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RESEARCH PAPER

Identification of α_{1L}-adrenoceptor in mice and its abolition by α_{1A}-adrenoceptor gene knockout

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Background and purpose: The α_{1L}-adrenoceptor has pharmacological properties that distinguish it from three classical α_1-adrenoceptors (α_{1A}, α_{1B} and α_{1D}). The purpose of this was to identify α_{1L}-adrenoceptors in mice and to examine their relationship to classical α_1-adrenoceptors.
Experimental approach: Radioligand binding and functional bioassay experiments were performed on the cerebral cortex, vas deferens and prostate of wild-type (WT) and α_{1A}-, α_{1B}- and α_{1D}-adrenoceptor gene knockout (AKO, BKO and DKO) mice.
Key results: The radioligand [^{3}H]-silodosin bound to intact segments of the cerebral cortex, vas deferens and prostate of WT, BKO and DKO but not of AKO mice. The binding sites were composed of two components with high and low affinities for prazosin or RS-17053, indicating the pharmacological profiles of α_{1A}-adrenoceptors and α_{1L}-adrenoceptors. In membrane preparations of WT mouse cortex, however, [^{3}H]-silodosin bound to a single population of prazosin high-affinity sites, suggesting the presence of α_{1A}-adrenoceptors alone. In contrast, [^{3}H]-prazosin bound to two components having α_{1A}-adrenoceptor and α_{1B}-adrenoceptor profiles in intact segments of WT and DKO mouse cortices, but AKO mice lacked α_{1A}-adrenoceptor profiles and BKO mice lacked α_{1B}-adrenoceptor profiles. Noradrenaline produced contractions through α_{1L}-adrenoceptors with low affinity for prazosin in the vas deferens and prostate of WT, BKO and DKO mice. However, the contractions were abolished or markedly attenuated in AKO mice.
Conclusions and implications: α_{1L}-Adrenoceptors were identified as binding and functional entities in WT, BKO and DKO mice but not in AKO mice, suggesting that the α_{1L}-adrenoceptor is one phenotype derived from the α_{1A}-adrenoceptor gene.
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Keywords: α_{1L}-adrenoceptor; α_{1A}-adrenoceptor; vas deferens; prostate; cerebral cortex; knockout mouse

Abbreviations: AKO, α_{1A}-adrenoceptor gene knockout; B_{max}, maximum binding capacity; BKO, α_{1B}-adrenoceptor gene knockout; BMY 7378, (8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4,5]decane-7,9-dione dihydrochloride; DKO, α_{1D}-adrenoceptor gene knockout; RS-17053, *N*-[2-(2-cyclopropylmethoxyphenoxy)ethyl]-5-chloro-α,α-dimethyl-1*H*-indole-3-ethamine hydrochloride

Introduction

In the α_1-adrenoceptors, three distinct subtypes (α_{1A}-, α_{1B}- and α_{1D}-adrenoceptors; nomenclature follows Alexander *et al*., 2008) have been cloned and are known to be involved in various physiological functions (Lomasney *et al*., 1991; Hieble *et al*., 1995; Zhong and Minneman, 1999; Michelotti *et al*., 2000). The three classical α_1-adrenoceptors have high (subnanomolar) affinity for prazosin, a prototypic, selective α_1-adrenoceptor antagonist, but show distinct pharmacological profiles for several antagonists. For example, silodosin, 5-methylurapidil and RS-17053 (*N*-[2-(2-cyclopropylmethoxyphenoxy)ethyl]-5-chloro-α,α-dimethyl-1*H*-indole-3-ethamine hydrochloride) are selective for α_{1A}-adrenoceptors, and BMY-7378 (8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4,5]decane-7,9-dione-dihydrochloride) is selective for α_{1D}-adrenoceptors (Lomasney *et al*., 1991; Hieble *et al*., 1995; Ford *et al*., 1996; Murata *et al*., 1999; Piao *et al*., 2000). Tamsulosin is a potent antagonist for the three α_1-adrenoceptor subtypes or shows slightly lower affinity for the α_{1B} subtype than for the other two subtypes (Ford *et al*., 1996; Morishima *et al*., 2008).

In addition to these classical α_1-adrenoceptors, the presence of another subtype (the putative α_{1L}-adrenoceptor) has been proposed (Flavahan and Vanhoutte, 1986; Muramatsu

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et al., 1990; Ford *et al*., 1996). The α_{1L}-adrenoceptor shows a unique pharmacological profile: low affinity for prazosin, 5-methylurapidil, RS-17053 and BMY 7378, but high affinity for silodosin and tamsulosin (Muramatsu *et al*., 1995, 1998; Ford *et al*., 1996; Murata *et al*., 1999). The α_{1L}-adrenoceptors have been identified primarily by functional studies: rabbit thoracic aorta (Oshita *et al*., 1993), rabbit iris (Nakamura *et al*., 1999; Suzuki *et al*., 2002), rat small mesenteric arteries (Stam *et al*., 1999), canine subcutaneous arteries (Argyle and McGrath, 2000), rat and human vas deferens (Ohmura *et al*., 1992; Amobi *et al*., 2002), and rabbit, rat, mouse and human prostate (Ford *et al*., 1996; Van der Graaf *et al*., 1997; Hiraoka *et al*., 1999; Gray and Ventura, 2006; Morishima *et al*., 2007a; Su *et al*., 2008). However, the gene corresponding to the α_{1L}-adrenoceptor has not yet been cloned, even though many trials of candidate genes have been carried out, including splicing variants of α_1-adrenoceptor genes and heterodimeric expression of different subtypes (Ramsay *et al*., 2004). Subsequently, it has been considered that the α_{1L}-adrenoceptor may be a functional phenotype of the α_{1A}-adrenoceptor because the functional studies with recombinant α_{1A}-adrenoceptors have revealed a relatively low affinity for prazosin (Ford *et al*., 1997; Daniels *et al*., 1999).

Recently, we have demonstrated in radioligand binding studies that α_{1L}-adrenoceptors occur as a distinct entity from α_{1A}-, α_{1B}- and α_{1D}-adrenoceptors (Hiraizumi-Hiraoka *et al*., 2004; Morishima *et al*., 2007a, 2008). However, the α_{1L}-adrenoceptors were detected only under intact tissue conditions and the pharmacological profile converted to that of the α_{1A}-adrenoceptor after homogenization (Morishima *et al*., 2008; Su *et al*., 2008). This line of evidence strongly suggests that the α_{1L}-adrenoceptor may be a phenotypic subtype of α_1-adrenoceptors (probably the α_{1A}-adrenoceptor) rather than a genetically different subtype (Su *et al*., 2008).

The aims of this study were to identify α_{1L}-adrenoceptors in mice by radioligand binding and bioassay approaches and to examine the effects of gene knockout of the classical α_1-adrenoceptor subtypes. The present results show that binding and functional profiles of α_{1L}-adrenoceptors can be clearly detected in wild-type (WT) mice, but that the α_{1L}-adrenoceptor is selectively abolished in α_{1A}-adrenoceptor gene knockout (AKO) mice. A part of this study was presented in the Satellite Meetings (Pharmacology of Adrenoceptors, Beijing, China, 2006) of the 15th International Congress of Pharmacology and the 80th Annual Meeting of The Japanese Pharmacological Society (Morishima *et al*., 2007b).

