Universal Imaging) software was used for image processing and final presentation. Orthogonal (cross and transverse) sectioning was performed by loading a full TIFF series in IMARIS (Bitplane) software and selecting an *xz* and *yz* section line to produce sectional views of the data.

### Drugs

Acetylcholine HCl, CGP 12177, CL 316243, isoprenaline, $N^G$-nitro-L-arginine methylester, papaverine, phenylephrine HCl, propranolol HCl, salbutamol, 5-HT and SR 59230A were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.); BODIPY-TMR-CGP 12177 and QAPB (quinazolinyl piperazine-borate-dipyrromethene, marketed under the name of BODIPY FL-prazosin) from Molecular Probes (Eugene, OR, U.S.A.) (an Invitrogen company); BAAM (bromoacetylalprenolol menthane) from Research BiochemicaI Incorporated (RBI, Poole, Dorset, U.K.); BRL 37344 from Tocris (Northpoint, Fourth Way, Avonmouth, U.K.); U 46619 from Calbiochem (Merck Biosciences Ltd, Beeston, Nottingham, U.K.) BAAM (10 mM) and U 46619 were dissolved in absolute ethanol and diluted with physiological salt solution. BODIPY-TMR-CGP 12177 (10 mM) was diluted in DMSO, aliquoted and stored at $-20$°C; further dilutions were made with physiological salt solution on the day of the experiment. All other chemicals used were of analytical grade.

### Data and statistical analysis

Location parameters were estimated for each experiment as pEC$_{50}$ (the negative logarithm of the concentration required to cause 50% of the maximum response). Agonist concentration ratios (CR) were calculated from the EC$_{50}$ values and, where appropriate, estimates of antagonist affinities expressed as p$K_B$ values were obtained from the equation p$K_B =$ log (agonist CR$-1$)$-$log [antagonist]. Data were compared using either unpaired Student's *t*-test (pEC$_{50}$) or two-way analysis of variance (ANOVA, CRC) as appropriate. Differences were considered statistically significant when $P < 0.05$. '$n$' is indicated in brackets in the figures and represents the number of animals used. Only one ring/animal/agonist was used.

## Results

### $\beta$-AR-mediated vasodilatation

The nonselective $\beta$-AR agonist isoprenaline, the $\beta_2$-AR agonist salbutamol, the atypical partial $\beta$-AR agonist CGP 12177 and the selective $\beta_3$-AR agonist BRL 37344 fully relaxed the phenylephrine preconstricted MRA in a concentration-related manner (Figure 1a). In contrast, the $\beta_3$-AR agonist CL 316243 failed to relax the phenylephrine precontracted vessel (Figure 1a). The pEC$_{50}$ values and the maximum relaxation for all $\beta$-AR agonists used are displayed in Table 1. Salbutamol, CGP 12177 and BRL 37344 lay to the right 144, 100 and 263 times, respectively, in relation to isoprenaline.

U 46619 (1 nM–1 $\mu$M) and 5-HT (1–30 $\mu$M) contracted the MRA in a concentration-related manner. The pEC$_{50}$ value obtained was $6.8 \pm 0.14$ ($n = 8$) for U 46619 and $5.20 \pm 0.2$ ($n = 4$) for 5-HT. The maximum contraction attained was $78 \pm 5.3$ and $83 \pm 5.4\%$ of 100 mM KCl for U 46619 and 5-HT, respectively. Isoprenaline was able to relax MRA preconstricted by either 5-HT (Figure 1b) or U 46619 (Figure 1c) in a concentration-related manner. When the vessel was contracted with U 46619, the values for the pEC$_{50}$ and the maximum relaxation were similar to those obtained when the vessels were contracted with phenylephrine (Table 1). The midrange sensitivity but not the maximum relaxation to isoprenaline was slightly reduced with 5-HT precontracted vessels. Salbutamol, CGP12177A, BRL 37344 and CL 316243 failed to relax U 46619 or 5-HT precontracted vessels (Figure 1b and c).

Removal of endothelium partly inhibited the CRC to isoprenaline and papaverine (Figure 2) in phenylephrine precontracted vessels. Responses to isoprenaline in U 46619 were also similarly affected by endothelium removal (results not shown). L-NAME (30 $\mu$M) failed to modify the CRC to isoprenaline *versus* phenylephrine precontraction (Figure 2).

### Inhibition of $\beta$-AR-mediated vasodilatation

The CRC to isoprenaline (pEC$_{50}$: $7.70 \pm 0.1$; $n = 14$) was shifted to the right by 0.1 $\mu$M propranolol (pEC$_{50}$: $6.52 \pm 0.2$;
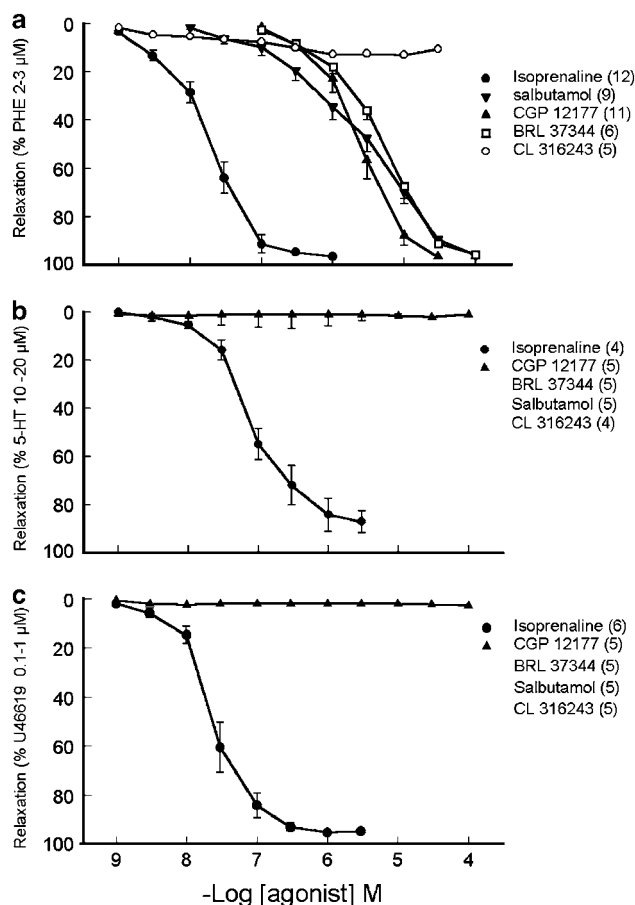
**Figure 1** Concentration–response curves to several $\beta$-AR agonists in phenylephrine (a), serotonine (b) and U 46619 (c) precontracted MRA. Results are expressed as mean $\pm$ s.e.m. The number of animals used is indicated in brackets. ***$P < 0.001$ for all agonists *versus* isoprenaline by two-way ANOVA.
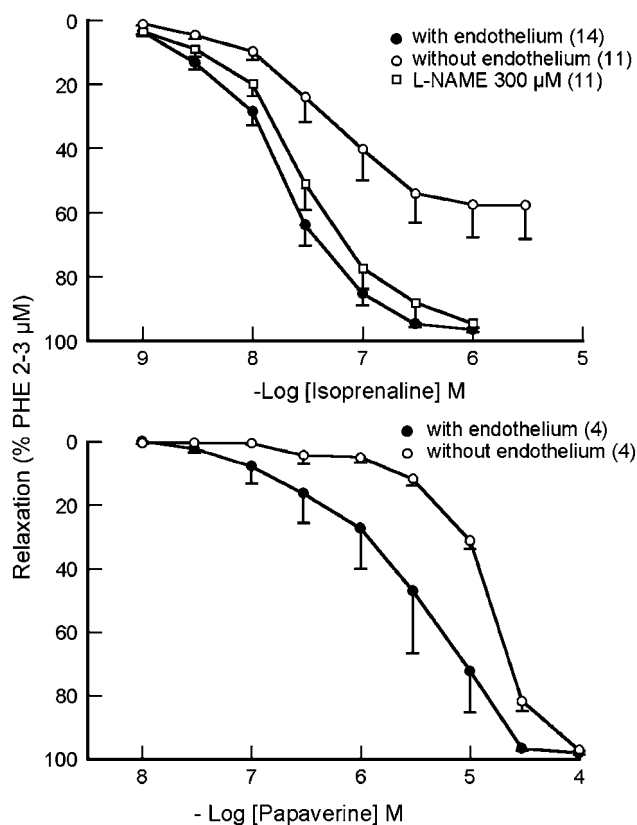


