

RESEARCH PAPER

$\alpha_{1A/B}$ -Knockout mice explain the native α_{1D} -adrenoceptor's role in vasoconstriction and show that its location is independent of the other α_1 -subtypes

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Background and purpose: Theoretically, three α_1 -adrenoceptor subtypes can interact at the signalling level to alter vascular contraction or at the molecular level to alter each other's cellular location. The $\alpha_{1A/B}$ -adrenoceptor knockout mouse ($\alpha_{1A/B}$ -KO) was used to study the isolated α_{1D} -adrenoceptor to consider these potential interactions in native tissue.

Experimental approach: Pharmacological analysis of carotid and mesenteric arteries employed wire myography and fluorescent ligand binding (α_1 -adrenoceptor ligand BODIPY FL-prazosin, QAPB).

Key results: $\alpha_{1A/B}$ -KO carotid had clear α_{1D} -adrenoceptor-induced contractions. In WT carotid α_{1D} -adrenoceptor dominated but all three α_1 -subtypes participated. $\alpha_{1A/B}$ -KO mesenteric had α_{1D} -adrenoceptor responses with high sensitivity and small maximum, explaining how α_{1D} -adrenoceptor could determine agonist sensitivity in WT. In both arteries $\alpha_{1A/B}$ -KO fluorescence levels were reduced but pharmacologically more consistent with 'pure' α_{1D} -adrenoceptors. α_{1D} -Adrenoceptor binding in $\alpha_{1A/B}$ -KO was observed on the cell surface and intracellularly and was present in a high proportion of smooth-muscle cells in both strains, regardless of artery type.

Conclusions and implications: 'Pure' α_{1D} -adrenoceptor pharmacology in $\alpha_{1A/B}$ -KO provides a quantitative standard. Functionally, the α_{1D} - and α_{1A} -adrenoceptors produce additive responses and do not significantly compensate for each other. α_{1D} -Adrenoceptor contributes to sensitivity even in resistance arteries. In $\alpha_{1A/B}$ -KO, the loss of α_{1A} - and α_{1B} -adrenoceptors is reflected by a general decrease in fluorescence, but similar binding distribution to WT indicates that the α_{1D} -adrenoceptor location in native smooth-muscle cells is not influenced by other α_1 -adrenoceptors. Equivalent levels of receptors did not correspond to equivalent responses. In conclusion, α_1 -subtypes do not interact but provide independent alternative signals for vascular regulation.

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Abbreviations: A - 61603, N - (5 - [4, 5 - dihydro - 1H - imidazol - 2 - y] - 2 - hydroxy - 5, 6, 7, 8 - tetrahydronaphthalen - 1 - yl)methanesulphonamide; $\alpha_{1A/B}$ -KO, $\alpha_{1A/B}$ -adrenoceptor knockout; BMY 7378, 8-(2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl)-8-azaspiro[4.5]decane-7,9-dione dihydrochloride; KO, knockout; QAPB, BODIPY FL-prazosin; RS100-329, 5-methyl-3-[3-[4-[2-(2,2,2-trifluoroethoxy)phenyl]-1-piperazinyl]propyl]-2,4-(1H,3H)-pyrimidinedione hydrochloride; WT, wild type

Introduction

Three α_1 -adrenoceptor subtypes (α_{1A} , α_{1B} and α_{1D}) exist, as defined by pharmacological analysis and molecular cloning (Alexander *et al.*, 2008). With more than one receptor present in each tissue (Hrometz *et al.*, 1999; Daly *et al.*, 2002; Deighan *et al.*, 2005) and the limited number of highly selective

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compounds available, the analysis of the α_1 -adrenoceptors in native tissue, and ultimately their role in adrenergic regulation of blood pressure, is complex.

α_{1D} -Adrenoceptor-mediated contraction has been examined previously in arteries in which it predominates, such as the mouse carotid (Daly *et al.*, 2002; Deighan *et al.*, 2005) and aorta (Yamamoto and Koike, 2001; Daly *et al.*, 2002). However, the presence of the other α_1 -subtypes may influence potency estimates of agonists and antagonists in these preparations. In arteries in which another α_1 -subtype predominates, the contribution of the α_{1D} -adrenoceptor can be obscured. By isolating the α_{1D} -adrenoceptor in the double α_{1A}/α_{1B} -adrenoceptor knockout mouse ($\alpha_{1A/B}$ -KO), the present study will examine its role as a 'minor' subtype in the mesenteric artery, in addition to obtaining potency values for agonists and antagonists at the dominant α_{1D} -adrenoceptor in the carotid.

Some studies of receptor localization in isolated cells have found that, while receptors are found both on the external plasma membrane and in the endoplasmic reticulum, the main cellular location of the α_{1A} - and α_{1D} -adrenoceptors is intracellular (Mackenzie *et al.*, 2000; Chalothorn *et al.* 2002; Hague *et al.* 2004; Wright *et al.* 2008) and the α_{1B} -adrenoceptor is predominantly found on the cell surface (McCune *et al.*, 2000; Hague *et al.*, 2004). However, other studies in isolated cells have shown that all three α_1 -subtypes are located at both sites with no apparent differences (Daly *et al.*, 1998; Hrometz *et al.*, 1999; Pediani *et al.*, 2000; Morris *et al.*, 2004). Thus, there is no obvious guide as to what to expect in native tissues. We recently showed that receptor localization can be studied in native arterial tissue by using fluorescent ligand binding and that, in live, unfixed arteries from the $\alpha_{1B/D}$ -KO, the α_{1A} -adrenoceptor was present on the cell surface and intracellularly (Methven *et al.*, 2009). Of the three α_1 -adrenoceptors, our understanding of the cellular location of the α_{1D} -adrenoceptor has been most limited. The present study provides the opportunity to clarify this and relate receptor localization to function. This is particularly important because immunocytochemistry has not been proved of great utility for α_1 -adrenoceptor localization, and two recent papers have challenged their specificity (Jensen *et al.*, 2009; Pradidarcheep *et al.*, 2009).

Thus, the aim of the present study was to employ the $\alpha_{1A/B}$ -KO to establish the pharmacological properties of the α_{1D} -adrenoceptor in native tissue and, by referral to the wild-type mouse (WT), whether the other α_1 -subtypes interact with the α_{1D} -adrenoceptor to alter vascular contraction or cellular location.

Methods

Animals used and vessel preparation

All animal care and this investigation conformed to the provisions of the UK Animals (Scientific procedures) Act 1986. Experimental protocols and rationale have been described in detail previously (Methven *et al.*, 2009).

$\alpha_{1A/B}$ -KO mice were generated by cross-breeding single knockout mice of the α_{1A} -adrenoceptor (α_{1A} -KO) and α_{1B} -adrenoceptor (α_{1B} -KO), as described previously (O'Connell

et al., 2003). WT littermates were used as control mice, and all mice were of C57Bl6/J background and were bred and genotyped at the University of Glasgow.

It should be noted that pregnancy rate in knockouts of the α_{1A} -adrenoceptor was markedly reduced. This is in agreement with Sanbe *et al.* (2007), who reported that the reduction in pregnancies was a result of male infertility, by impaired contraction of the vas deferens, and ruled out sperm abnormalities or differences in sexual behaviour as potential causes.

Male mice, 4–5 months old, weighing between 27 and 46 g (WT: 30–38 g; α_{1A} -KO: 29–41 g; α_{1B} -KO: 31–46 g; $\alpha_{1A/B}$ -KO: 27–45 g), were used. Mice were maintained on a 12:12-h light/dark schedule at 22–25°C with 45–65% humidity and fed *ad libitum* on a standard rodent diet and provided drinking water. The mice were killed by carbon dioxide asphyxiation before first-order mesenteric, and carotid arteries were isolated by using a dissection microscope.

Experimental protocols for functional pharmacology

Two-millimetre sections of artery were mounted in physiological salt solution at 37°C on a four-chamber wire myograph (Danish Myotechnology, Aarhus, Denmark). Following equilibration periods and application of 250-mg passive tension, a start-up procedure using 10 μ M of one of the three agonists [the 'subtype non-selective' α_1 -adrenoceptor agonist, phenylephrine; an agonist 'selective' for the α_{1A} -adrenoceptor, A-61603 (N-(5-[4,5-dihydro-1H-imidazol-2-yl]-2-hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl)methanesulphonamide) (Knepper *et al.*, 1995); or 5-hydroxytryptamine] was performed. The viability of the endothelium was tested by using 3 μ M acetylcholine. Data were recorded by using Powerlab and Chart (version 5.0) (ADInstruments, Chalgrove, UK).