Materials and methods

Animals

All animal procedures and this study were performed according to the Guidelines for Animal Experiments, University of Fukui and University of California.

AKO (Rokosh and Simpson, 2002), α_{1B}-adrenoceptor gene knockout (BKO) (Cavalli *et al*., 1997), α_{1D}-adrenoceptor gene knockout (DKO) (Sadalge *et al*., 2003) and WT mice were all of the C57Bl/6J background. Mice of both sexes were used, aged 1–6 months (mean 3). Mice were housed under a 12-h light-dark cycle and had free access to standard laboratory food and tap water. The room temperature and relative humidity were regulated at 22 °C and 40–70%, respectively.

Mice were anaesthetized with isoflurane or halothane and killed by cervical dislocation or removal of the heart under deep anaesthesia. The brain cortex, vas deferens and prostate were rapidly excised and placed in a modified Krebs–Henseleit solution (120.7 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 2.0 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 25.5 mM $NaHCO_3$ and 11.5 mM D-glucose). The modified Krebs–Henseleit solution was aerated with a mixture of 95% O_2 and 5% CO_2 and maintained at 4 °C (pH 7.4).

Tissue segment binding experiments with mouse cerebral cortex, vas deferens and prostate

Tissue segment binding experiments with mouse cerebral cortex, vas deferens and prostate were performed as described earlier (Muramatsu *et al*., 2005; Morishima *et al*., 2008). The cerebral cortex was cleaned from the pia mater and substantia alba, and cut into small pieces (approximately $2 \times 2 \times 1$ mm). The whole vas deferens was cleared of connective tissue and the epithelium, and then the muscle layer was cut into small pieces (approximately 3 mm long and 1–2 mm wide, 16–20 segments from one mouse). The prostate was cleared of connective tissue and separated into four to five fragments (approximately 1–2 mm long and 1 mm wide) without damaging the lobules. Each segment of cerebral cortex, vas deferens and prostate was incubated with [^{3}H]-silodosin for 16 h at 4 °C in 1 mL of a Krebs incubation buffer containing 135.7 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 2.0 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 10.5 mM $NaHCO_3$ and 11.5 mM D-glucose (pH 7.4). On samples of the cerebral cortex, [^{3}H]-prazosin was also used. In binding saturation experiments, [^{3}H]-silodosin or [^{3}H]-prazosin at concentrations 50–1000 pM were used. Binding competition experiments were performed with [^{3}H]-silodosin and [^{3}H]-prazosin at 500 pM. After incubation, the pieces were gently washed and then solubilized in 1 mL of 0.3 M NaOH solution to estimate the radioactivity and protein content. The specific binding was determined by subtracting the nonspecific binding estimated in the presence of 30 μM phentolamine from the total radioactivity bound per milligram protein. Experiments with the cerebral cortex were carried out in duplicate at each radioligand concentration for saturation experiments or with a competing ligand for competition binding experiments. The abundance of α_1-adrenoceptors in mouse cerebral cortex was represented as maximum binding capacity per milligram of total tissue protein (fmol per mg of total tissue protein). In the case of vas deferens, each segment was used for a single concentration of [^{3}H]-silodosin in saturation experiments or with a competing ligand for competition binding experiments. In the experiments with prostate segments, binding of only 500 pM [^{3}H]-silodosin was examined because of the small number of segments.

Membrane binding experiments with mouse cerebral cortex

Mouse cortex was minced with scissors and homogenized in 40 volumes (v/w) of Krebs incubation buffer containing proteinase inhibitors (Complete, EDTA-free tablet, Roche,

Penzberg, Germany) using a Polytron homogenizer at 4 °C. The tissue homogenate was subjected to centrifugation at 1000 *g* for 10 min at 4 °C, and then the supernatant was centrifuged at 80 000 *g* for 30 min at 4 °C. The resulting pellet was resuspended in ice-cold Krebs incubation buffer without proteinase inhibitors and used as the crude membrane preparations for conventional membrane binding experiments. The incubation was carried out for 4 h at 4 °C in 1 mL Krebs incubation buffer. For saturation binding experiments, 30–1000 pM [^{3}H]-silodosin was used. For competition binding experiments, the membranes were incubated with 500 pM [^{3}H]-silodosin in the absence or presence of unlabelled competing ligands. Nonspecific binding of [^{3}H]-silodosin was defined as the binding in the presence of 30 μM phentolamine. Experiments were carried out in duplicate. The protein contents of the homogenates before centrifugation and of the crude membrane fractions were measured using a Bio-Rad Protein Assay kit (Bio-Rad Japan, Tokyo, Japan). The abundance of α_1-adrenoceptors in the crude membranes was represented as maximum binding capacity per milligram of total tissue protein (fmol per mg of total tissue protein), but not of crude membrane protein after centrifugation.

Functional studies with mouse vas deferens and prostate

Functional studies were performed as described earlier (Ohmura *et al*., 1992). Briefly, mouse vas deferens (epididymal portion) and prostate strips were placed at 37 °C in organ baths containing a modified Krebs–Henseleit solution composed of 120.7 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 2.0 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 25.5 mM $NaHCO_3$ and 11.5 mM D-glucose. Noradrenaline was applied non-cumulatively to the vas deferens and cumulatively to the prostate, and the isometric tension changes were recorded through a force transducer. Desipramine (0.3 μM), deoxycorticosterone acetate (5 μM) and propranolol (1 μM) were added to inhibit neural and extraneural reuptake of noradrenaline and to block β-adrenoceptors, as described elsewhere (Muramatsu *et al*., 1995). Antagonists were used 60 min before and during the evaluation of contractile responses to noradrenaline. The tissue responsiveness to α,β-methylene ATP was also examined. The concentrations of α,β-methylene ATP were 1 μM in vas deferens, which produced roughly the same amplitude of contraction as 100 μM noradrenaline, and 10 μM in prostate, which corresponded to the maximum concentration.

Data analysis

Data are presented as the mean ± s.e.mean with the number of experiments or mice. Data from WT, AKO, BKO and DKO mice were statistically analysed by ANOVA followed by Scheffe's *post hoc* test.

Binding data in saturation and competition experiments were analysed using PRISM software (version 3, GraphPad, San Diego, CA, USA). The number of α_1-adrenoceptors was presented as the maximal binding capacity per milligram of total tissue protein (B_{max}: fmol per mg of total tissue protein). That is, in the case of conventional binding experiments with membrane fractions of cerebral cortex, the proteins in the homogenates before fractionation were measured as total protein. In saturation binding studies, data were fitted by a one-site saturation-binding isotherm. In competition studies, the data were first fitted to a one- and then a two-site model, and if the residual square sums were significantly less for a two-site fit of the data than for a one-site fit (P-value <0.05 as determined by F-test), then a two-site model was accepted. Slopes of pseudo-Hill plots were also determined for some competitors to validate one- or two-site fitting. For pseudo-Hill plot analyses, Origin (version 7.5, Origin Lab Co., Northampton, MA, USA) was used.