**Figure 2** Concentration–response curves to isoprenaline (top) and papaverine (bottom) in the presence and the absence of endothelium in phenylephrine precontracted MRA. The influence of L-NAME on isoprenaline response is also shown (top). Results are expressed as mean $\pm$ s.e.m. The number of animals used is indicated in brackets. ***$P < 0.001$ for isoprenaline and **$P < 0.01$ for papaverine in the absence *versus* the presence of endothelium by two-way ANOVA.

**Table 1** Relaxant effects of $\beta$-adrenoceptor agonist in rat mesenteric resistance arteries

| $\beta$-AR agonist | $pEC_{50}$ | % Maximum response | n |
|---|---|---|---|
| Isoprenaline | $7.7 \pm 0.10$ | $97.26 \pm 0.72$ | 11 |
| CGP 12177 | $5.7 \pm 0.04$*** | $96.20 \pm 0.87$ | 10 |
| Salbutamol | $5.54 \pm 0.07$*** | $96.20 \pm 0.87$ | 12 |
| BRL 37344 | $5.28 \pm 0.07$*** | $95.75 \pm 1.07$ | 7 |

***$P < 0.001$ *versus* isoprenaline by unpaired Student's *t*-test.

$n = 6$; $P < 0.0001$) and by $1 \mu$M atenolol ($pEC_{50}$: $7.1 \pm 0.08$; $n = 5$; $P < 0.01$) with no reduction in the maximum relaxation (Figure 3a). However, ICI 118551 ($0.1 \mu$M) did not modify the CRC to isoprenaline ($pEC_{50}$: $7.65 \pm 0.08$, $n = 5$; Figure 3a). Estimation of the magnitude of the shift from the $EC_{50}$ values gave a 15- and a 3.9-fold shift for propranolol and atenolol, which corresponds to a $pK_B$ values of 8.17 (propranolol) and 7.47 (atenolol).

The relaxations of phenylephrine-induced tone that were induced by BRL 37344 (Figure 3b) and CGP 12177 (Figure 3c) were shifted to the right by SR 59230A (0.1 and $0.3 \mu$M), a selective $\beta_3$-AR antagonist. The use of a higher concentration

of SR 59230A ($1 \mu$M) was precluded because this compound drastically reduced the maximum contraction to phenylephrine. The nonselective $\beta$-AR antagonist propranolol ($0.1 \mu$M) had no effect on the CRC for BRL 37344 and CGP 12177 (results not shown).

*Effect of SR 59230A, CGP 12177, BRL 37344 and CL 316243 on the CRC curves to phenylephrine*

Phenylephrine ($0.1$–$100 \mu$M) contracted the MRA in a concentration-dependent manner ($pEC_{50}$: $5.69 \pm 0.05$; $n = 7$). Preincubation with SR 59230A (Figure 4a), CGP 12177 (Figure 4b) and BRL 37344 (Figure 4c) shifted the CRC to phenylephrine to the right in a concentration-related manner. The estimated $pK_B$ was 7.1, 5.16 and 4.6 for SR 59230A, CGP 12177A and BRL 37344, respectively. In contrast, CL 316243 was without effect on the CRC to phenylephrine (Figure 4d).

*$\beta$-AR agonist and antagonist capacity of the fluo-ligand BODIPY-TMR-CGP 12177*