Cumulative concentration–response curves were produced for each agonist (half-log increments 1 nM to 30 μ M or 100 μ M). Subtype selective antagonists were incubated for 30 min: prazosin 1 nM, 10 nM and 100 nM (non-selective α_1 -adrenoceptor antagonist); BMY7378 (8-(2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl)-8-azaspiro[4.5]decane-7,9-dione dihydrochloride) 10 nM, 30 nM and 100 nM [α_{1D} -adrenoceptor-selective antagonist (Goetz *et al.*, 1995; Saussy *et al.* 1996)]; and RS100-329 (5-methyl-3-[3-[4-[2-(2,2,2-trifluoroethoxy)phenyl]-1-piperazinyl]propyl]-2,4-(1H,3H)-pyrimidinedione hydrochloride) 10 nM and 100 nM (α_{1A} -adrenoceptor-selective antagonist (Williams *et al.*, 1999).

Experimental protocols for visualization of smooth muscle cell α_1 -adrenoceptors

BODIPY FL-prazosin (QAPB) incubations. In the WT and $\alpha_{1A/B}$ -KO, 3- to 5-mm segments of live unfixed arteries were incubated with QAPB (100 nM for 120 min) at room temperature (21°C).

Non-fluorescent antagonists were used to compete for QAPB binding sites in segments from the same animals as QAPB controls. Once QAPB equilibrium was established (60 min Pediani *et al.*, 2005), segments were coincubated with an antagonist for 60 min: prazosin (100 nM); rauwolscine (100 nM); RS100-329 (100 nM) or BMY7378 (100 nM). The concentration of each antagonist was consistent with the

concentration of those selected previously (Methven *et al.*, 2009).

Arteries were mounted in the incubation solutions with a coverslip (No. 1.5) on top. The mesenteric artery was mounted intact, while the carotid artery was sliced open with a single-edged razor blade and laid flat (Miquel *et al.*, 2005).

Optical sections were collected on a Bio-Rad Radiance 2100 Confocal Laser Scanning System (Bio-Rad, Hemel Hempstead, Hertfordshire, UK) at an excitation/emission of 488/515 nm for QAPB. Arteries were visualized by using $\times 40$ oil immersion objective (numerical aperture 1.0). Optimal laser intensity, gain (contrast) and offset (brightness) were determined and then kept constant for each artery. Photomultiplier sensitivities were set such that all pixels fell within the range 0 (black) to 255 (white). Carotid arteries were imaged at high power (zoom 8) to include only smooth-muscle cells and avoid areas containing elastic lamina in the media. Mesenteric artery images were collected at low power (zoom 3), which allows coverage of a wider area of exclusively smooth-muscle cells. Arteries were scanned by Z-series (0.5 μm slices) from the internal elastic lamina through the media, producing a stack of approximately 20–30 μm in depth. Each procedure was carried out in triplicate on four different mice per strain.

Data analysis and statistical procedures

All graphical and statistical analysis was performed by using Graph Pad Prism (version 5.0, Graphpad Software, San Diego, CA, USA). Mean data were compared across mouse strains and in the presence and absence of the selected antagonists by using either one-way ANOVA and Bonferroni's *post-test* or Student's *t-test*.

Functional pharmacology. Data are expressed as a percentage of the control curve maximum and represent the mean value \pm SEM. When a maximum response was achieved, pEC_{50} values were calculated for control curves and agonist curves in the presence of antagonists.

Quantitative confocal microscopy. Image analysis was performed as described in Methven *et al.* (2009) using Meta-morph software (version 6.1, Molecular Devices, Downingtown, PA, USA). A single plane at a depth of 4–6 μm from the internal elastic lamina of each z-series was selected for analysis to capture only smooth-muscle cells. Average pixel intensity was measured for the entire plane (96 \times 96 μm

for mesenteric arteries; 36 \times 36 μm for carotid arteries) and individual smooth-muscle cells (smooth-muscle cells were outlined and measurements made as a Region of Interest). The average pixel intensity correlated to a greyscale range of 0 (black) to 255 (white), with intermediate intensities being assigned an appropriate grey level.

Drugs and chemicals

Physiological salt solution was of the following composition (in mM): 119 NaCl, 4.7 KCl, 1.2 $\text{MgSO}_4 \cdot \text{H}_2\text{O}$, 1.2 KH_2PO_4 , 24.9 NaHCO_3 , 2.5 CaCl_2 and 11.1 glucose.

All analytical grade drugs were dissolved and diluted to the required concentration in distilled water (unless otherwise stated). Drugs supplied by Sigma Aldrich (Poole, UK) were phenylephrine, acetylcholine, prazosin, 5-hydroxytryptamine, BMY 7378 (8-(2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl)-8-azaspiro[4.5]decane-7,9-dione dihydrochloride), by Tocris (Bristol, UK) A-61603 (N-(5-[4,5-dihydro-1H-imidazol-2-yl]-2-hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl)methanesulphonamide), RS100-329 (5-methyl-3-[3-[14-(2-(2,2,2-trifluoroethoxy)phenyl)-1-piperazinyl]propyl]-2,4-(1H,3H)-pyrimidinedione hydrochloride), rauwolscine, and by Invitrogen (Paisley, UK) QAPB; this was dissolved in dimethyl sulfoxide and diluted in freshly gassed physiological salt solution at room temperature of 21°C.

All drug and molecular target nomenclature conforms to *The British Journal of Pharmacology's* 'Guide to Receptors and Channels' (Alexander *et al.*, 2008).

Results

Functional pharmacology

Carotid arteries. In the WT and all three knockout strains, the maximum response and sensitivity to 5-hydroxytryptamine were similar (Table 1). This eliminates generic structural or smooth muscle sensitivity changes as a basis for differences subsequently found using drugs acting via α_1 -adrenoceptors.

Phenylephrine (non-selective α_1 -adrenoceptor agonist) produced concentration-dependent contractions in all mouse strains (Figure 1). In the α_{1A} -KO and α_{1B} -KO, the maximum response was not significantly different compared with the WT but was significantly reduced in the $\alpha_{1A/B}$ -KO (Table 1). Sensitivity to phenylephrine was not significantly different between mouse strains. These data not only confirm that the

Table 1 Agonist responses in mouse carotid arteries

| Mouse | Phenylephrine | | A-61603 | | 5-HT | |
|---------------------|----------------------|----------------|----------------------|-----------------|----------------------|----------------|
| | Maximum response (g) | pEC_{50} | Maximum response (g) | pEC_{50} | Maximum response (g) | pEC_{50} |
| WT | 0.32 \pm 0.03 | 6.9 \pm 0.13 | 0.23 \pm 0.02 | 6.1 \pm 0.13 | 0.28 \pm 0.04 | 6.7 \pm 0.29 |
| α_{1A} -KO | 0.29 \pm 0.02 | 7.0 \pm 0.06 | 0.15 \pm 0.01* | 5.7 \pm 0.09* | 0.26 \pm 0.04 | 7.0 \pm 0.14 |
| α_{1B} -KO | 0.27 \pm 0.03 | 7.0 \pm 0.17 | 0.23 \pm 0.03 | 6.4 \pm 0.14 | 0.28 \pm 0.05 | 6.9 \pm 0.16 |
| $\alpha_{1A/B}$ -KO | 0.21 \pm 0.01* | 7.1 \pm 0.08 | 0.12 \pm 0.02** | 5.6 \pm 0.11* | 0.27 \pm 0.04 | 7.2 \pm 0.12 |

Data are expressed as mean \pm SEM.

* $P < 0.05$; ** $P < 0.01$ compared to WT (one-way ANOVA, Bonferroni's *post test*).

α_{1A} -KO, α_{1A} -adrenoceptor knockout; $\alpha_{1A/B}$ -KO, $\alpha_{1A/B}$ -adrenoceptor knockout; α_{1B} -KO, α_{1B} -adrenoceptor knockout; WT, wild type mice.