In functional studies, antagonist affinity estimates (pK_B values) were obtained by plotting the data according to the Schild analysis. When the straight lines had a slope of unity, the pA_2 value estimated was taken as the pK_B value. When a single concentration of an antagonist was tested, the pK_B value was also determined for a single concentration of the antagonist by the concentration ratio method (Furchgott, 1972).

Drugs

Silodosin (KMD-3213) and tamsulosin were donated from Kissei Pharmaceutical Co., Ltd. (Matsumoto, Japan). [^{3}H]-silodosin (specific activity 1.92 TBq mmol^{-1}) was manufactured by GE Healthcare UK (Buckinghamshire, UK), and provided by Kissei Pharmaceutical Co., Ltd. The other drugs were obtained from commercially available sources.

Results

[^{3}H]-silodosin bindings in the mouse cerebral cortex

[^{3}H]-silodosin (50–1000 pM) bound to tissue segments and membrane preparations of the WT mouse cerebral cortex in a concentration-dependent manner (Figure 1a). The Hill coefficients were 1.00 ± 0.04 for tissue segments and 1.05 ± 0.05 for membrane preparations, and it was assumed that [^{3}H]-silodosin bound to a single class of sites. The dissociation constants and maximal binding capacities were 470 ± 35 pM and 228 ± 12 fmol per mg total tissue protein in the segments ($n=6$) and 390 ± 20 pM and 193 ± 15 fmol per mg total tissue protein in the membrane preparations ($n=6$), respectively. Thus, there was no significant difference in the abundance of [^{3}H]-silodosin binding sites between tissue segments and membrane preparations of the WT mouse cerebral cortex (Figure 1b).

In contrast, [^{3}H]-silodosin binding was negligible in tissue segments and membrane preparations of the AKO mouse cortex ($B_{max}<20$ fmol per mg protein, $n=5$; Figures 1a and b), whereas the same B_{max} values as that of WT mouse were estimated in tissue segments of BKO and DKO mice (Figure 1b).

The pharmacological profiles of [^{3}H]-silodosin binding sites in the tissue segments and the membrane preparations of WT mouse cortex were examined in competition binding studies using several drugs. Competition curves for prazosin and RS-17053 in the tissue segments were shallow, better fitting a two-site model in computer analysis (Figures 2a and b). The slope factors in the pseudo-Hill plot analyses

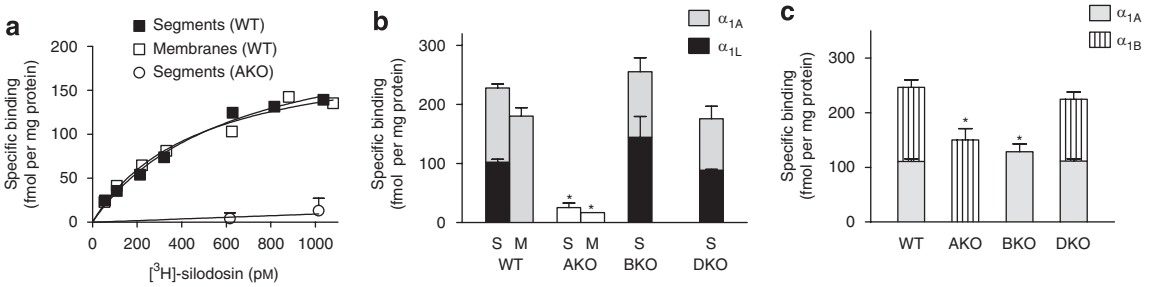

Figure 1 Binding of [^{3}H]-silodosin and [^{3}H]-prazosin to mouse cerebral cortex. (**a**) Saturation curves for specific binding of [^{3}H]-silodosin in tissue segments and membrane preparations of WT mouse cortex and in tissue segments of AKO mouse cortex. (**b**) Binding density of [^{3}H]-silodosin estimated from saturation experiments with WT, AKO, BKO and DKO mouse cortices. The saturation experiments were conducted with intact tissue segments (S) and membranes (M) of cerebral cortex. High- and low-affinity sites for prazosin at [^{3}H]-silodosin binding sites were represented as α_{1A} and α_{1L}, respectively. (**c**) Binding density of [^{3}H]-prazosin estimated from saturation experiments with intact segments of WT, AKO, BKO and DKO mouse cortices. High- and low-affinity sites for silodosin at [^{3}H]-prazosin-binding sites were represented as α_{1A} and α_{1B}, respectively. The number of experiments was six in WT, five in AKO, four in BKO and three in DKO. *Significantly different from WT mouse ($P<0.01$). AKO, α_{1A}-adrenoceptor gene knockout; BKO, α_{1B}-adrenoceptor gene knockout; DKO, α_{1D}-adrenoceptor gene knockout; WT, wild type.

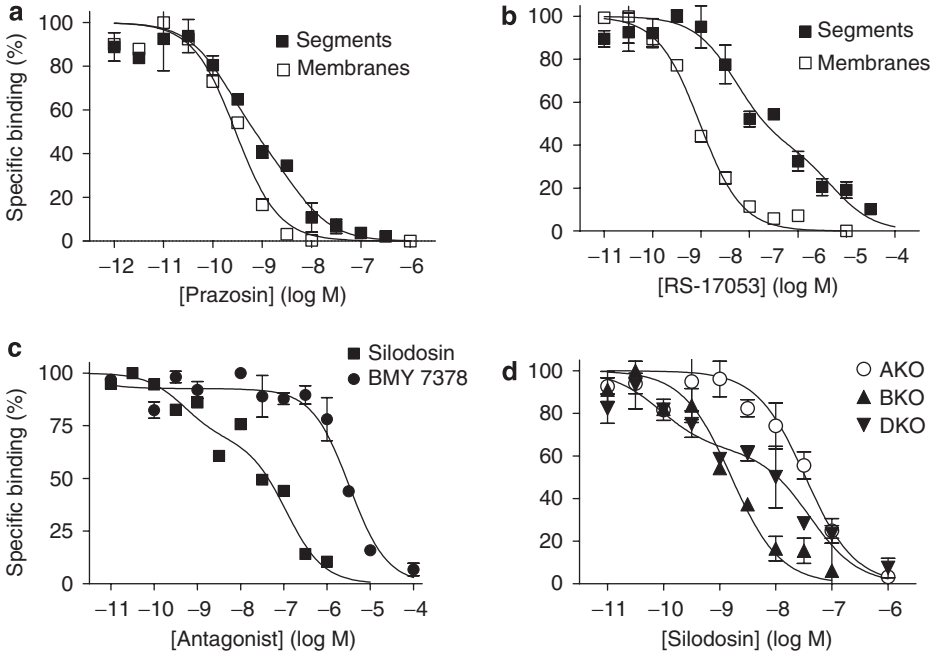

Figure 2 Competition curves for various antagonists in mouse cerebral cortex. (**a** and **b**) Competition curves for prazosin and RS-17053 at 500 pM [^{3}H]-silodosin-binding sites in the intact segments and membranes of the WT mouse cortex. (**c**) Competition curves for silodosin and BMY 7378 at 500 pM [^{3}H]-prazosin-binding sites in the intact segments of the WT mouse cortex. (**d**) Competition of silodosin in the intact segments of AKO, BKO, and DKO mouse cortices at 500 pM [^{3}H]-prazosin-binding sites. Each curve is representative of similar results obtained in 2–5 experiments. AKO, α_{1A}-adrenoceptor gene knockout; BKO, α_{1B}-adrenoceptor gene knockout; DKO, α_{1D}-adrenoceptor gene knockout; WT, wild type.

were -0.67 ± 0.03 for prazosin and -0.56 ± 0.07 for RS-17053, which also supported the existence of two affinity sites for these antagonists. The proportion of high-affinity sites was approximately 55% (Table 1). However, in the membrane preparations, prazosin and RS-17053 showed monophasic competition curves with high affinity (Figures 2a and b, Table 1). Tamsulosin, silodosin and BMY 7378 competed monotonically for the [^{3}H]-silodosin binding in either tissue segments or membrane preparations (Table 1).