BODIPY-TMR-CGP 12177 failed to relax the phenylephrine precontracted MRA (Figure 5a). A slight relaxation was seen with the highest concentration used and can be ascribed to DMSO (Figure 5a). The CRC to isoprenaline (Figure 5b) was
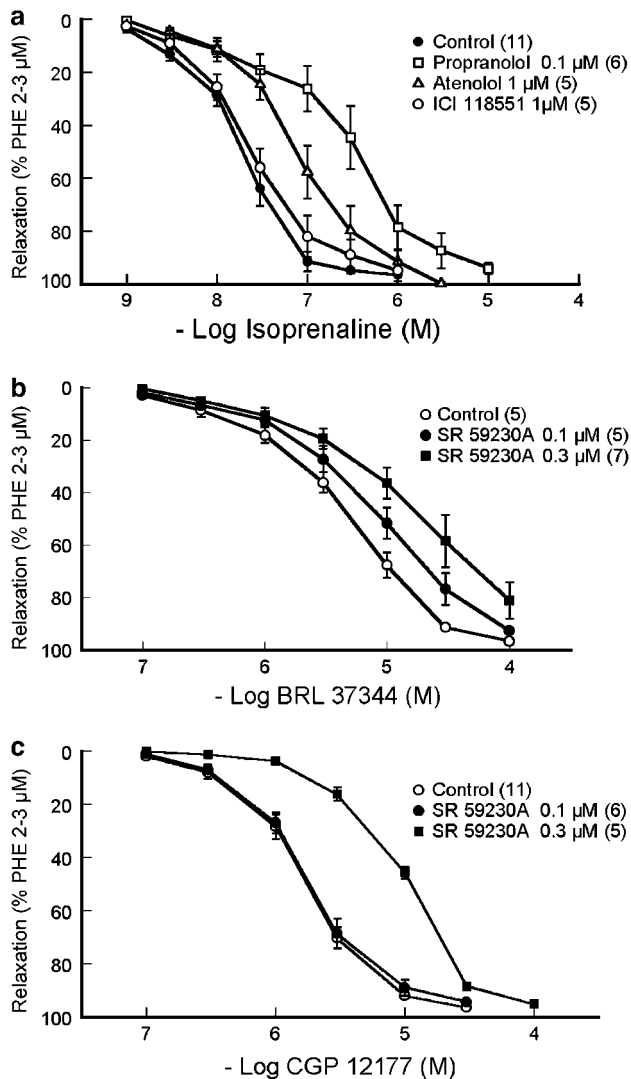
**Figure 3** Effect of different $\beta$-AR antagonists on isoprenaline-mediated vasodilatation (a), BRL 37344 (b) and CGP 12177 (c) on phenylephrine precontracted MRA. Results are expressed as mean $\pm$ s.e.m. The number of animals used is indicated in brackets. ***$P < 0.001$ for isoprenaline in presence *versus* absence of propranolol and for BRL 37344 and CGP 12177 in the presence *versus* absence of SR 59230A (0.3 $\mu$M); **$P < 0.01$ for isoprenaline in presence *versus* absence of atenolol by two-way ANOVA.

inhibited in a concentration-related manner by BODIPY-TMR-CGP 12177. Lower concentrations (1–10 nM) significantly shifted the CRC to isoprenaline to the right with no significant inhibition of maximum relaxation. However, the incubation with higher concentrations of BODIPY-TMR-CGP 12177 (0.1–1 $\mu$M) significantly decreased the maximum relaxation induced by this agonist. Incubation with BODIPY-TMR-CGP 12177 (1 $\mu$M; 30 min) followed by a 30 min washout abolished the CRC to isoprenaline (Figure 5c).

*Binding-induced fluorescence of BODIPY-TMR-CGP 12177*

BODIPY-TMR-CGP 12177 did not exhibit significant fluorescence in solution. Therefore, experiments could be performed at equilibrium and without washing. BODIPY-TMR-

CGP 12177 bound to adventitial (Figure 6a), smooth muscle (Figure 6c) and endothelial (Figure 6e) cells. The fluo-ligand bound to smooth muscle cells within the media of the MRA showing circumferential orientation of the cells (Figure 6c). Binding of BODIPY-TMR-CGP 12177 to endothelial cells could not be seen in whole (live) vessels. However, it was clearly visualised in fixed vessel rings (Figure 6e). This is possibly because $\beta$-ARs have low expression levels on the surface of endothelial cells and this is difficult to visualise when scanning through the entire thickness of the vessel wall. Incubation of the vessels with the irreversible $\beta$-AR antagonist BAAM decreased the fluorescence in adventitia (Figure 6b), media (Figure 6d) and endothelium (Figure 6f).

Examination of smooth muscle binding was achieved using orthogonal (cross) sectioning. Within the vascular media, binding appeared to be both perinuclear and surface bound (Figure 7a inset). Resectioning the image volumes in the *yz* (Figure 7b) and *xz* (Figure 7c) axes reveals the cell profiles within the wall. In most cases, the fluorescence in individual smooth muscle cells appears either as a 'ring' or 'spot' (see arrows in Figure 7b), this is indicative of surface or intracellular binding, respectively.

In the adventitia, BODIPY-TMR-CGP 12177 binds to cells (probably fibrocytes) and elongated structures likely to be nerves (Figure 6c). The intensity of binding was reduced in the presence of BAAM (Figure 6d), indicating that these sites are $\beta$-ARs. Intense adventitial binding is also apparent when viewing the orthogonal sections in Figure 7b and c. Orthogonal sectioning did not reveal any endothelial binding, possibly due to the physical limitations of laser depth penetration and the relatively thin architecture of the endothelial cells. However, endothelial cells could be clearly visualised in the fixed cut ring sections shown in Figure 6e. Close inspection of the adventitial and endothelial cells in Figure 6a and e, respectively, shows perinuclear and thus probable intracellular binding. It is thus possible that failure to see binding on intact cells is due to the inability of the ligand to penetrate live endothelial cells.

The pattern of neuronal binding was examined by comparing the binding of the $\beta$-AR ligand (BODIPY-TMR-CGP 12177, 1 $\mu$M) with an $\alpha$-AR ligand (QAPB, 1 $\mu$M) (Figure 8). In general, the two ligands bound to the same types of structures but the relative proportions differed. QAPB bound strongly to nerves at the adventitia-medial border, identified as sympathetic by their characteristic plexus arrangement and position (Figure 8, top left). BODIPY-TMR-CGP 12177 also bound to these nerves although colocalisation with QAPB was incomplete (Figure 8, bottom left panel), suggesting different phenotypes among the sympathetic nerve terminals. In the outer adventitial layer, there are neurone-like structures, which, although binding both ligands to some extent, displayed high density of BODIPY-TMR-CGP 12177 binding (Figure 8, bottom right panel). As well as showing differential distribution of the receptors, these data confirm the lack of signal crossover and the selectivity of binding of each fluo-ligand.