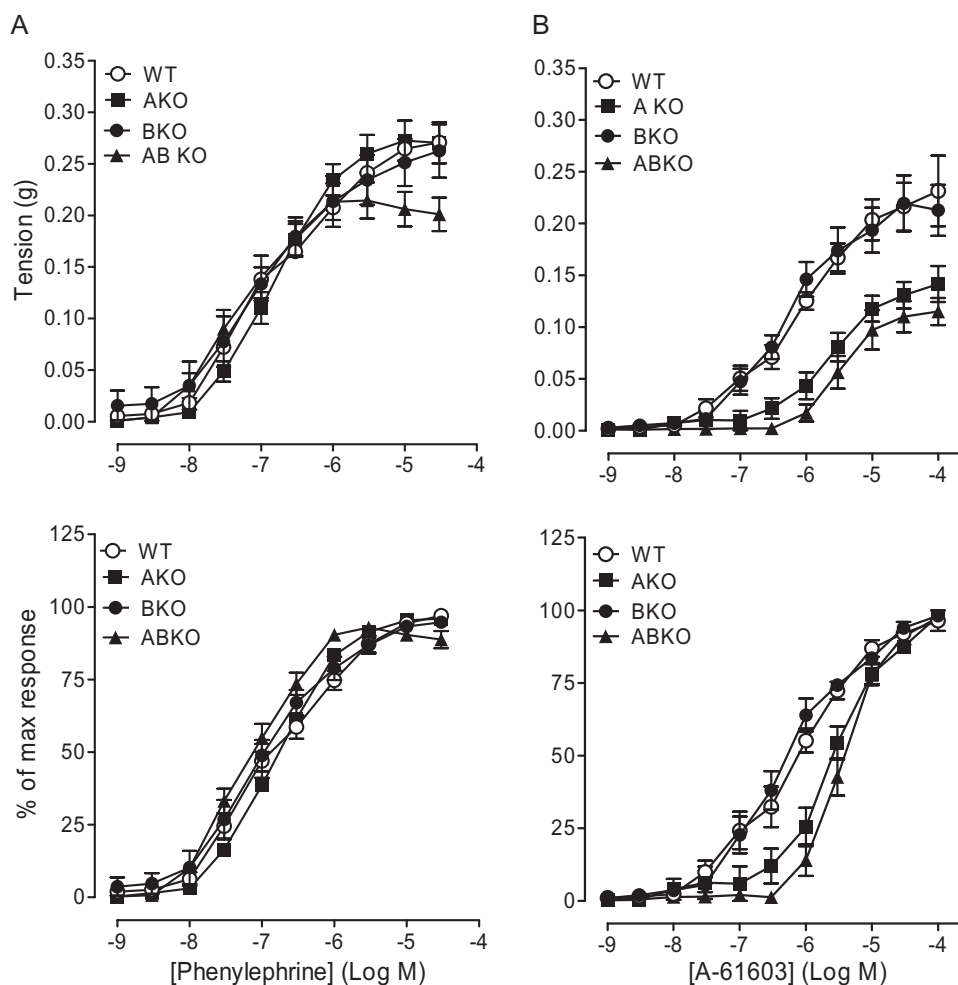


Figure 1 Contractile responses to (A) phenylephrine ($n = 6$) and (B) A-61603 ($n = 6$) in carotid arteries of wild type (WT), α_{1A} -adrenoceptor knockout (AKO), α_{1B} -adrenoceptor knockout (BKO) and $\alpha_{1A/B}$ -adrenoceptor knockout (ABKO) mice. Data points are expressed in g tension or percentage of control curve maximum as mean \pm SEM.

α_{1D} -adrenoceptor is the main contractile α_1 -adrenoceptor in the WT but also suggest that the α_{1A} - and/or the α_{1B} -adrenoceptor modulates the phenylephrine-induced response at high concentrations, although this effect was small.

A-61603-induced, concentration-related contractile response curves in all mouse strains (Figure 1). Both the maximum response and sensitivity to A-61603 were significantly reduced in the α_{1A} - and $\alpha_{1A/B}$ -KO compared with those in the WT and α_{1B} -KO (Table 1). These data suggest that the α_{1A} -adrenoceptor-selective agonist response in the WT consists of α_{1A} - and α_{1D} -adrenoceptor components.

Prazosin competitively antagonized the phenylephrine-induced response in the $\alpha_{1A/B}$ -KO, producing a pA_2 consistent with an α_1 -adrenoceptor-mediated response (Figure 2, Table 2). In the α_{1A} -KO, the phenylephrine response was inhibited by prazosin (Figure 2); 10 nM acted competitively (Table 2) and 100 nM significantly reduced the maximum response achieved within the agonist concentration range used.

BMY 7378 competitively antagonized the phenylephrine response in the α_{1A} -KO and $\alpha_{1A/B}$ -KO (Figure 3; Table 2). The high pK_B (9.0) and pA_2 (9.4) produced in the α_{1A} -KO and

$\alpha_{1A/B}$ -KO, respectively, showed that the response was mediated by α_{1D} -adrenoceptors. BMY 7378 (10 nM) produced a parallel rightward shift of the A-61603 curve in the $\alpha_{1A/B}$ -KO (Table 2), suggesting that A-61603 produces at least part of its action via α_{1D} -adrenoceptors.

In the $\alpha_{1A/B}$ -KO, the phenylephrine-induced response was not significantly affected by RS100-329 (10 nM), but 100 nM shifted the curve rightwards (Figure 3). The consequent low pK_B of 7.8 (Table 2) shows the potency of RS100-329 at the α_{1D} -adrenoceptor and is consistent with ligand binding estimates. RS100-329 100 nM was tested further against the A-61603 curve in the $\alpha_{1A/B}$ -KO but had no significant effect (Figure 3). This provides further evidence to show that the action of A-61603 in the $\alpha_{1A/B}$ -KO is via the α_{1D} -adrenoceptor.

Mesenteric arteries. 5-Hydroxytryptamine produced concentration-related contractile responses in all three knockout strains, showing no significant differences to the WT (Table 1). This suggests that there are no generic structural or smooth-muscle sensitivity changes in the knockout mice.

In the WT and α_{1B} -KO, a concentration-related contractile response to phenylephrine was observed, with a significantly

higher maximum response than in both the α_{1A} -KO and $\alpha_{1A/B}$ -KO (Figure 4; Table 3). Sensitivity to phenylephrine was significantly higher in both the α_{1A} -KO and $\alpha_{1A/B}$ -KO than in the WT and α_{1B} -KO. This not only suggests that, in the mesenteric artery, the α_{1A} -adrenoceptor is the main contractile α_1 -adrenoceptor but also indicates that the α_{1D} -adrenoceptor but not the α_{1B} -adrenoceptor has a significant contractile role at low concentrations.

Concentration-dependent contractions were obtained to A-61603 in mesenteric arteries of the WT and α_{1B} -KO, but contractions to A-61603 were produced only at high concen-

trations in the $\alpha_{1A/B}$ -KO and α_{1A} -KO (Figure 4; Table 3). Both maximum response and sensitivity to A-61603 were significantly reduced in the α_{1A} -KO and $\alpha_{1A/B}$ -KO compared with both the WT and α_{1B} -KO. This elimination of the response confirms the dominant α_{1A} -adrenoceptor-mediated contraction in this artery in the WT.

Prazosin (1 nM) caused a rightward displacement of the phenylephrine concentration–response curve without reducing the maximum response in the $\alpha_{1A/B}$ -KO (Figure 5), producing a pK_B of 10.3 indicative of an α_1 -adrenoceptor-mediated response. However, 10 nM and 100 nM produced a non-competitive blockade. Similarly, prazosin (100 nM) reduced the maximum response in the α_{1A} -KO. Because of the low potency of the phenylephrine-induced response, no further antagonist study was performed in the mesenteric artery of either the α_{1A} -KO or the $\alpha_{1A/B}$ -KO.

Visualization of α_1 -adrenoceptors using the fluorescent ligand QAPB

Carotid artery. Visually, fluorescent binding to smooth-muscle cells was comparable in the WT and $\alpha_{1A/B}$ -KO, showing a wide distribution throughout the media (Figure 6). However, the intensity of fluorescence of the $36 \times 36 \mu\text{m}$ plane was significantly reduced in the $\alpha_{1A/B}$ -KO compared with the WT (Table 4). Furthermore, the intensity of fluorescence of smooth-muscle cells, when measured separately, was significantly lower in the $\alpha_{1A/B}$ -KO than in the WT (Table 4), indicative of a general decrease in fluorescence in the knock-out strains. In both mouse strains, QAPB was observed on the cell surface and perinuclear sites, with evidence of both diffuse and punctate binding. In the $\alpha_{1A/B}$ -KO, this demonstrates that the α_{1D} -adrenoceptor is present in both locations.