From these results, it was suggested that [^{3}H]-silodosin binding sites in WT mouse cortex are composed of two components having different affinities for prazosin and RS-17053 in the intact tissue segments, but that both components change the pharmacological profiles to that of a single component having high affinity for prazosin and RS-17053 after homogenization. These results are identical to those recently obtained in rat cerebral cortex (Morishima *et al*., 2008), suggesting the presence of α_{1A} and α_{1L} subtypes (see Introduction and Discussion for receptor subclassification). From the estimated proportions of high- and low-affinity sites for prazosin in the intact tissue segments, the densities of both α_{1A} and α_{1L} subtypes were extrapolated. Figure 1b shows that, in addition to total density, the density of high- or low-affinity sites for prazosin (α_{1A} or α_{1L} subtypes) is roughly the same among WT, BKO and DKO mouse cortex samples.

Table 1 Pharmacological characteristics of [^{3}H]-silodosin and [^{3}H]-prazosin-binding sites in the WT mouse cerebral cortex

Antagonist	*[^{3}H]-silodosin binding*			*[^{3}H]-prazosin binding*	
	Segments		*Membranes*	*Segments*	
	$pK_{i_{high}}$ *(%)*	$pK_{i_{low}}$	pK_i	$pK_{i_{high}}$ *(%)*	$pK_{i_{low}}$
Prazosin	10.1 ± 0.2 (55)	8.5 ± 0.2	9.9 ± 0.1	—	
Silodosin	9.7 ± 0.2		9.5 ± 0.1	9.9 ± 0.3 (38)	7.4 ± 0.2
Tamsulosin	9.9 ± 0.1		10.3 ± 0.1	—	
BMY 7378		6.3 ± 0.2	6.4 ± 0.1	5.7 ± 0.1	
RS-17053	8.4 ± 0.1 (55)	6.3 ± 0.1	9.3 ± 0.1	8.8 ± 0.3 (40)	7.7 ± 0.2

Abbreviations: RS-17053, *N*-[2-(2-cyclopropylmethoxyphenoxy)ethyl]-5-chloro-α,α-dimethyl-1*H*-indole-3-ethamine hydrochloride; WT, wild type.
% indicates proportion of high-affinity sites.
$pK_{i_{high}}$ and $pK_{i_{low}}$: negative logarithm of the equilibrium constants (pK_i) at high- and low-affinity sites for tested drugs.
Competitive binding experiments with intact tissue segments and crude membrane preparations were carried out at 500 pM [^{3}H]-silodosin or [^{3}H]-prazosin. Data represent mean ± s.e.mean of 4–6 experiments.

[^{3}H]-prazosin bindings in the mouse cerebral cortex

As [^{3}H]-silodosin recognized two distinct components of α_1-adrenoceptors in tissue segments of the cerebral cortex, binding of [^{3}H]-prazosin was also examined in the tissue segments of WT and KO mouse cortices. In saturation binding experiments, [^{3}H]-prazosin bound to α_1-adrenoceptors ($K_D = 455 \pm 56$ pM and $B_{max} = 237 \pm 32$ fmol per mg of total tissue protein, $n = 5$). The same density as that in WT mice was estimated in DKO mice, but the abundance in AKO and BKO mice was significantly lower than in WT mice (Figure 1c).

In competition experiments with WT mouse cortex segments, silodosin biphasically inhibited 500 pM [^{3}H]-prazosin binding; approximately 40% were high-affinity sites for silodosin (Figure 2c, Table 1). A similar biphasic competition curve for silodosin was observed in DKO mice (Figure 2d). However, the bindings in AKO and BKO mouse cortices showed monotonic competition for silodosin with low and high affinities, respectively (p$K_i = 7.7 \pm 0.1$ in AKO mice and 9.2 ± 0.2 in BKO mice, $n = 3$; Figure 2d). On the basis of the proportions of these different affinity sites for silodosin, the densities of both components were extrapolated in [^{3}H]-prazosin binding sites and are shown in Figure 1c, where high- and low-affinity sites for silodosin are represented as α_{1A} and α_{1B} subtypes. Both α_{1A} and α_{1B} subtypes were identified in WT and DKO mice, but the α_{1B} subtype only occurred in the AKO mouse cortex and α_{1A} subtype only occurred in the BKO mouse cortex. BMY 7378 competed with [^{3}H]-prazosin binding monotonically with low affinity in WT mouse cortex (Figure 2c, Table 1). These results suggest that α_{1A} and α_{1B} subtypes showing different affinities for silodosin occurred in the [^{3}H]-prazosin binding sites of WT and DKO mouse cortex segments, but that each α_{1A} or α_{1B} subtype was selectively abolished by AKO or BKO mice. The prazosin low-affinity sites corresponding to α_{1L} subtype were not detected in the binding study with [^{3}H]-prazosin.

[^{3}H]-silodosin bindings in the mouse vas deferens and prostate

As [^{3}H]-silodosin recognized low-affinity sites for prazosin (α_{1L} subtype) in mouse cerebral cortex segments, the binding approach with this radioligand was applied to tissue segments of vas deferens and prostate. Because both tissues were too small to provide a large number of tissue segments, very limited experiments were conducted. [^{3}H]-silodosin specifically bound to tissue segments of the WT mouse vas deferens ($K_D = 130 \pm 60$ pM and $B_{max} = 153 \pm 18$ fmol per mg protein, $n = 5$) (Figure 3a). However, specific binding of [^{3}H]-silodosin was not detected in AKO mice. The binding capacities of 500 pM [^{3}H]-silodosin in the vas deferens of BKO and DKO mice were not significantly different from that of WT mice (Figure 3c).

In competition experiments, the specific binding of [^{3}H]-silodosin to WT mouse vas deferens was a biphasic competition with prazosin (p$K_i = 9.9$ and 8.1) (Figure 3b) and RS-17053 (p$K_i = 8.9$ and 6.8), suggesting the presence of the two distinct α_{1A} and α_{1L} subtypes, as in the mouse cortex. From the binding capacities of 500 pM [^{3}H]-silodosin in the absence or presence of 1 nM prazosin, the proportion of α_{1A} and α_{1L} subtypes was evaluated. Figure 3c shows that both subtypes occur at a similar ratio in tissue segments of WT, BKO and DKO mouse vas deferentia, but are absent in AKO mice.