## Discussion

The present study shows that $\beta$-ARs that mediate vasodilatation in the MRA belong to the $\beta_1$-AR subtype and are located
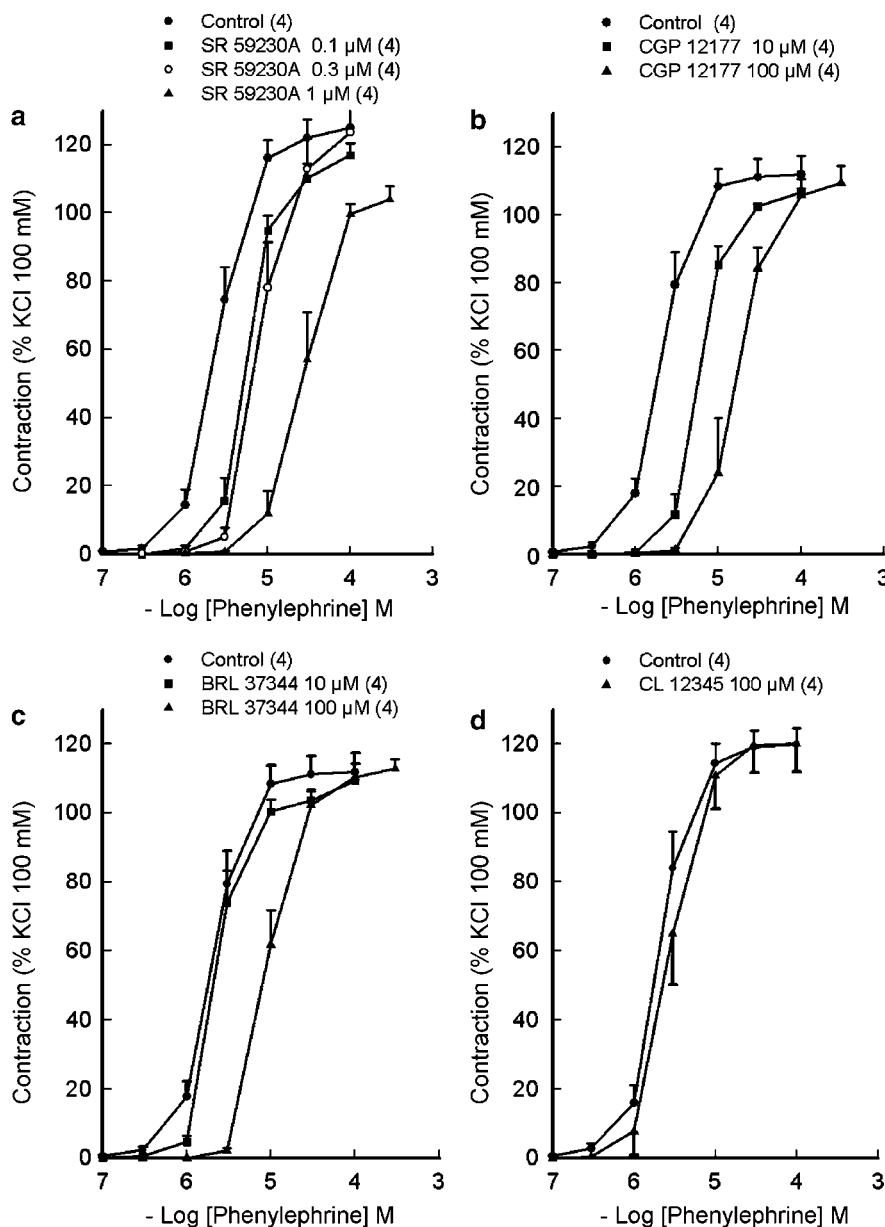
**Figure 4** Concentration response curves for phenylephrine-induced contraction in the absence and presence of (a) SR 59230A, (b) CGP 12177, (c) BRL 37344 and (d) CL 316243 in MRA. Results are expressed as mean $\pm$ s.e.m. The number of animals used is indicated in brackets. \*\*$P < 0.001$ for phenylephrine in presence *versus* absence of SR 59230A, CGP 12177A and BRL 37344 by two-way ANOVA.

intracellularly throughout the smooth muscle cell layer. Pharmacological evidence against a vasodilator effect of $\beta_2$- and $\beta_3$-ARs is reported. In addition, we have shown that the fluo-ligand BODIPY-TMR-CGP 12177 behaves as an irreversible $\beta_1$-AR antagonist (Figure 4c) with no agonist action at this receptor subtype.

Previous studies have concluded that the main population of $\beta$-ARs responsible for vasodilatation in MRA is the $\beta_1$-AR subtype (Graves & Poston, 1993; White *et al*., 2001). Nevertheless, the presence of the $\beta_2$-AR subtype has been inferred, based on the effect of salbutamol (Zwaveling *et al*., 1996). The evidence from our present study and others is heavily against this. In tissues with a high proportion of $\beta_2$-ARs (Schreurs *et al*., 1980; Granger *et al*., 1985; Priest *et al*., 1997),

salbutamol has a $pD_2$ value ranging from 7.0 to 8.3. The low $pEC_{50}$ value (5.5) of salbutamol in the MRA (Zwaveling *et al*., 1996; White *et al*., 2001; Smits *et al*., 2002; present study), as in other blood vessels (Oriowo, 1994; Shafiei & Mahmoudian, 1997), in our opinion, is not consistent with stimulation of $\beta_2$-ARs. We found that salbutamol produced relaxation of phenylephrine-induced tone, albeit 150 times less potent than isoprenaline. This was a false positive effect since salbutamol did not relax tone to other agents while isoprenaline did.

In summary, the presence of $\beta_1$- rather than $\beta_2$-ARs is based on several experimental observations: (i) the potency ratio between isoprenaline and salbutamol is more than 10 times greater than the usual potency ratio observed in tissues that express mainly $\beta_2$-ARs (Arch *et al*., 1984; Priest *et al*., 1997);
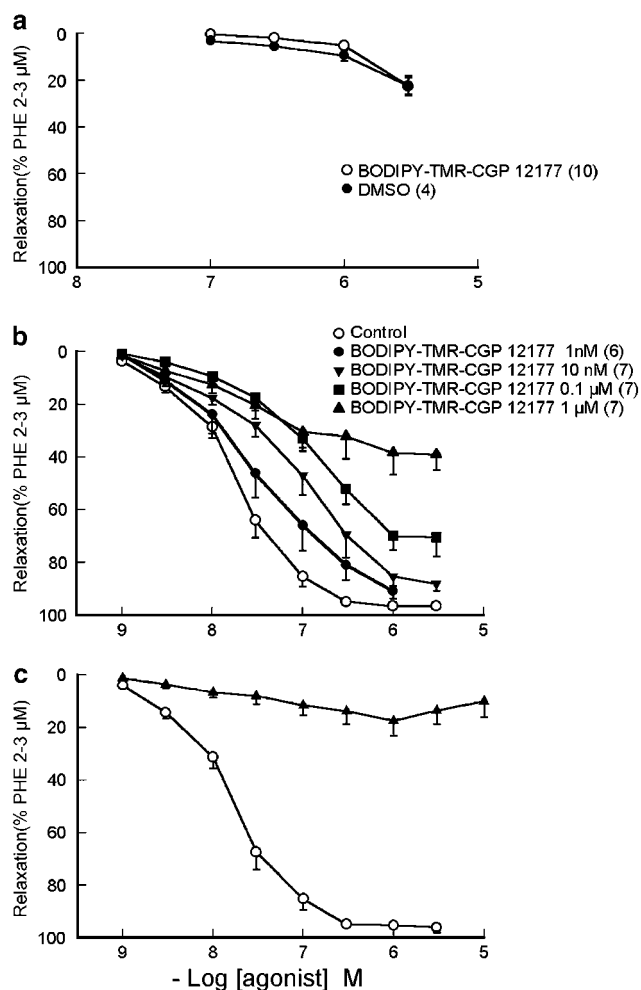
**Figure 5** Functional pharmacology of BODIPY-TMR-CGP 12177 in MRA precontracted with phenylephrine. (a) Concentration–response curves (CRC) to BODIPY-TMR-CGP 12177 and DMSO. (b) CRC to isoprenaline in the absence and presence of increasing concentrations of BODIPY-TMR-CGP 12177. (c) CRC to isoprenaline in the absence and presence of 1 $\mu$M BODIPY-TMR-CGP 12177 (30 min) followed by a washout period (every 5 min over 30 min). Results are expressed as mean $\pm$ s.e.m. The number of animals used is indicated in brackets. \*\*\*$P < 0.001$ for isoprenaline *versus* BODIPY-TMR-CGP by two-way ANOVA.