The non-selective α_1 -adrenoceptor antagonist, prazosin (100 nM), competed with QAPB to significantly reduce fluorescent binding to a background level in the WT and $\alpha_{1A/B}$ -KO (Figure 6; Table 5). No significant difference in fluorescent ligand binding was detected in the presence of the non-selective α_2 -adrenoceptor antagonist rauwolscine (100 nM) (Figure 6; Table 5). These findings confirm that, at 100nM, QAPB is a selective ligand for α_1 -adrenoceptors.

At 100 nM, BMY 7378 should selectively compete for α_{1D} -adrenoceptors but not the α_{1A} - or α_{1B} -adrenoceptor. QAPB binding was significantly reduced by BMY 7378 in the WT and reduced to a background level in the $\alpha_{1A/B}$ -KO (Figure 6; Table 5), confirming that the α_{1D} -adrenoceptor is present in smooth-muscle cells of this artery in both strains and that only the α_{1D} -adrenoceptor is present in the $\alpha_{1A/B}$ -KO.

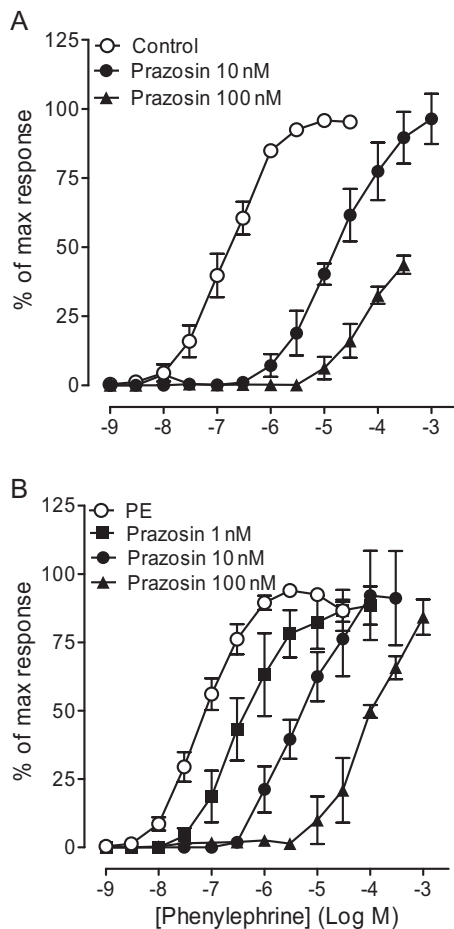


Figure 2 Effects of prazosin on contractile response to phenylephrine in carotid arteries of (A) α_{1A} -adrenoceptor knockout (α_{1A} -KO) ($n = 6$) and (B) $\alpha_{1A/B}$ -adrenoceptor knockout ($\alpha_{1A/B}$ -KO) ($n = 7$) mice. Data points are expressed as percentage of control curve maximum as mean \pm SEM.

Table 2 Potency values of antagonists against phenylephrine and A-61603 in mouse carotid arteries

| Antagonist | Phenylephrine | | | | A-61603 | |
|------------|-------------------|-------|---------------------|---------------|---------------------|-------|
| | α_{1A} -KO | | $\alpha_{1A/B}$ -KO | | $\alpha_{1A/B}$ -KO | |
| | pA_2/pK_B | Slope | pA_2/pK_B | Slope | pA_2/pK_B | Slope |
| Prazosin | 9.3 at 10 nM | – | 9.4 | 1.3 (0.9–1.6) | – | – |
| BMY 7378 | 9.0 at 100 nM | – | 9.4 | 0.8 (0.3–1.8) | 8.5 at 10 nM | – |
| RS100-329 | – | – | 7.8 at 100 nM | – | No shift at 100 nM | – |

α_{1A} -KO, α_{1A} -adrenoceptor knockout; $\alpha_{1A/B}$ -KO, $\alpha_{1A/B}$ -adrenoceptor knockout; α_{1B} -KO, α_{1B} -adrenoceptor knockout; WT, wild type mice.

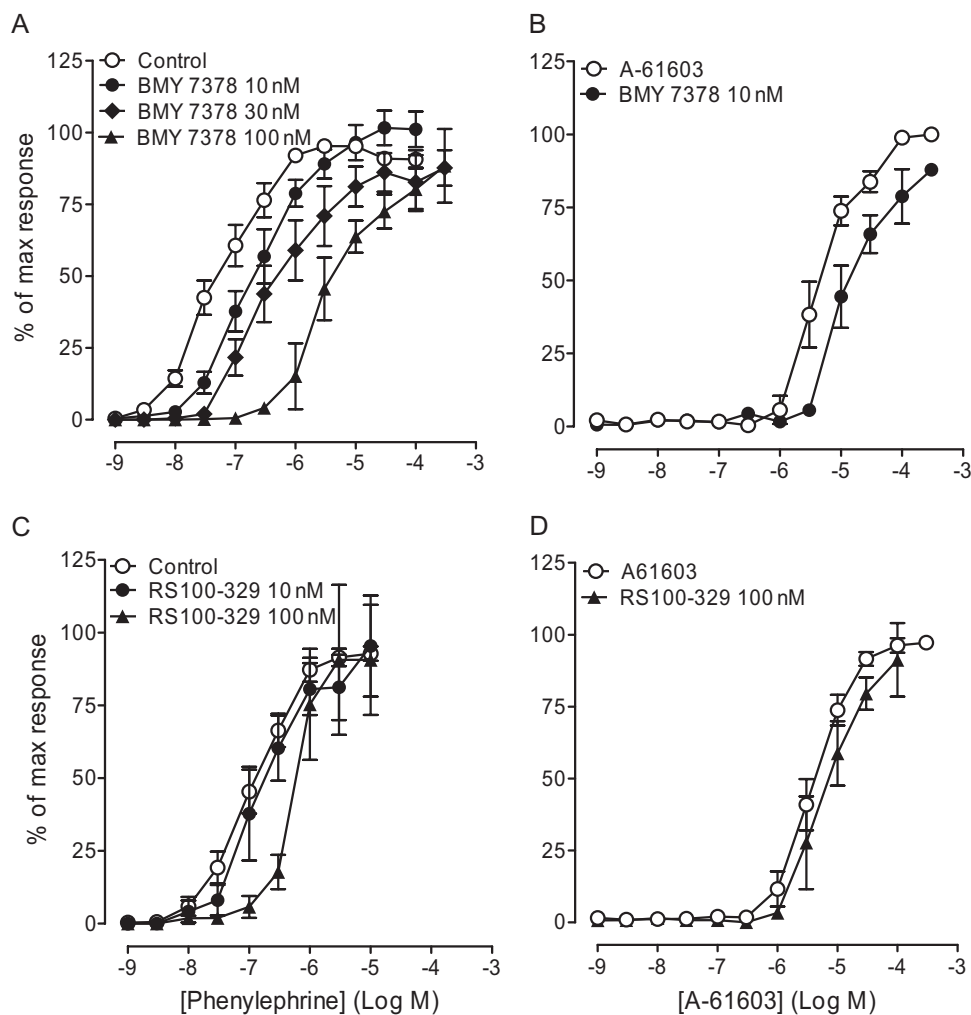


Figure 3 Effects of subtype selective antagonists in mouse carotid arteries of $\alpha_{1A/B}$ -KO. Antagonizing effect of BMY 7378 on contractile response to (A) phenylephrine ($n = 7$) and (B) A-61603 ($n = 6$). Antagonizing effect of RS100-329 on contractile response to (C) phenylephrine ($n = 6$) and (D) A-61603 ($n = 6$). Data points are expressed as percentage of control curve maximum as mean \pm SEM.

In the presence of RS100-329 (100 nM), fluorescent ligand binding was significantly reduced in the WT but not in the $\alpha_{1A/B}$ -KO (Figure 6; Table 5). This is in agreement with the α_{1A} -adrenoceptor being present in the WT and RS100-329 100 nM not having an effect on the α_{1D} -adrenoceptor.