[^{3}H]-silodosin also bound to the prostate segments. Because only four to five segments were prepared from each mouse, only the binding capacities at 500 pM [^{3}H]-silodosin were examined. As shown in Figure 3d, the densities were estimated at 60 fmol per mg total tissue protein in prostrate of WT, BKO and DKO mice, but binding was negligible in AKO mice. In WT mouse prostate, the specific binding of 500 pM [^{3}H]-silodosin was reduced to 39 ± 4% in the presence of 1 nM prazosin ($n = 4$).

Contractile responses to noradrenaline in mouse vas deferens and prostate

In the vas deferentia isolated from WT mice, non-cumulative application of noradrenaline (1–100 μM) produced a transient contraction. This contraction was relatively insensitive to prazosin, and thus not inhibited by prazosin at concentrations less than 10 nM (p$K_B = 7.7 \pm 0.2$, $n = 5$). However, the contraction was strongly attenuated by low concentrations of silodosin (p$K_B = 9.7 \pm 0.2$, $n = 5$, Figure 4). The contractile responses to noradrenaline also showed low sensitivity to RS-17053, 5-methylurapidil and BMY 7378 (Figure 4b, Table 2). These results suggested that the contractile responses in the WT mouse vas deferens were mediated through prazosin

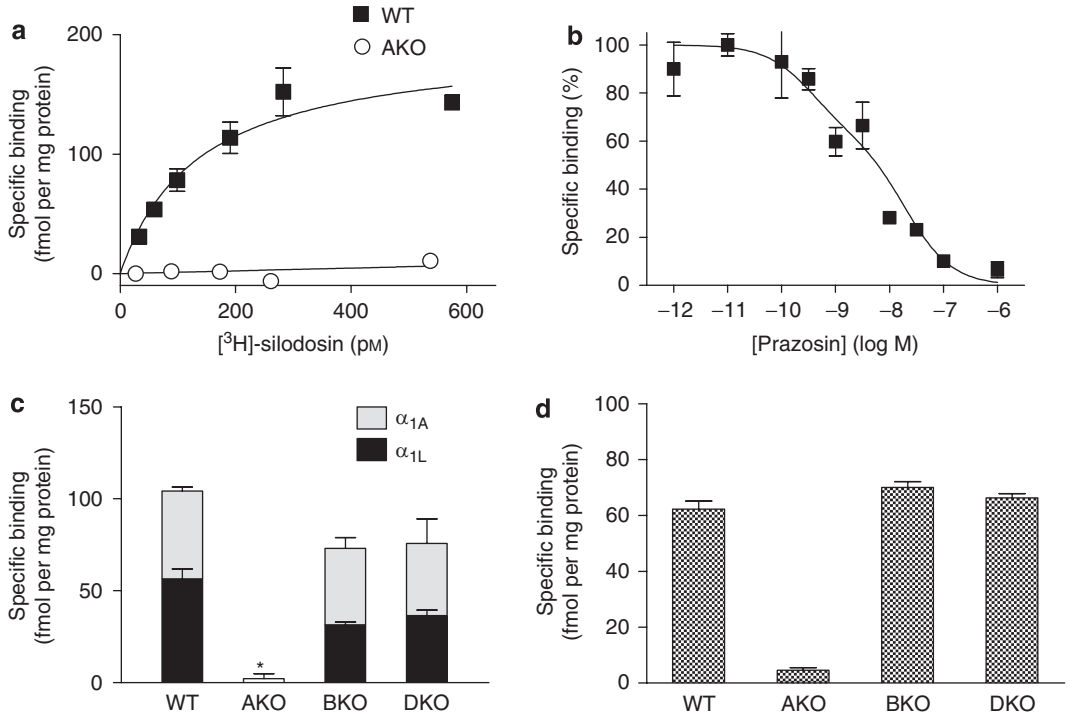

Figure 3 Binding of [^{3}H]-silodosin to mouse vas deferens and prostate. (**a**) Representative saturation curves for specific binding of [^{3}H]-silodosin in tissue segments of vas deferens isolated from WT and AKO mice. (**b**) Representative competition curve for prazosin at 500 pM [^{3}H]-silodosin-binding sites in tissue segments of the WT mouse vas deferens. (**c** and **d**) Binding capacities of 500 pM [^{3}H]-silodosin in the intact segments of WT, AKO, BKO and DKO mouse vas deferentia (**c**) and prostate (**d**). In panel c, high- and low-affinity sites for prazosin at [^{3}H]-silodosin-binding sites were represented as α_{1A} and α_{1L}, respectively. *Significantly different from WT mice ($P<0.01$). Mean ± s.e.mean of 3–6 experiments.

low-affinity α_1-adrenoceptors (α_{1L} subtype). In AKO mice, the response of the vas deferens to noradrenaline was abolished, whereas the same amplitude of contraction was produced by 100 μM noradrenaline in BKO and DKO mouse vas deferentia (Figure 5). The contractile responses to 10 μM noradrenaline in BKO and DKO mouse vas deferentia were not affected by treatment with 10 nM prazosin or 100 nM BMY 7378, but were attenuated by 3 nM silodosin to less than 20% ($n=3$ in BKO and DKO mice, respectively). α,β-Methylene ATP (1 μM, a P2X purinoceptor agonist) also produced a transient contraction, which was not affected by gene targeting of the three α_1-adrenoceptors (Figure 5b).

In WT mouse prostate, the cumulative application of noradrenaline produced concentration-dependent contraction (maximum amplitude of contraction = 53 ± 3 mg, $pEC_{50} = 6.5 \pm 0.2$, $n=15$). The contraction was relatively insensitive to prazosin, RS-17053 and 5-methylurapidil; low affinities were estimated (Figure 6, Table 2). However, the responses to noradrenaline were inhibited by low concentrations of silodosin ($pK_B = 9.6 \pm 0.3$, $n=5$) (Figure 6b). Furthermore, the response of the prostate was markedly attenuated in AKO mice, but not in BKO or DKO mice (Figures 7a and c). The residual contractile response to noradrenaline in AKO mice was not inhibited by 3 nM silodosin or 10 nM BMY 7378, but was insurmountably inhibited by 1 nM prazosin (Figure 7b). Amplitudes of contraction similar to those of WT mouse prostate were produced in prostate of BKO and DKO mice (Figure 7c), and the pEC_{50} values for noradrenaline were 6.5 ± 0.1 ($n=4$) in BKO mice and 6.4 ± 0.2 ($n=3$) in DKO mice. The concentration–response curves for noradrenaline in BKO and DKO mice were not attenuated by 1 nM prazosin and 10 nM BMY 7378, but were shifted to the right and downwards by 3 nM silodosin (pK_B value = 9.4 ± 0.2 in BKO mice and 9.5 ± 0.2 in DKO mice, $n=3$). The amplitudes of contractions induced by α,β-methylene ATP (10 μM) were not significantly different among WT, AKO, BKO and DKO mice (Figure 7c).