(ii) the estimated p$K_B$ values obtained for propranolol (8.17) and atenolol (6.6), a $\beta_1$-AR selective antagonist, *versus* isoprenaline CRCs were similar to those previously reported in the rat MRA (White *et al.*, 2001) and aorta (Doggrell & Henderson, 1998) and agree with the recently reported (Baker, 2005) p$K_D$ for both agonists at $\beta_1$-ARs; (iii) ICI 118551, a highly selective $\beta_2$-AR antagonist (Baker, 2005) did not modify the CRC to isoprenaline and (iv) no relaxation to salbutamol was found when the precontractile agent was U 46619 or 5-HT instead of phenylephrine.

We found 'false positive' evidence for $\beta_3$-ARs that can be explained by $\alpha$-AR antagonist effects of some key $\beta_3$-AR ligands, as demonstrated by Brahmadevara *et al.* (2004) in rat aorta. BRL 37344, a compound employed as a selective $\beta_3$-AR agonist (Arch *et al.*, 1984), and CGP 12177, an 'atypical $\beta$-AR' partial agonist with $\beta_1$- and $\beta_2$-AR antagonistic properties (Mohell & Dicker, 1989), behaved as would be expected of full agonists in respect of relaxation of phenylephrine-induced tone. As potential $\beta_3$-AR agonists, these compounds are less potent in MRA (present study) and in other blood vessels (Mohell & Dicker, 1989; Oriowo, 1994; 1995; Sooch & Marshall, 1997; Trochu *et al.*, 1999; Brawley *et al.*, 2000a; Brahmadevara *et al.*, 2003; Matsushita *et al.*, 2003) than in the colon (Bloom *et al.*, 1992; Kaumann & Molenaar, 1996; Brahmadevara *et al.*, 2003). In addition, CL 316243, a potent and selective $\beta_3$-AR agonist (Kaumann & Molenaar, 1996; Manara *et al.*, 1996; Baker, 2005) failed to relax phenylephrine preconstricted vessels, reflecting a similar observation in rat aorta (Brahmadevara *et al.*, 2003).

In the present study, the CRC to BRL 37344 and CGP 12177 was antagonised by low concentrations of SR 59230A, a condition that seems to fit with the presence of $\beta_3$-ARs (Manara *et al.*, 1996). Brahmadevara *et al.* (2003) reported an inhibition by SR 59230A of the phenylephrine-induced tone in rat aorta suggesting an antagonism of $\alpha_1$-ARs. We found that phenylephrine CRC was shifted to the right by SR 59230A indicating antagonism of $\alpha_1$-ARs. CGP 12177 and BRL 37344 but not CL 12177A also exhibited $\alpha_1$-AR antagonism. These results are very similar to those observed in rat aorta (Brahmadevara *et al.*, 2004) and intrapulmonary artery (Leblais *et al.*, 2004). Note that in rat aorta, the $\alpha_1$-ARs are mainly $\alpha_{1D}$ and in MRA there are mainly $\alpha_{1A}$. Therefore, these compounds are nonsubtype selective $\alpha_1$-AR antagonists.

It has been suggested that the effects of $\beta$-AR agonists differ according to the agent used to contract the vessel. In the present study, the '$\beta_3$-AR agonists', BRL 37344 and CGP 12177, but not CL 316243, relaxed phenylephrine-induced tone *via* antagonism of $\alpha_1$-ARs. Consequently, their failure to relax contraction induced by U 46619 or 5-HT indicates an absence of $\beta_3$-ARs. Similarly with salbutamol, low potency relaxation of phenylephrine-induced tone might have implied $\beta_2$-ARs but failure to relax tone to other agents adds to the evidence for a lack of $\beta_2$-ARs. To our knowledge, there is no contrary evidence in this vessel. In the main mesenteric artery, the presence of atypical $\beta$-ARs and the lack of $\beta_3$-ARs have been reported (Kozlowska *et al.*, 2003).

There is long-standing controversy over the possibility that the endothelium might influence $\beta$-AR-mediated vasorelaxation. This is not straightforward because there are several putative endothelial factors that can be released and can influence smooth muscle tone, and there is a further possibility that an action on smooth muscle can be transmitted chemically or electrically to endothelial cells, which, in turn, can release factors that influence the smooth muscle.