Mesenteric artery. In the WT and $\alpha_{1A/B}$ -KO, the QAPB binding to smooth-muscle cells was similar and was observed throughout the media (Figure 7). In both strains, smooth-muscle cells showing QAPB binding fluoresced brightly, but fluorescence varied between individual cells. Quantitative analysis showed that the intensity of fluorescence of the $96 \times 96 \mu\text{m}$ plane was significantly reduced in the $\alpha_{1A/B}$ -KO compared with the WT (Table 4). The fluorescence of individual smooth-muscle cells was significantly lower in the $\alpha_{1A/B}$ -KO than in the WT (Table 4), consistent with a general decrease of fluorescence in the knockout mice. Evidence of fluorescent ligand binding was observed on the cell membrane and intracellular compartments of smooth-muscle cells, with both diffuse and punctate binding observed intracellularly.

In both mouse strains, the non-selective α_1 -adrenoceptor antagonist, prazosin (100 nM), abolished visible QAPB binding and significantly reduced measured fluorescence (Figure 7, Table 5). This, together with the finding that the non-selective α_2 -adrenoceptor antagonist, rauwolscine (100 nM), had no significant effect (Figure 7, Table 5), showed that QAPB is binding to α_1 -adrenoceptor sites on smooth-muscle cells.

Following competition with the selective α_{1D} -adrenoceptor antagonist, BMY 7378 (100 nM), QAPB binding was significantly reduced in the WT but was reduced to background levels in the $\alpha_{1A/B}$ -KO (Figure 7; Table 5), showing that the α_{1D} -adrenoceptor is present in the media of both strains of mice.

The selective α_{1A} -adrenoceptor antagonist, RS100-329 (100 nM), significantly reduced fluorescent ligand binding in the WT (Figure 7; Table 5). RS100-329 (100 nM) appeared to be selective for α_{1A} -adrenoceptor sites because the same concentration had no significant effect on QAPB binding in the absence of the α_{1A} -adrenoceptor in the $\alpha_{1A/B}$ -KO.

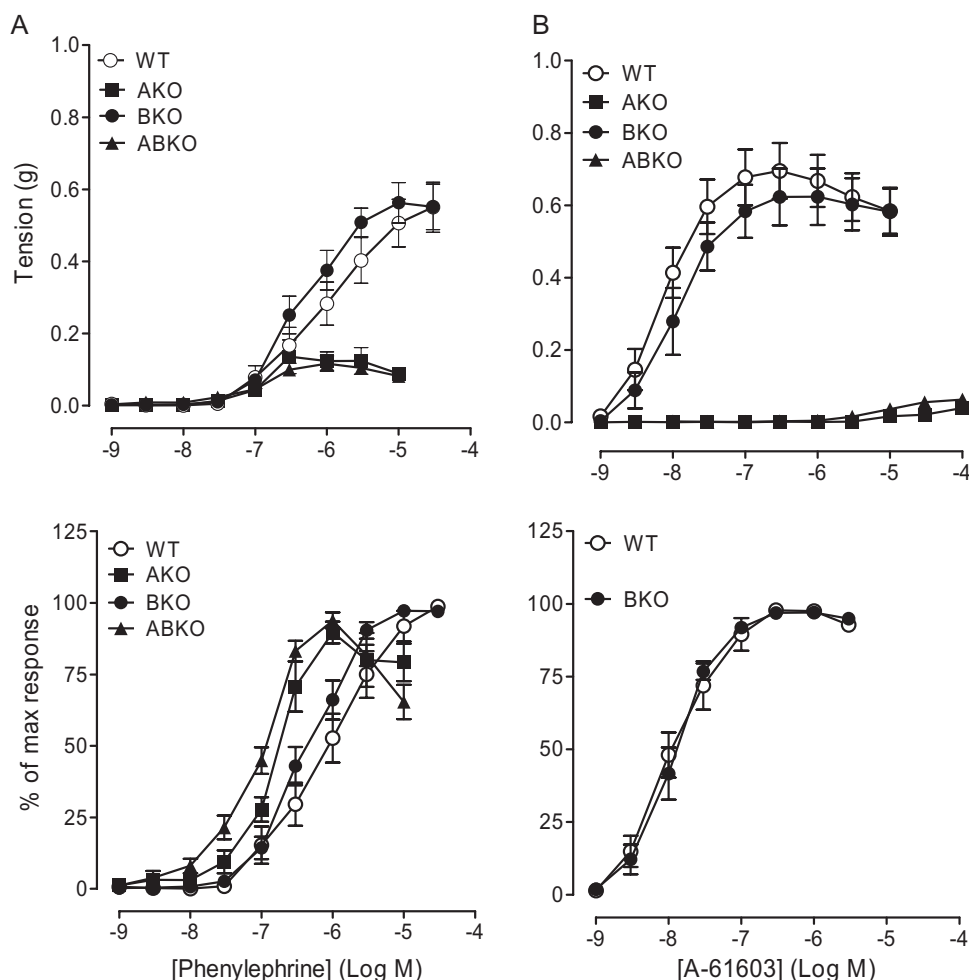


Figure 4 Contractile responses to (A) phenylephrine ($n = 7$) and (B) A-61603 ($n = 6$) in mesenteric arteries of wild type (WT), α_{1A} -adrenoceptor knockout (AKO), α_{1B} -adrenoceptor knockout (BKO) and $\alpha_{1A/B}$ -adrenoceptor knockout (ABKO) mice. Data points are expressed in g tension or percentage of control curve maximum as mean \pm SEM. (A-61603 data for AKO and ABKO mice have not been expressed as % of own maximum as a result of the small magnitude of the response).

Table 3 Agonist responses in mouse mesenteric arteries

| Mouse | Phenylephrine | | A-61603 | | 5-HT | |
|---------------------|----------------------|-------------------|----------------------|-------------------|----------------------|----------------|
| | Maximum response (g) | pEC_{50} | Maximum response (g) | pEC_{50} | Maximum response (g) | pEC_{50} |
| WT | 0.56 \pm 0.07 | 6.1 \pm 0.19 | 0.64 \pm 0.10 | 7.9 \pm 0.14 | 0.29 \pm 0.03 | 6.7 \pm 0.06 |
| α_{1A} -KO | 0.19 \pm 0.04*** | 6.7 \pm 0.07* | 0.04 \pm 0.01*** | 5.0 \pm 0.12*** | 0.23 \pm 0.03 | 6.5 \pm 0.03 |
| α_{1B} -KO | 0.58 \pm 0.06 | 6.4 \pm 0.10 | 0.64 \pm 0.08 | 7.9 \pm 0.15 | 0.27 \pm 0.02 | 6.8 \pm 0.17 |
| $\alpha_{1A/B}$ -KO | 0.13 \pm 0.02*** | 7.0 \pm 0.24*** | 0.07 \pm 0.01*** | 5.2 \pm 0.17*** | 0.20 \pm 0.03 | 6.9 \pm 0.06 |

Data are expressed as mean \pm SEM.

* $P < 0.05$; *** $P < 0.001$ compared with WT (one-way ANOVA, Bonferroni's *post test*).

α_{1A} -KO, α_{1A} -adrenoceptor knockout; $\alpha_{1A/B}$ -KO, $\alpha_{1A/B}$ -adrenoceptor knockout; α_{1B} -KO, α_{1B} -adrenoceptor knockout; WT, wild type mice.

Discussion and conclusions

The arteries from $\alpha_{1A/B}$ -KO displayed a 'pure' α_{1D} -adrenoceptor pharmacology that provides a quantitative standard for interpreting studies in other strains of mouse or other species, which are commonly compromised by the presence of other α_1 -subtypes. The α_{1D} -adrenoceptor-selective antagonist, BMY 7378, became highly potent, and the α_{1A} -adrenoceptor-

selective antagonist RS100-329 showed a low potency, in both cases consistent with radioligand binding studies of recombinant receptors.