Discussion

[^{3}H]-Silodosin has very high apparent affinities for both α_{1A}-adrenoceptor and α_{1L}-adrenoceptor subtypes (Murata *et al*., 1999; Morishima *et al*., 2008). In recent radioligand binding studies with [^{3}H]-silodosin, it has been demonstrated that α_{1L}-adrenoceptors are pharmacologically distinct from the three classical α_1-adrenoceptors (α_{1A}, α_{1B} and α_{1D}) in the intact segments of the rat cerebral cortex (Morishima *et al*., 2008), rabbit ear artery (Hiraizumi-Hiraoka *et al*., 2004) and human and rabbit prostate (Morishima *et al*., 2007a; Su *et al*., 2008). In the present binding study with [^{3}H]-silodosin, the α_{1L}-adrenoceptor was recognized as a distinct site showing low affinity for prazosin, RS-17053 and BMY 7378, but high affinity for silodosin and tamsulosin in the intact tissue segments of the cerebral cortex, vas deferens and prostate of WT mouse, along with the α_{1A}-adrenoceptor, which showed high affinity for prazosin, RS-17053, silodosin and tamsulosin. The same results were also obtained in BKO and DKO mice, whereas the [^{3}H]-silodosin binding sites

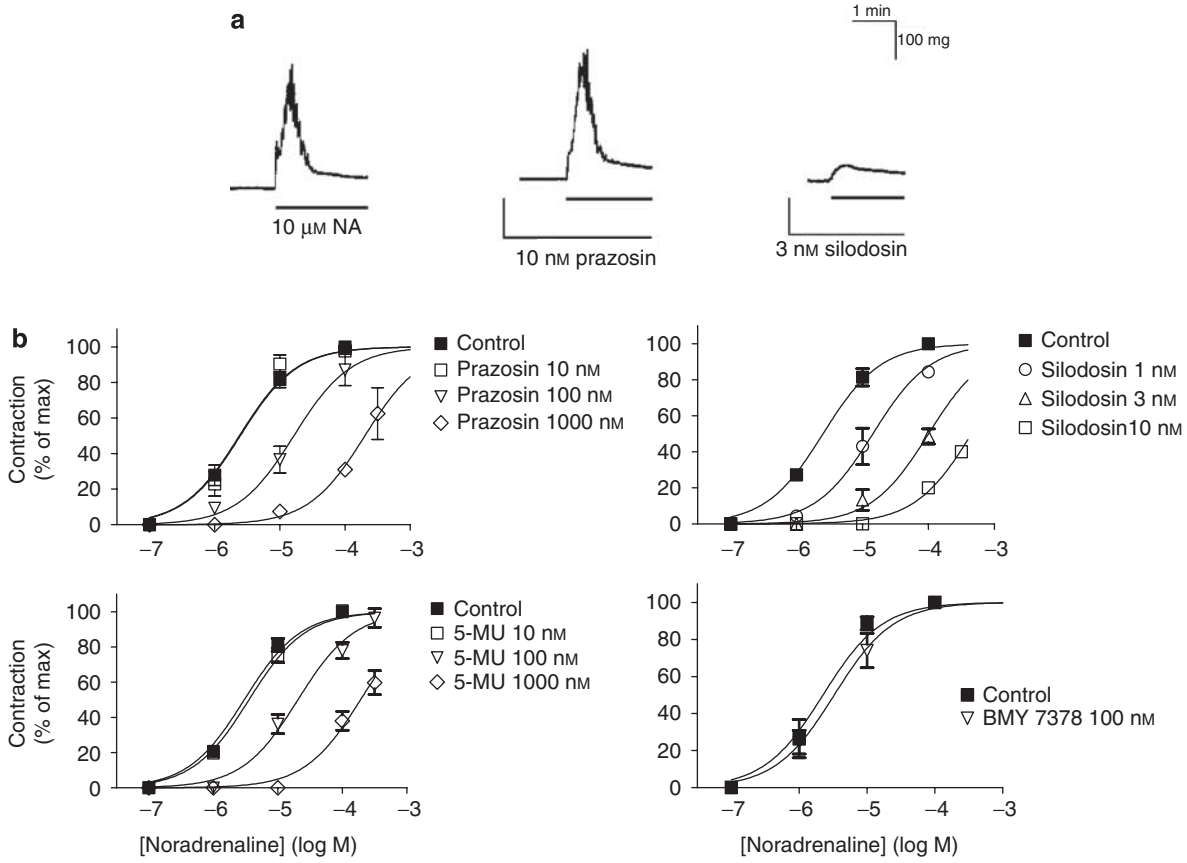

Figure 4 Contractile responses to noradrenaline in mouse vas deferens (epididymal portion). (**a**) Effects of prazosin and silodosin on the contractile responses to 10 μM noradrenaline (NA) in the vas deferens isolated from WT mice. In contrast to silodosin (3 nM), prazosin (10 nM) failed to inhibit the contractile response to noradrenaline. (**b**) Effects of prazosin, silodosin, 5-methylurapidil (5-MU) and BMY 7378 on the concentration–response curves for noradrenaline in the WT mouse vas deferens. Mean ± s.e.mean of 4–6 experiments. WT, wild type.

Table 2 Pharmacological characteristics of noradrenaline-induced contractions in WT mouse vas deferens and prostate

Antagonist	pK_B	
	Vas deferens	*Prostate*
Prazosin	7.7 ± 0.2	8.2 ± 0.3
Silodosin	9.7 ± 0.2[a]	9.6 ± 0.3[a]
RS-17053	NI	6.9 ± 0.2[a]
5-Methylurapidil	7.6 ± 0.2	7.4 ± 0.3
BMY 7378	NI	NI

Abbreviations: RS-17053, *N*-[2-(2-cyclopropylmethoxyphenoxy)ethyl]-5-chloro-α, α-dimethyl-1*H*-indole-3-ethamine hydrochloride; WT, wild type.
Mean ± s.e.mean of 4–6 experiments.
NI indicates no inhibition at 0.1 μM BMY 7378 and 1 μM RS-17053.
pK_B values were calculated by the Schild analysis.
[a]Estimated at 1 nM silodosin or 1 μM RS-17053 by the concentration ratio method (Furchgott, 1972).

disappeared in AKO mice. Furthermore, the functional studies with mouse vas deferens and prostate revealed that the α_1-adrenoceptors mediating contractile responses to noradrenaline showed an α_{1L}-adrenoceptor profile and that the contractions were specifically abolished in AKO mice, but not in BKO and DKO mice. Adrenergic contractions in vas deferens and prostate have been reported through α_{1L}-adrenoceptors in many mammalian species including mice (Ohmura *et al*., 1992; Hiraoka *et al*., 1999; Ford *et al*., 1996; Van der Graaf *et al*., 1997; Amobi *et al*., 2002; Gray and Ventura, 2006; Morishima *et al*., 2007a; Su *et al*., 2008). Thus, the present results strongly suggest that α_{1L}-adrenoceptors occur and function independently from other α_1-adrenoceptor subtypes, but that the expression of the α_{1L}-adrenoceptor is closely related to that of the α_{1A}-adrenoceptor gene, and that the α_{1B}-adrenoceptor and the α_{1D}-adrenoceptor are not involved in manifestation of the α_{1L}-adrenoceptor phenotype.