The general paradigm in resistance arteries is that endothelium-derived hyperpolarizing factor (EDHF) together with NO plays an important role on the response to vasodilators (Hwa *et al.*, 1994; Shimokawa *et al.*, 1996; Sekiguchi *et al.*, 2002; Chauhan *et al.*, 2003). With regard to the $\beta$-AR-mediated response, we first excluded an influence of NO in the action of isoprenaline by the absence of an effect of L-NAME. This is also consistent with a theoretical extrapolation of $\beta$-AR-mediated signalling, since it seems unlikely that activation of adenylyl cyclase, the (so far) ubiquitous mechanism for $\beta$-AR, would depolarise the endothelial cells and release endothelial factors. Nevertheless, we did find that the vasorelaxation by isoproterenol was attenuated by physical damage to the endothelium.
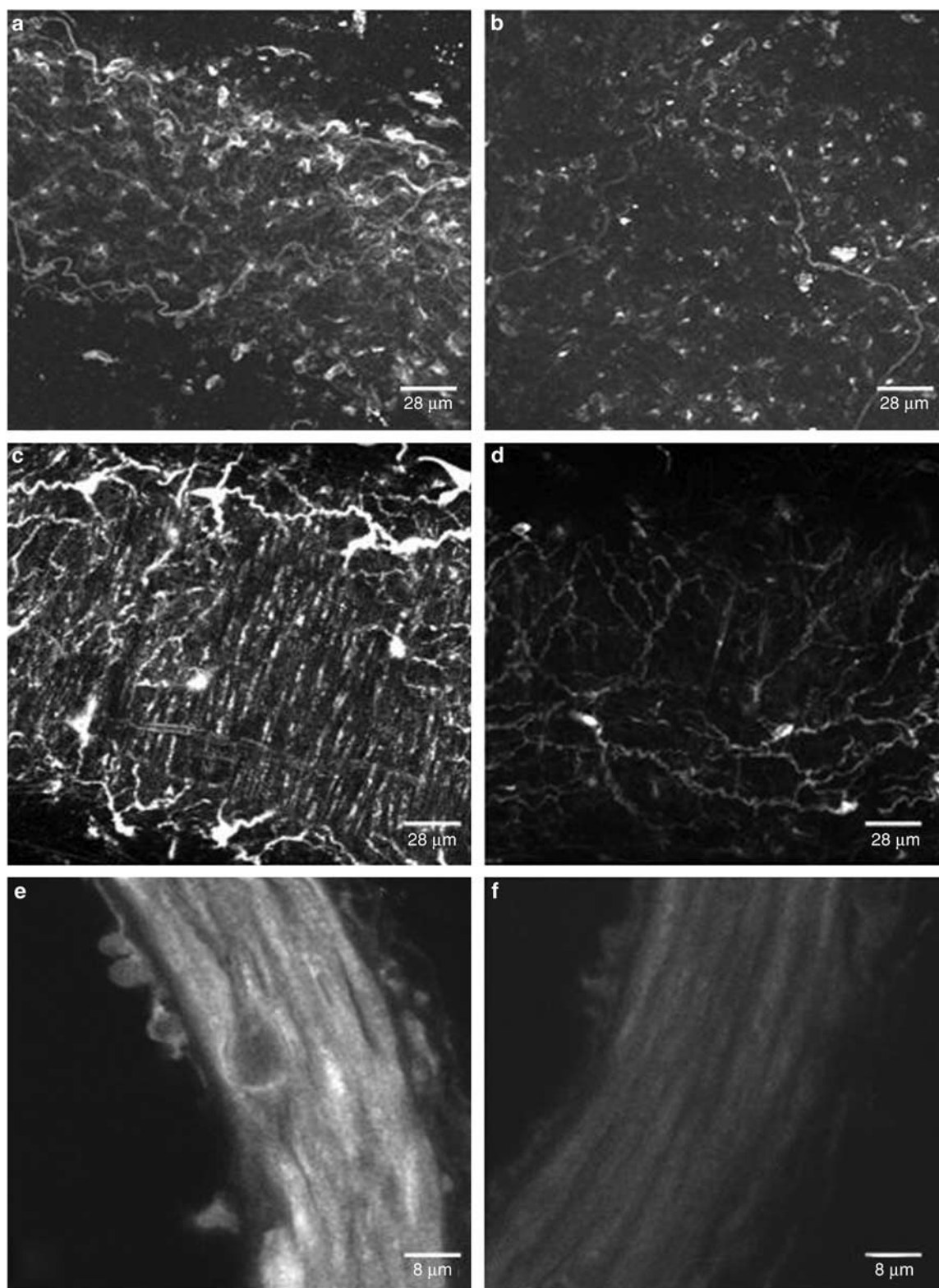
**Figure 6** Binding of BODIPY-TMR-CGP 12177 (1 $\mu$M) to the adventitial (a, b), smooth muscle (c, d) and endothelial (e, f) cells in the absence (left pannels) and presence (right pannels) of BAAM (10 $\mu$M). Smooth muscle derived fluorescence (c, d) is apparent as elongated structures running vertically in the images. Images were constructed by assembling serial (confocal) sections, of the vascular media, and are displayed as extended focus views using Metamorph image processing software.

A decrease in the relaxation responses to $\beta$-AR agonists after removal of endothelium has been used to argue for the presence of endothelial $\beta$-ARs (Brawley *et al.*, 2000b; Vanhoutte, 2001). However, endothelial cells can release several vasoactive factors, sometimes spontaneously and thus, when the endothelium is removed, the loss of basal release of
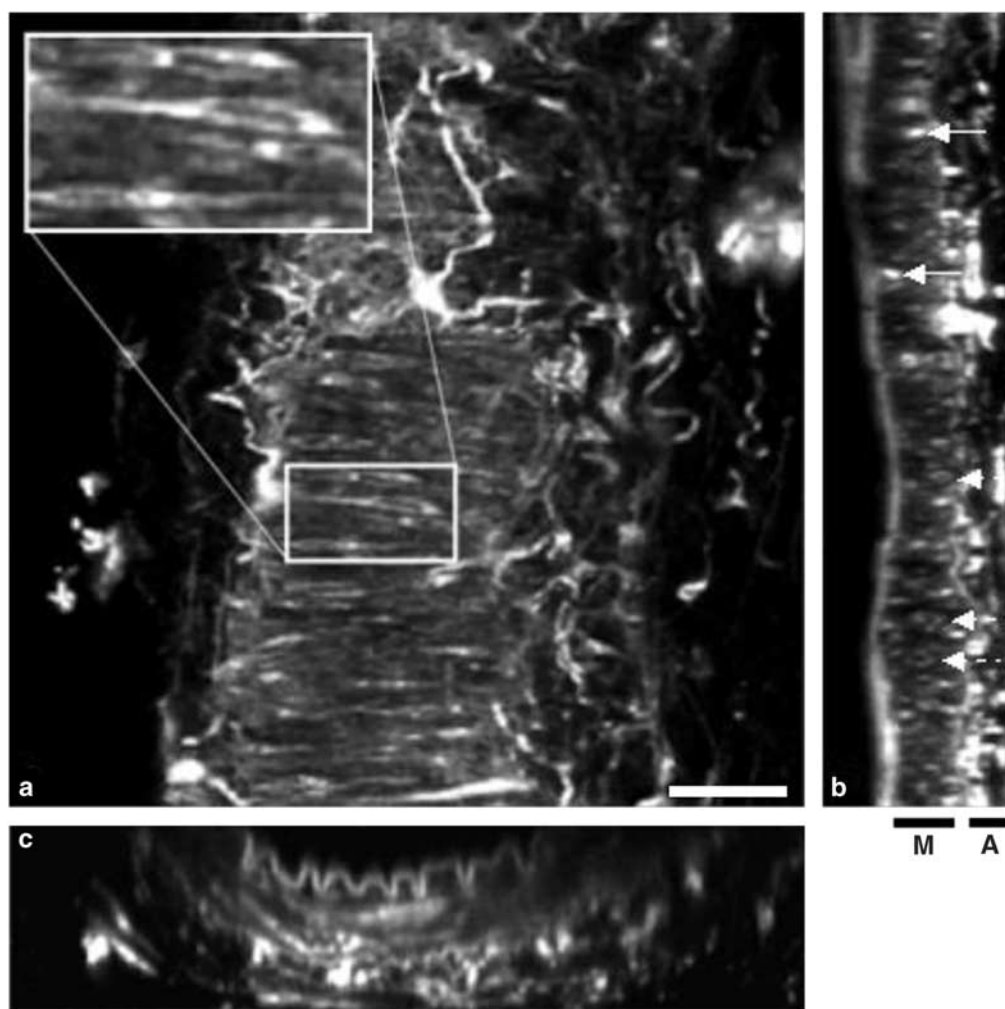
**Figure 7** Orthogonal sectioning of a rat MRA in the presence of BODIPY-TMR-CGP 12177 (1 $\mu$M). (a) The image shows a single *xy* plane of a full data volume (image *z*-series). A magnification of one selected area shows CGP binding in a perinuclear region. (b) The *yz* section is equivalent to a virtual transverse section of the wall and shows the profile of medial smooth muscle cells. A 'ring' of fluorescence (dashed arrow) is indicative of binding to the smooth muscle cell surface. Bright spots (full arrow) indicate intracellular binding. (c) The *xz* section is equivalent to a virtual transverse cross-section showing the convoluted internal elastic lamina and fluorescence binding in smooth muscle cells close to the adventitia and intima. Strong adventitial binding is apparent on both orthogonal sections. Calibration bar represents 50 $\mu$m.