'Pure' α_{1D} -adrenoceptor pharmacology in the $\alpha_{1A/B}$ -KO

α_{1D} -Adrenoceptor pharmacology was very clear in the carotid, where BMY 7378 had a pA_2 of 9.4 against phenylephrine, in

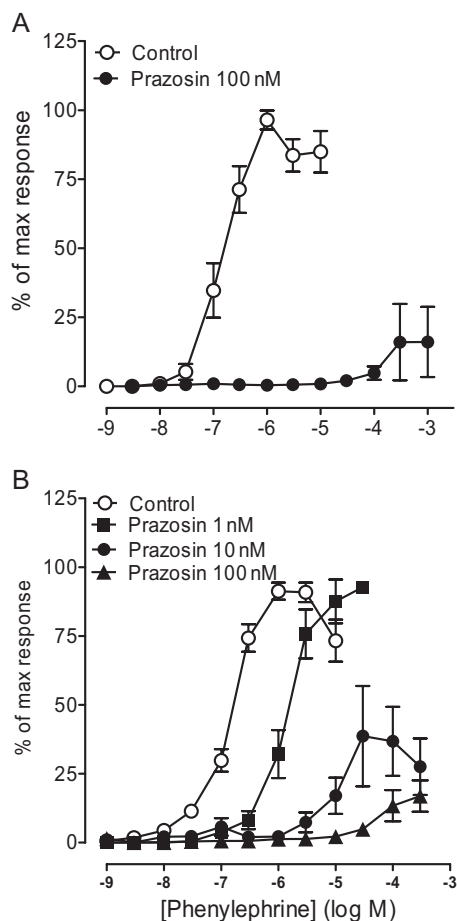


Figure 5 Effects of prazosin on contractile response to phenylephrine in mesenteric arteries of (A) α_{1A} -adrenoceptor knockout (α_{1A} -KO) ($n = 6$) and (B) $\alpha_{1A/B}$ -adrenoceptor knockout ($\alpha_{1A/B}$ -KO) ($n = 7$). Data points are expressed as percentage of control curve maximum as mean \pm SEM.

line with pK_i values at recombinant α_{1D} -adrenoceptors (pK_i 8.1–9.3) (Goetz *et al.*, 1995; Hieble *et al.*, 1995; Kenny *et al.*, 1995; Saussy *et al.*, 1996; Piascik *et al.*, 1997; Testa *et al.*, 1997; Mackenzie *et al.*, 2000; Yoshio *et al.*, 2001; Deighan *et al.*, 2004) and shows that the lower pK_B of 8.3 previously published for the WT (Deighan *et al.*, 2005) was a result of phenylephrine activating multiple α_1 -subtypes. Similarly, the potent and selective α_{1A} -adrenoceptor antagonist, RS100-329, had the relatively low pK_B of 7.8. This is an accurate reflection of this antagonist's potency at the α_{1D} -adrenoceptor and is comparable to its pK_i of 7.9 at the cloned α_{1D} -adrenoceptor (Williams *et al.*, 1999; Deighan *et al.* 2004) and its pK_B of 7.9 obtained against phenylephrine in the WT (Methven *et al.*, 2009). Thus, the α_1 -adrenoceptor-mediated response in the $\alpha_{1A/B}$ -KO carotid artery appears to be representative of a 'pure' α_{1D} -adrenoceptor population.

Complex α_1 -adrenoceptor pharmacology in the WT is explained by multiple subtypes

In the carotid artery, the most straightforward way to interpret the pharmacological data in the WT is to work back from the effects of the non-selective α_1 -adrenoceptor agonist, phe-

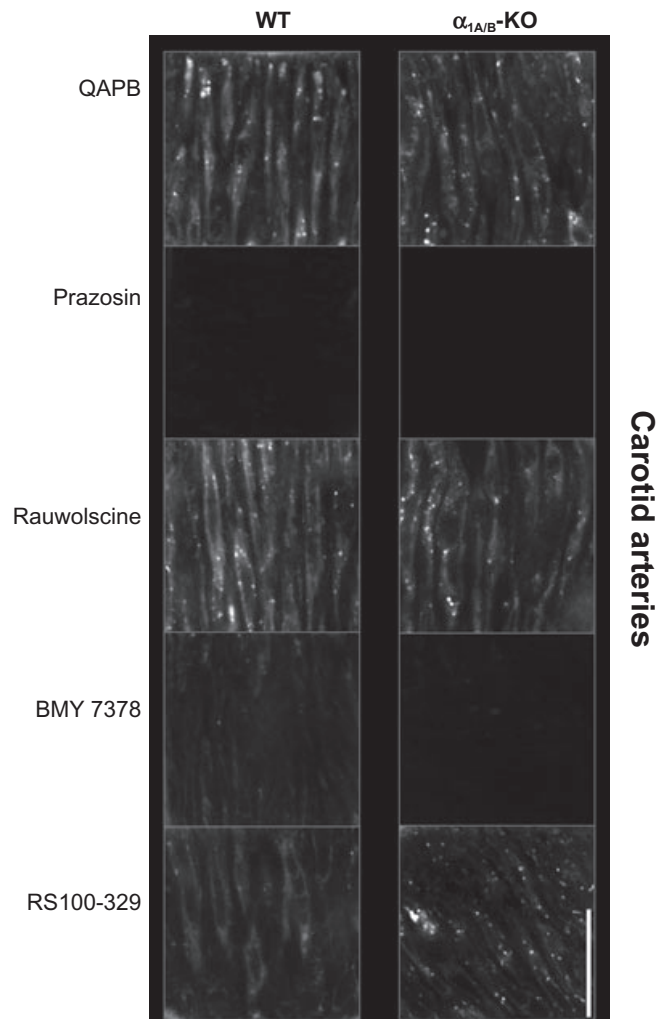


Figure 6 BODIPY FL-prazosin (QAPB) (100 nM) fluorescent ligand binding in mouse carotid arteries. Comparison of wild type (WT) and $\alpha_{1A/B}$ -adrenoceptor knockout ($\alpha_{1A/B}$ -KO) mice and effect of selective antagonists on QAPB fluorescent binding. Non-fluorescent antagonists (100 nM) were used to compete with QAPB after QAPB equilibrium was established. All images were collected under identical conditions of laser intensity, photomultiplier gain (contrast) and offset (brightness). Imaging was performed at high power (zoom 8) to include only smooth-muscle cells and avoid areas containing elastic lamina in the media. An image size of 512×512 pixels produced a field size of $36 \mu\text{m} \times 36 \mu\text{m}$. Calibration bar indicates $10 \mu\text{m}$. Each image is representative of those generated from four mice.

nylephrine, in the $\alpha_{1A/B}$ -KO. Sensitivity to phenylephrine was highest in the $\alpha_{1A/B}$ -KO, providing evidence that this agonist has higher potency at the α_{1D} -adrenoceptor than at the α_{1A} -adrenoceptor so that measured sensitivity is reduced when both are present.

Although knocking out the α_{1A} -adrenoceptor alone had no significant effect on the phenylephrine-induced response, knocking out both the α_{1A} - and α_{1B} -adrenoceptors reduced the maximum and the responses to supramaximal concentrations. This was the only evidence from the present study, albeit indirect and a small effect, for any influence of the α_{1B} -adrenoceptor in either artery studied.

The mesenteric arteries of the $\alpha_{1A/B}$ -KO also showed clear characteristics of an α_{1D} -adrenoceptor population, but, in this

Table 4 Comparison of average pixel intensity of entire image and smooth-muscle cells (SMC) in wild-type (WT) and $\alpha_{1A/B}$ -adrenoceptor knockout ($\alpha_{1A/B}$ -KO) mice

| Mouse strain | Carotid arteries (n = 4) | | Mesenteric arteries (n = 4) | |
|---------------------|---------------------------------------------------------|--------------------------------|---------------------------------------------------------|--------------------------------|
| | Average pixel intensity of 36 \times 36 μ m plane | Average pixel intensity of SMC | Average pixel intensity of 96 \times 96 μ m plane | Average pixel intensity of SMC |
| WT | 50.7 \pm 2.0 | 65.5 \pm 2.4 | 73.6 \pm 3.7 | 93.4 \pm 3.5 |
| $\alpha_{1A/B}$ -KO | 30.7 \pm 1.2*** | 39.1 \pm 1.7** | 28.7 \pm 2.2*** | 63.1 \pm 3.1** |

Data are expressed as mean \pm SEM.

Mesenteric arteries were imaged at low power (zoom 3), which allows coverage of a wider area of exclusively smooth-muscle cells; carotid arteries were imaged at high power (zoom 8) to include only smooth-muscle cells and avoid areas containing elastic lamina in the media.

Individual smooth-muscle cells from a plane from each z-stack were outlined as a Region of interest to measure average pixel intensity.