More recently, we reported in a study with the rat cerebral cortex that the α_{1L}-adrenoceptor could be detected in the intact segments, but that the pharmacological profile disappeared after homogenization (Morishima *et al*., 2008). The same phenomenon was observed in this study with WT mouse cortex. Interestingly, in both species, the total binding density of [^{3}H]-silodosin in the tissue segments of cerebral cortex was equal to the density of α_{1A}-adrenoceptors detected in the membrane preparations (Figure 1b). Because contamination with α_{1B}-adrenoceptors and α_{1D}-adrenoceptors is negligible in the present [^{3}H]-silodosin binding sites (Murata *et al*., 1999; Morishima *et al*., 2008), it seemed that the pharmacological profile of the α_{1L}-adrenoceptors converted to that of the α_{1A}-adrenoceptors by homogenization

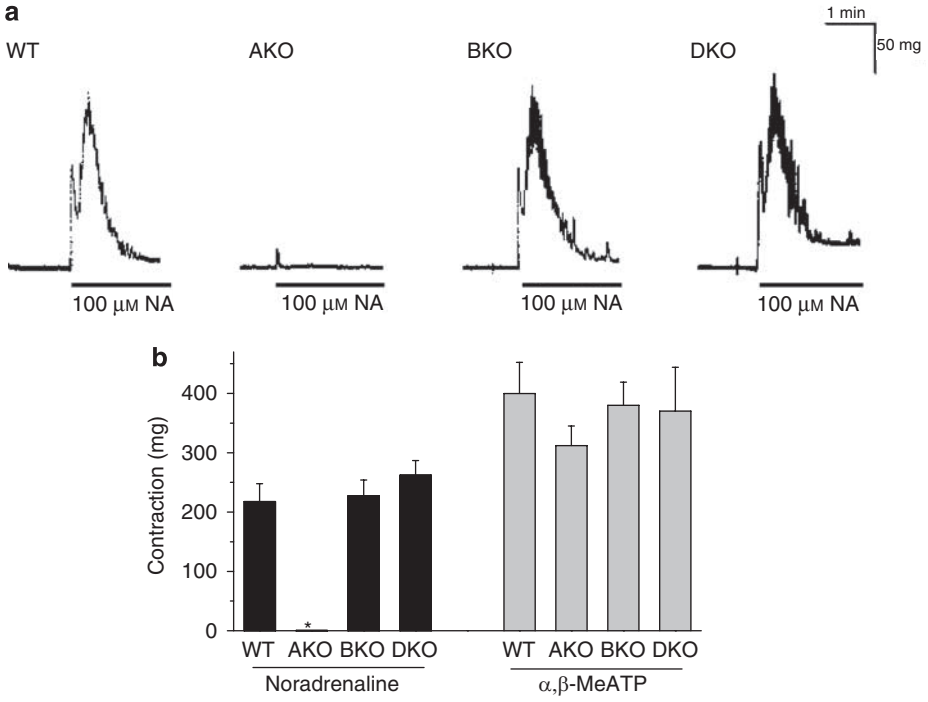

Figure 5 Effects of α_1-adrenoceptor gene knockout on the contractile responses in mouse vas deferens (epididymal portion). (**a**) Representative responses to 100 μM noradrenaline (NA) in WT, AKO, BKO and DKO mice. (**b**) Contractile responses induced by 100 μM noradrenaline and 1 μM α, β-methylene ATP (α,β-Me ATP). Mean ± s.e.mean of 3–6 experiments. *Significantly different from other columns ($P<0.01$). AKO, α_{1A}-adrenoceptor gene knockout; BKO, α_{1B}-adrenoceptor gene knockout; DKO, α_{1D}-adrenoceptor gene knockout; WT, wild type.

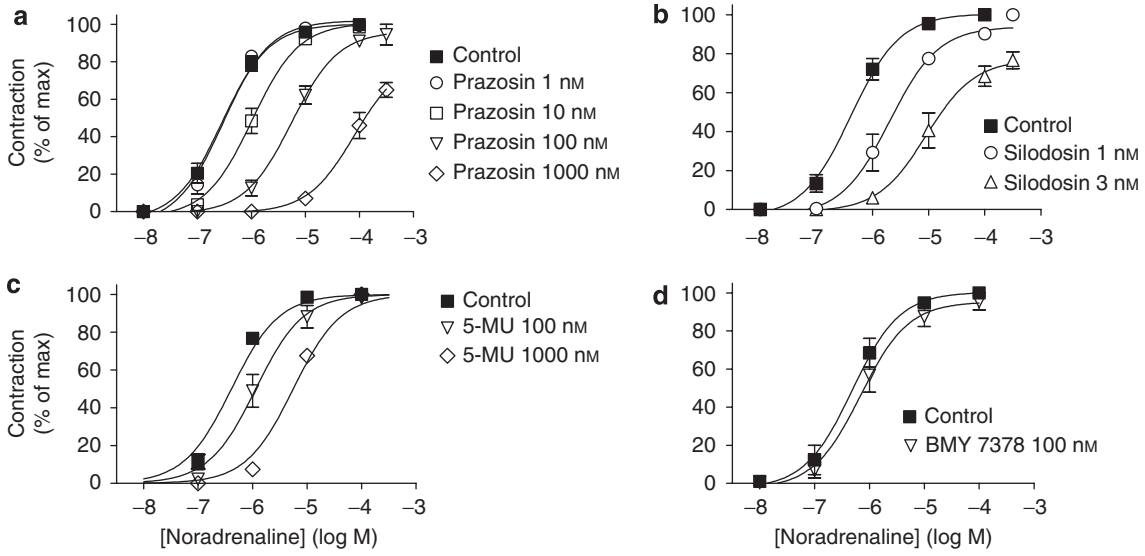

Figure 6 Effects of prazosin (**a**), silodosin (**b**), 5-methylurapidil (**c**; 5-MU) and BMY 7378 (**d**) on the concentration–response curves for noradrenaline in WT mouse prostate. Mean ± s.e.mean of 4–6 experiments. WT, wild type.

without a loss of yield. Moreover, the α_{1A}-adrenoceptors in the membrane preparations were abolished in AKO mice. These results show that both α_{1A}-adrenoceptor and α_{1L}-adrenoceptor are derived from the same α_{1A}-adrenoceptor gene, and further suggest that α_{1L}-adrenoceptor is a pharmacological phenotype of α_{1A}-adrenoceptor (Su *et al*., 2008).