endothelial factors could influence vascular tone. To test this concept, we studied the effects of a wide range of vasorelaxant agents (data not shown) in this preparation and consistently found that vasodilator effects were attenuated by endothelial destruction, even including such nonspecific agents as papaverine (Figure 2b). This suggests a nonspecific constitutive influence of the endothelium on the smooth muscle cells rather than an endothelial $\beta$-AR-mediated action. Thus, although we have demonstrated the presence of $\beta$-AR on endothelium using BODIPY-TMR-CGP 12177, we believe that the function of these receptors remains unknown. In contrast, the presence of a $\beta$-AR-mediated vasodilatation that survived endothelial destruction provides positive evidence for a vasodilator function for the $\beta$-AR that we visualised on vascular smooth muscle.

One objective of this study was to show the cellular distribution of $\beta$-ARs through the vessel wall since, surprisingly, this is not known in any detail. The fluo-ligand, BODIPY-TMR-CGP 12177, has been shown to be a long-

acting $\beta_2$-AR agonist in cells expressing $\beta_2$-AR (Baker *et al*., 2003) but its other pharmacological properties have not been analysed. It is essential, when using a fluoligand, to know its affinity and selectivity for the receptors of interest (Daly & McGrath, 2003). In our hands, BODIPY-TMR-CGP 12177 was without effect on phenylephrine precontracted MRA indicating that, in this tissue, it does not behave as a $\beta$-AR agonist or as an $\alpha_1$-antagonist. Furthermore, since it has been shown to be a $\beta_2$-AR agonist (Baker *et al*., 2003), we can reinforce the concept that this preparation does not respond to $\beta_2$-AR agonists. Relaxation by isoprenaline was significantly inhibited by BODIPY-TMR-CGP 12177 in a concentration-dependent manner. These results, together with the blockade of isoprenaline-induced relaxation after incubation and wash out of 1 $\mu$m BODIPY-TMR-CGP 12177 (Figure 4a), indicate that this compound behaves as a slowly dissociating antagonist at $\beta_1$-ARs (and in practical terms is irreversible). The absence of smooth muscle cell fluorescence induced by BODIPY-TMR-CGP 12177 in the presence of, or after incubation
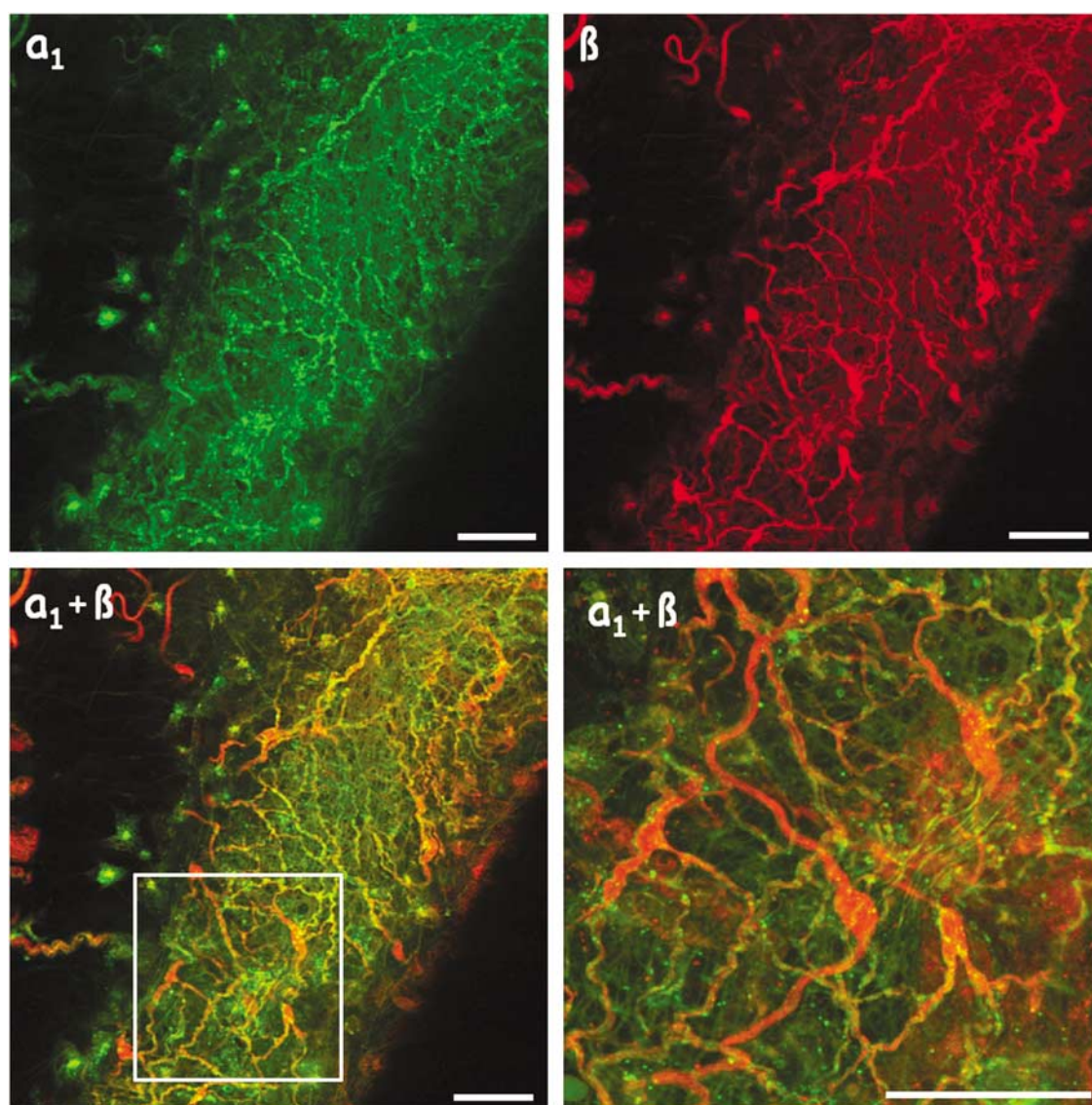
**Figure 8** Combined binding of QAPB and BODIPY-TMR-CGP 12177. Top panels show the binding of the fluorescent $\alpha$-AR ligand (QAPB 1 $\mu$M; left) and BODIPY-TMR-CGP 12177 (1 $\mu$M; right) to a segment of rat MRA. The combination of both top panels (bottom left and right) shows the degree of colocalisation of the fluorescent ligands, which if coincident shows as yellow. Bottom left panel shows the colocalisation image of the two top panels (showing the inner most region of adventitia). Bottom right panel show a colocalisation image (at higher magnification) of the region outlined in the bottom left. This magnified view is composed of image planes comprising the inner and outer layers of adventitia, in order to incorporate the most superficial nerves. All images were collected using confocal microscopy and represent an 'extended focus' view where several planes are merged. Calibration bar represents 50 $\mu$m.