** $P < 0.01$; *** $P < 0.001$ compared with WT (Student's *t*-test).

Table 5 Effect of subtype-selective antagonists on fluorescent ligand binding in carotid and mesenteric arteries

| Antagonist | Average pixel intensity | | | |
|--------------|--------------------------|-----------------------------|--------------------------|-----------------------------|
| | WT | | $\alpha_{1A/B}$ -KO | |
| | Carotid arteries (n = 4) | Mesenteric arteries (n = 4) | Carotid arteries (n = 4) | Mesenteric arteries (n = 4) |
| QAPB control | 50.7 \pm 2.0 | 73.6 \pm 3.7 | 30.7 \pm 1.2 | 30.7 \pm 1.2 |
| Prazosin | 4.4 \pm 0.8*** | 6.8 \pm 1.2*** | 7.2 \pm 1.3*** | 7.2 \pm 1.3*** |
| Rauwolscine | 54.4 \pm 2.4 | 67.8 \pm 4.5 | 27.7 \pm 4.1 | 27.7 \pm 4.1 |
| BMY 7378 | 16.2 \pm 4.3*** | 27.8 \pm 3.8*** | 7.9 \pm 0.8*** | 7.9 \pm 0.8*** |
| RS100-329 | 24.0 \pm 2.5*** | 21.9 \pm 1.9*** | 27.2 \pm 3.7 | 27.2 \pm 3.7 |

Data are expressed as mean \pm SEM.

Mesenteric arteries were imaged at low power (zoom 3), which allows coverage of a wider area of exclusively smooth-muscle cells; carotid arteries were imaged at high power (zoom 8) to include only smooth-muscle cells and avoid areas containing elastic lamina in the media.

*** $P < 0.001$ compared to QAPB control (one-way ANOVA, Bonferroni's post test).

$\alpha_{1A/B}$ -KO, $\alpha_{1A/B}$ -adrenoceptor knockout; WT, wild type mice.

case, the responses were greatly reduced compared with WT, reflecting the greater contribution of the α_{1A} -adrenoceptor to the maximum contraction. However, responses to phenylephrine in the α_{1A} -KO and α_{1AB} -KO, although reduced in size, showed higher sensitivity than in the WT. This shows that an additional component for phenylephrine in the WT arises from the α_{1D} -adrenoceptor, and explains the complex pharmacology of antagonists versus phenylephrine in the WT.

Thus, although the dominant α_1 -subtype differed between the conductance and resistance arteries, the response in both vessels contained a significant α_{1D} -adrenoceptor component.

Relevance to the α_{1L} / α_{1H} -adrenoceptor hypothesis

The tissues studied did not demonstrate low affinity to prazosin. In another study of α_1 -KO mice, low affinity to prazosin was found in the vas deferens and prostate of the WT, α_{1B} -KO and α_{1D} -KO, and these showed an increased affinity in the α_{1A} -KO (Muramatsu *et al.*, 2008), suggesting that the presence of the α_{1A} -adrenoceptor leads to low affinity for prazosin. However, in our study of the $\alpha_{1A/B}$ -KO, prazosin was highly potent at α_{1D} -adrenoceptors in both mesenteric and carotid arteries, and this was also found at α_{1A} -adrenoceptors in the $\alpha_{1B/D}$ -KO (Methven *et al.*, 2009). This does not support the idea that either of these two receptor subtypes represents a low-affinity ' α_{1L} -adrenoceptor'.

Interestingly, in the $\alpha_{1A/B}$ -KO and α_{1A} -KO mesenteric arteries, prazosin produced a non-competitive block against the

residual phenylephrine-induced response at concentrations of 10 nM and higher. A similar phenomenon was observed in the $\alpha_{1B/D}$ -KO and α_{1D} -KO carotid artery (Methven *et al.*, 2009) and in the α_{1A} -KO prostate, with prazosin (1 nM) against noradrenaline (Muramatsu *et al.*, 2008). In all of these tissues, prazosin shows insurmountable antagonism when the main contractile α_1 -adrenoceptor is absent. A possible mechanism for such an effect was indicated in a study of recombinant α_{1A} -adrenoceptors, where antagonism exhibited by prazosin (and WB4101) was compatible with a model in which the dissociation of antagonists from the receptor was very slow and was a major factor in their inhibition of the transient phase (intracellular release of Ca^{2+}) of the agonist-mediated response, leading to the appearance of insurmountable antagonism (Pediani *et al.*, 2000). This is characteristic of a system with a low receptor reserve. Because the existence of such a phasic response in smooth muscle was one of the original pointers to α_1 -subtypes and ligand-directed signalling (McGrath, 1982, 1983, 1985), this may explain part of the mechanism involved.

The resulting exaggerated blockade of the minor contributor could be another explanation for the very varied potency of prazosin in a wide variety of tissues that has been ascribed to the putative presence of receptors with varying affinity for prazosin termed α_{1L} - and α_{1H} -adrenoceptors (Flavahan and Vanhoutte, 1986; Muramatsu *et al.* 1990).

The difficulty in ascribing any of the cloned α_1 -adrenoceptors to either of these classes may be explained

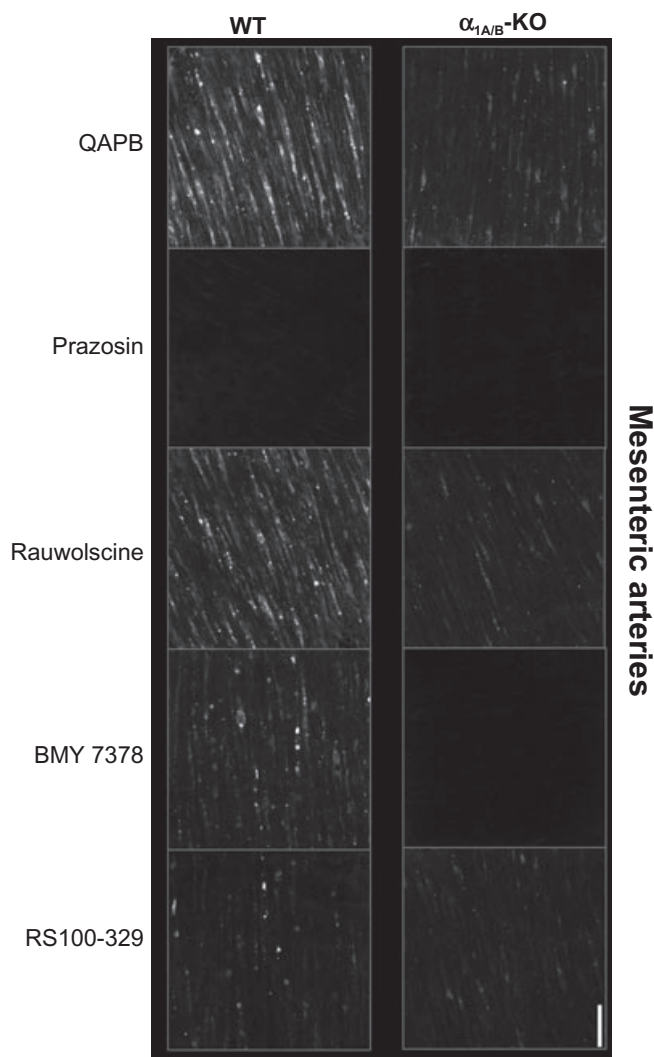


Figure 7 BODIPY FL-prazosin (QAPB) (100 nM) fluorescent ligand binding in mouse mesenteric arteries. Comparison of wild type (WT) and $\alpha_{1A/B}$ -adrenoceptor knockout ($\alpha_{1A/B}$ -KO) mice and effect of selective antagonists on QAPB fluorescent binding. Non-fluorescent antagonists (100 nM) were used to compete with the fluorescent ligand QAPB after QAPB equilibrium was established. All images were collected under identical conditions of laser intensity, photomultiplier gain (contrast) and offset (brightness). Imaging was performed at low power (zoom 3), which allows coverage of a wide area of exclusively smooth-muscle cells. An image size of 512×512 pixels produced a field size of $96 \mu\text{m} \times 96 \mu\text{m}$. Calibration bar indicates $10 \mu\text{m}$. Each image is representative of those generated from four mice.

by the variation in the identity of the more susceptible receptor according to the tissue.