[^{3}H]-prazosin also bound to the intact segments of WT mouse cortex with a high affinity and the binding sites were divided into two components by silodosin but not by BMY 17053. A similar population of silodosin high- and low-affinity sites and the same densities were observed in DKO mouse cortex (Figure 1c). However, sites for silodosin were selectively abolished in AKO mice (no high-affinity sites) or

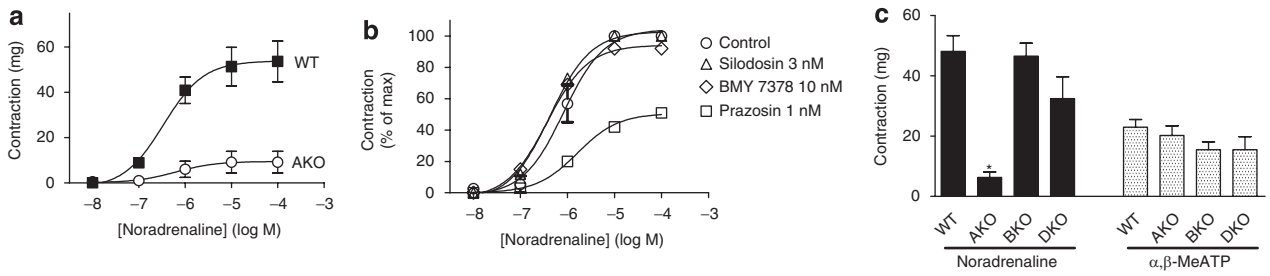

Figure 7 Contractile responses to noradrenaline in mouse prostate. (**a**) Concentration–response curves for noradrenaline in WT and AKO mouse prostate. Mean ± s.e.mean of 4–6 experiments. (**b**) Effects of silodosin, BMY 7378 and prazosin on the contractile response to noradrenaline in the AKO mouse prostate. This is a representative result obtained from three AKO mice. The response induced by 100 μM noradrenaline in the control was equivalent to 18.5 mg, which was taken as 100%. Prazosin (1 nM) produced insurmountable inhibition. (**c**) Contractile responses induced by 100 μM noradrenaline and 10 μM α,β-methylene ATP (α,β-Me ATP). Mean ± s.e.mean of 3–6 experiments. *Significantly different from other columns ($P<0.01$). AKO, α_{1A}-adrenoceptor gene knockout; WT, wild type.

BKO mice (no low-affinity sites), resulting in a complete loss of the corresponding subtypes. These results show that [^{3}H]-prazosin at the concentrations used in this experiment can selectively recognize α_{1A}-adrenoceptors and α_{1B}-adrenoceptors in the intact segments of WT and DKO mouse cortices, as reported in the rat cerebral cortex (Morrow and Creese, 1986; Morishima *et al*., 2008).

The α_{1A}-, α_{1B}- and α_{1L}-adrenoceptor subtypes were distinctly identified in the [^{3}H]-silodosin and [^{3}H]-prazosin binding sites in WT mouse, whereas in AKO mice, the α_{1A} and α_{1L} subtypes were specifically abolished and the α_{1B} subtype selectively disappeared in BKO mice. Thus, we conclude that no quantitative/qualitative compensation by other subtypes of the same α_1-adrenoceptor group (for example, upregulation or supersensitivity) is caused in knockout mice, which supports previous *in vivo* and *in vitro* studies with AKO, BKO and DKO mice (Cavalli *et al*., 1997; Rokosh and Simpson, 2002; Tanoue *et al*., 2002; Simpson, 2006). This conclusion also accounts for a complete loss of α_{1L}-adrenoceptor-mediated responses in the vas deferens and prostate of AKO mice and no significant shift of pEC_{50} values for noradrenaline between WT and BKO or DKO mouse prostate. It can be noted that the expression of each α_1-adrenoceptor subtype is differentially regulated in cardiac myocytes (Rokosh *et al*., 1996).

In contrast to AKO mouse vas deferens, a small but significant contraction was elicited by noradrenaline in AKO mouse prostate (Figure 7a). This residual contraction was insensitive to silodosin (1 nM, a specific concentration for α_{1A} and α_{1L}-adrenoceptors) and BMY 17053 (10 nM, a specific concentration for α_{1D}-adrenoceptor) but potently inhibited by prazosin (1 nM, a specific concentration for α_{1A}-, α_{1B}-, or α_{1D}-adrenoceptors) (Figure 7b). Therefore, a part of the contractile response to noradrenaline in mouse prostate may be mediated through α_{1B}-adrenoceptors, although the major component is still α_{1L}-adrenoceptor-mediated.

This study clearly revealed that two different phenotypes (α_{1A} and α_{1L}-adrenoceptors) originate from a single α_{1A}-adrenoceptor gene, yet no evidence explains why multiple phenotypes are produced from a single gene. However, it is interesting, in this context, to note the history of the β_4-adrenoceptor. Like α_{1L}-adrenoceptors, β_4-adrenoceptors were originally identified as functional receptors resistant to several β-adrenoceptor antagonists, such as propranolol (Kaumann and Molenaar, 1996; Sarsero *et al*., 1998). However, the β_4-adrenoceptor was absent after β_1-adrenoceptor gene knockout (Kaumann *et al*., 2001). At present, the β_1-adrenoceptor has been considered to have two distinct binding sites within the same receptor, orthosteric and allosteric sites with high and low affinity for catecholamines or propranolol (Konkar *et al*., 2000; Baker and Hill, 2007). The β_4-adrenoceptor reflects the pharmacological profiles of an allosteric site of β_1-adrenoceptors with low affinity for propranolol. Thus, it may be possible that the α_{1L} phenotype represents the presence of an allosteric site in the α_{1A}-adrenoceptor.

Another interesting finding in this study was that, even though α_{1L}-adrenoceptors occurred as a separate entity, independent of α_{1A}-adrenoceptors, in the intact segments, the pharmacological profile completely changed to that of classical α_{1A}-adrenoceptors upon homogenization. Such a change in profile upon homogenization was not seen in β_4-adrenoceptors (Kaumann *et al*., 2001; Joseph *et al*., 2003). Thus, if the α_{1L}-adrenoceptor is an allosteric site variant of the α_{1A}-adrenoceptor, it is likely that a more drastic conformational change is caused by homogenization in α_{1A}-adrenoceptors than in β_1-adrenoceptors, resulting in a greater change in profile. Alternatively, the α_{1L}-adrenoceptor may be a phenotype constructed with some associated proteins and the construct may be disrupted by homogenization. This has been demonstrated in the receptor for calcitonin gene-related peptide, in which different phenotypes are produced by association with different receptor-modifying proteins (McLatchie *et al*., 1998; Poyner *et al*., 2002). Moreover, the α_{1L}-adrenoceptor might be constructed under a particular environment with specific parameters, such as microdomains, relation to the cytoskeleton and extracellular ion environment, without involving an associated protein. Recently, it has been suggested that pharmacological properties may not necessarily remain constant between tissues expressing the same receptors or between different assay conditions (Kenakin *et al*., 1995; Muramatsu *et al*., 2005; Nelson and Challiss, 2007). Thus, the molecular mechanisms underlying the expression of the α_{1L}-adrenoceptor phenotype should be explored in future studies.

In summary, this study using knockout mice demonstrated that α_{1L}-adrenoceptors can be identified as binding and functional entities in mice, and strongly suggested that two

distinct phenotypes (α_{1A}-adrenoceptor and α_{1L}-adrenoceptor) are derived from the same α_{1A}-adrenoceptor gene.

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Conflict of interest

The authors state no conflict of interest.

Editor's note. Readers may note that the content of this paper is very similar to that of a paper by Gray *et al*. (Gray KT, Short JL, Ventura S (2008). The α_{1A}-adrenoceptor gene is required for the α_{1L}-adrenoceptor-mediated response in isolated preparations of the mouse prostate. *Br J Pharmacol* **155**: 103–109; doi:10.1038/bjp.2008.245) in this journal, and reaches similar conclusions. The first draft of this paper was received before acceptance of the paper by Gray *et al*., and the two manuscripts were handled independently by different editors and referees.

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