with, the irreversible $\beta$-AR antagonist BAAM shows that the binding is specific for $\beta$-ARs. Further evidence for the selectivity of BODIPY-TMR-CGP 12177 comes from its relative lack of colocalisation with the fluorescent ligand for $\alpha_1$-ARs (QAPB). Since BODIPY-TMR-CGP 12177 antagonised the vasodilator effect of isoprenaline at $\beta_1$-ARs, the fluorescence indicates the presence of the functional $\beta_1$-ARs on smooth muscle cells.

$\beta_1$-ARs were found on smooth muscle cells throughout the vascular medial layer with no obvious bias according to depth of location. However, in some cases, a particularly high density of binding was observed in individual smooth muscle-like cells, which requires further study. This level of detail had not previously been available. It was also possible to take the resolution to the subcellular level. This showed that the $\beta_1$-ARs

were located in a general distribution across the surface of the cells. There was a bias towards the central portion of the cells that may simply reflect that this is the thickest part of the cell. Receptors were also present inside the cells, particularly in the perinuclear region. This configuration is not uncommon and can be seen for both $\alpha$- and $\beta$-ARs as well as other receptor subtypes (Daly & McGrath, 2003). Agonist-induced cycling of $\beta$-AR in single cells is well documented (Von Zastrow & Kobilka, 1994). Therefore, it is not surprising that $\beta$-ARs are found at intracellular locations in vascular smooth muscle cells under normal conditions. QAPB is taken up into live cells by spontaneous endocytosis attached to $\alpha_{1A}$-AR (Pediani *et al.*, 2005), so it is possible that this applies also to the $\beta$-AR ligand, explaining its intracellular location in live cells.

The binding of BODIPY-TMR-CGP 12177 to endothelial cells and adventitial cell bodies was susceptible to the irreversible $\beta$-AR antagonist BAAM. This is strong evidence for the presence of $\beta$-AR in these locations. The subtype is not known since the ligand has affinity for both $\beta_1$-AR (present study) and $\beta_2$-AR (Baker *et al.*, 2003).

We discussed above our view that our functional pharmacological data produced no evidence in favour of a contribution of endothelial $\beta$-AR to the vasodilator action of isoprenaline. These receptors may, therefore, have another function. A long-standing literature suggests that endothelial $\beta$-AR may have a role in modulating vascular permeability (Zink *et al.*, 1993; Allen & Coleman, 1995; Weneger *et al.*, 1998). Thus, we can hypothesize that the functional role of endothelial $\beta$-ARs demonstrated with BODIPY-TMR-CGP 12177 would be related to vascular permeability rather than vasodilatation. The endothelial cells also have $\alpha$-AR that can be demonstrated with the fluorescent ligand QAPB. In that case, an analysis using receptor knockout mice and selective antagonists showed that these receptors comprise both $\alpha_1$-AR and $\alpha_2$-AR (Shafaroudi *et al.*, 2005). Since genetic manipulation is not available in the present rat study, it is safer to classify the receptors to which QAPB binds as $\alpha$-AR without further subdivision.

The presence of $\beta$-AR in the adventitia is a new observation. We recently described the presence of $\alpha$-AR in adventitial cells and on nerve terminals of mouse first-order mesenteric arteries (McGrath *et al.*, 2005). The present observations extend this to rat third-order mesenteric arteries and show that these possess both $\alpha$-AR and $\beta$-AR. The structures involved include freestanding fibrocyte/fibroblasts and two distinct types of nerve-like structure. One type of nerve is clearly the plexus of sympathetic nerve terminals that lies at the adventitia–medial interface. Here, the two-receptor types are present to some extent on most terminals and on a substantial population they are well colocalised. These presumably are the autoreceptors well known for modulation of transmitter release. The other structures consist of long processes, two or more of which are attached to their cell bodies. These lie further out in the adventitia and envelop the artery, running for considerable distances both around the circumference and along the length. These are possibly sensory nerves. Resistance arteries possess sensory nerves in the outer adventitia that may detect changes in the vessel's dimensions and take part in a local reflex adjustment (Scotland *et al.*, 2004) involved in the 'myogenic' response. If so, the presence of adrenoceptors, particularly $\beta$-AR, suggests adrenergic modulation of these reflexes and hence of autoregulation of blood flow.

In conclusion, in rat MRA, relaxation mediated by $\beta$-ARs is due mainly to the stimulation of $\beta_1$-ARs present throughout the smooth muscle cell layer, whose population has a partial intracellular location. Evidence against the participation of $\beta_2$- and $\beta_3$-ARs is also reported. We propose that the focus of research on $\beta$-AR, as regards vascular resistance, should be on smooth muscle $\beta_1$-ARs and should take their subcellular location into account. We also demonstrate the presence of adventitial, neuronal and endothelial $\beta$-ARs. This deserves further investigation.

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