Comparison of the isolated α_{1A} - and α_{1D} -adrenoceptors using double knockouts

The present study with the $\alpha_{1A/B}$ -KO provides a profile of the effects of agonists at α_{1D} -adrenoceptors. This, together with our previous findings in the $\alpha_{1B/D}$ -KO (Methven *et al.*, 2009), illustrates that the carotid artery has a dominant α_{1D} -adrenoceptor response combined with a small but significant α_{1A} -adrenoceptor response at high agonist concentrations.

The α_{1D} - and α_{1A} -adrenoceptors summate to produce the overall response, with no evidence of compensation when one or the other is lost. Because the α_{1A} -adrenoceptor has a relatively lower potency than the α_{1D} -adrenoceptor, the latter dominates all responses except at very high concentrations.

In the mesenteric artery, there is an almost converse depiction of a dominant α_{1A} -adrenoceptor response combined with a small but significant α_{1D} -adrenoceptor response, which summate to produce the overall response. However, because the α_{1D} -adrenoceptor-mediated response occurs at low concentrations, it may actually be the more important receptor for physiological concentrations of agonists. This would explain the unexpectedly large effect of α_{1D} -adrenoceptor antagonists against responses to perivascular nerve stimulation (Bexis *et al.*, 2008). It also leads to a confounding effect on selective antagonist potency, as mentioned earlier. Overall, our evidence suggests that, in native tissue, the responses to α_1 -subtypes are additive and can function independently of each other. So there was no evidence of interactions occurring between α_1 -adrenoceptors at the signalling level that could alter the function of a particular subtype.

Comparison of the double knockouts also provides insight into the differences between agonists at the receptor subtypes. At the α_{1A} -adrenoceptor, the responses to A-61603 were substantially greater than those to phenylephrine, indicating that phenylephrine is a partial agonist at this receptor. The converse occurs at the α_{1D} -adrenoceptor, where phenylephrine had greater efficacy than A-61603, and, thus, A-61603 is a partial agonist. Indeed, phenylephrine is as much a selective α_{1D} -adrenoceptor agonist as A-61603 is a selective α_{1A} -adrenoceptor agonist.

The α_{1B} -adrenoceptor remains enigmatic as far as function in arteries is concerned. In future studies, the $\alpha_{1A/D}$ -KO may illuminate the function, if any, of the α_{1B} -adrenoceptor in the vasculature. For the moment, a definite action of the α_{1B} -adrenoceptor remains unproven.

α_{1D} -Adrenoceptor location and potential interaction with other subtypes

Two recent papers by independent groups of laboratories (Jensen *et al.*, 2009; Pradidarcheep *et al.*, 2009) have concluded that the commercially available antibodies for α_1 -adrenoceptor subtypes are not specific. If this can be generalized throughout the literature, there is, in effect, no reliable information on the distribution of α_1 -adrenoceptors in native tissues. This greatly enhances the value of the present study, where the selectivity of the fluorescent ligand is proven by using selective antagonists. Thus, our data, here on α_{1D} -adrenoceptors and in the works of Methven *et al.* (2009) on α_{1A} -adrenoceptors, are the only reliable guide to α_1 -adrenoceptor distribution in blood vessels.

By isolating the α_{1D} -adrenoceptor in the $\alpha_{1A/B}$ -KO, its widespread distribution was evident. The smooth-muscle cells of the $\alpha_{1A/B}$ -KO had a reduced level of fluorescence but had a comparable binding pattern to the WT. This suggests that, in the $\alpha_{1A/B}$ -KO, the overall reduction in fluorescence is a result of the loss of the other α_1 -subtypes from at least some of the same cells. This provides indirect evidence that different subtypes are present in the same cells, a vital piece of information

if molecular interactions between α_1 -subtypes are to occur. Surprisingly, this has not been demonstrated previously. On the other hand, because the cellular distribution of the fluorescence was similar when the α_{1D} -adrenoceptor alone was present, this also suggests that the distribution of the α_{1D} -adrenoceptor is not regulated by either the α_{1A} - or α_{1B} -adrenoceptor. Furthermore, we found no evidence of an exaggerated internalization of the isolated α_{1D} -adrenoceptor as would be expected from the hypothesis that, through heterodimerization, the α_{1B} -adrenoceptor chaperones the α_{1D} -adrenoceptor to the cell surface (Uberti *et al.*, 2003; Hague *et al.*, 2004). Regardless of whether the α_{1D} -adrenoceptor has a major or minor role in a particular vessel, the α_{1D} -adrenoceptor was found at both the cell membrane and at intracellular sites. The detection of α_1 -subtypes at both cellular locations is consistent with studies in isolated cells (Daly *et al.*, 1998; Hrometz *et al.*, 1999; Pediani *et al.*, 2000; Morris *et al.*, 2004) and our previous study in native tissue from the $\alpha_{1B/D}$ -KO (Methven *et al.*, 2009). Indeed, it is difficult to see how the situation could be otherwise because α_1 -adrenoceptors are synthesized inside the cell and need to be located at the plasmalemmal membrane to function and constitutively cycle between the two locations (Morris *et al.*, 2004; Pediani *et al.*, 2005).

Collectively, these data show that the expression of the α_{1D} -adrenoceptor was not altered by the removal of the α_{1A} - and α_{1B} -adrenoceptors at either a cellular or subcellular level.

Relationship between receptor subtype location and function

There was no correlation between which subtype was dominant in mediating contraction and the amount of fluorescent binding detected when that subtype was isolated. Carotid and mesenteric arteries showed similar levels of both receptor subtypes but unequal functional responses. This confirms the poor correlation between mRNA or receptor expression and function. Despite the abundance of all three α_1 -adrenoceptors being similar, the contribution of each subtype to a functional response varies considerably (Piascik *et al.*, 1995; Yang *et al.*, 1997). There must be another explanation for the functional dominance of each receptor rather than its level of mRNA expression or functional protein. This most probably lies with the efficiency of the signalling process rather than the expression level of the receptor subtype.

Although its functional significance varied between artery types, the distribution of the α_{1D} -adrenoceptor was ubiquitous. This contrasts with the more heterogeneous distribution of the α_{1A} -adrenoceptor (Methven *et al.*, 2009). Stassen *et al.* (1998) demonstrated that the expression of the α_{1A} -adrenoceptor is dependent on the innervation of arteries, while, in poorly innervated arteries, such as the carotid artery, 'non- α_{1A} -adrenoceptors' were dominant. Thus, the distribution of the individual α_1 -adrenoceptor subtypes may be linked to the level of adrenergic innervation that the arteries receive. The α_{1D} -adrenoceptor-dominant carotid artery is poorly innervated and has a thick medium consisting of several layers of elastic lamina separating smooth-muscle cells layers. Thus, a high number of smooth-muscle cells expressing a functional population of α_{1D} -adrenoceptors would facilitate a cell-cell transmission of excitation. In contrast, the highly

innervated, α_{1A} -adrenoceptor-dominant mesenteric artery would be less reliant on a high number of smooth-muscle cells expressing the α_{1A} -adrenoceptor, as depolarization can easily spread through the thin media of this artery.

Therapeutic potential

The present study has demonstrated that the α_{1D} -adrenoceptor exists and is functional in examples of a conductance artery and a resistance artery. In particular, the highly sensitive contractile response of the α_{1D} -adrenoceptor in the mesenteric artery demonstrates a role for the α_{1D} -adrenoceptor in the vasculature and is supported by reduced vasoconstriction to noradrenaline and reduced blood pressure in the α_{1D} -KO (Tanoue *et al.*, 2002). The α_{1D} -adrenoceptor also has a role in the regulation of coronary vasoconstriction (Turnbull *et al.*, 2003). These functions, together with the presence of the α_{1D} -adrenoceptor being linked to the onset of hypertension (Villalobos-Molina and Ibarra, 1999), demonstrate the great importance of the development of more selective agonists and antagonists for research and ultimately for therapeutic use. Furthermore, α_1 -adrenoceptor antagonists remain extensively in use for the treatment of benign prostatic hyperplasia, without relevant regard to their subtype selectivity. This study provides powerful tools for overcoming the uncertainties of studies with immunocytochemistry or selective agonists or antagonists in native tissues. The $\alpha_{1A/B}$ -KO can be utilized to further our understanding of the expression and physiological or pathophysiological roles of α_{1D} -adrenoceptors and to assess the selectivity of existing and new compounds.

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