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Modulation of contraction by $\alpha_{2A/D}$-adrenoceptors in mouse aorta: evidence employing knockout technology

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1 We have investigated noradrenaline-evoked contractions in endothelium-denuded aorta from wild-type and $\alpha_{2A/D}$-adrenoceptor knockout mice. The maximum contraction to noradrenaline was significantly larger (1.36 ± 0.24 mN, $n=5$) in aorta from knockout than from wild-type animals (0.78 ± 0.14 mN, $n=12$, $P<0.05$), but there was no difference in potency of noradrenaline. There was no difference between groups in the contraction to KCl (80 mM) or $PGF_{2\alpha}$ (10 μM).

2 The contraction to noradrenaline (10 μM) was significantly larger in aorta from knockout animals, but yohimbine (1 μM) significantly increased this contraction (to $136 \pm 10\%$ of control, $n=6$) in aorta from wild-type but not from knockout ($97 \pm 10\%$, $n=6$, $P<0.05$).

3 In tissues precontracted with $PGF_{2\alpha}$ (10 μM), xylazine (1 μM) produced relaxations only in tissues from wild-type mice.

4 The K^+ channel blocker glibenclamide (1 μM) had no significant effects on contractions to noradrenaline in either group.

5 It is concluded that an $\alpha_{2A/D}$-adrenoceptor exerts an inhibitory modulation of contraction in mouse aorta.

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Abbreviations: CEC, chloroethylclonidine; $PGF_{2\alpha}$, prostaglandin F2α

Introduction

Contractions of rat aorta to noradrenaline involve mainly α_{1D}-adrenoceptors (Kenny *et al*., 1995; Piascik *et al*., 1995). However other α_1- and α_2-adrenoceptor genes are expressed (Ping & Faber, 1993; Rokosh *et al*., 1994). However, the possible participation of α_2-adrenoceptors in the myogenic responses of this vessel remains unclear. Although α_2-adrenoceptor agonists such as clonidine produces vasoconstriction of rat aorta, much of the effects involve α_1-adrenoceptors (Carrier & White, 1985; Iwanaga *et al*., 1998). Studies with the alkylating agent chloroethylclonidine (CEC) produced results consistent with the involvement of an α_2-adrenoceptor in contractions of rat aorta. In the presence of CEC, a component of the response to noradrenaline was resistant to blockade by the α_1-adrenoceptor antagonist prazosin (O'Rourke *et al*., 1995). This resistant component is prevented by pre-treatment with yohimbine prior to CEC, suggesting a role for α_2-adrenoceptors. Some authors have also proposed the existence of an α_2-adrenoceptor-induced vasodilator response of aortic smooth muscle cells through the opening of ATP-sensitive potassium channels (Fauaz *et al*., 2000).

Until now, studies investigating the involvement of α_2-adrenoceptors on vessel contraction, especially in the presence of a dominant α_1-adrenoceptor, based their findings on the effect of drugs with limited specificity. Currently, virtually all agonists and antagonists used to characterize α_2-adrenoceptors, have a significant affinity for both α_1- and α_2-adrenoceptors. For example, clonidine is usually used as an α_2-adrenoceptor 'specific' agonist because its affinity (pK_1 value) for α_2-adrenoceptors ($\approx$ 7.5) is greater than for α_1-adrenoceptors ($\approx$ 6.5) (Millan *et al*., 2000). However, there is always an ambiguity to assume that only α_2-adrenoceptors mediates the response to this agonist (see above). Similarly, the alkylating agent, CEC has a mixed action as well. It acts as an irreversible or reversible ligand at all subtypes of both α_1- and α_2-adrenoceptors (Michel *et al*., 1993) and is an irreversible agonist at α_2-adrenoceptors (Nunes & Guimaraes, 1993). The α_2-adrenoceptor antagonist, yohimbine, also acts as an antagonist on α_1-adrenoceptors in rat aorta with pA_2 close to 6.5 (Muramatsu *et al*., 1990; Aboud *et al*., 1993). Furthermore, the predominant α_1-adrenoceptor mediated component of the response induced by catecholamines on rat aorta makes the effects of activation of α_2-adrenoceptors difficult to establish.

Thus, the use of α_2-adrenoceptor knockout mice should allow observation of the consequences of the absence of these receptors on the regulation of vascular tone. *In vivo* experiments using $\alpha_{2A/D}$-adrenoceptor knockout mice have shown that following α_1 blockade, noradrenaline failed to induce any increase in blood pressure in the knockout mice (Duka *et al*., 2000). This effect was not observed in either α_{2B}-adrenoceptor and α_{2C}-adrenoceptor knockout. These authors concluded that α_2-adrenoceptor-mediated vasoconstriction is attributable to the $\alpha_{2A/D}$-subtype. However, α_{2C}-adrenoceptors mediate contractions of human saphenous vein (Gavin *et al*., 1997), and so represent a second vascular α_2-adrenoceptor subtype.

In this study, we initially compared endothelium-denuded aorta from wild-type and $\alpha_{2A/D}$-adrenoceptor knockout mice in their responsiveness to noradrenaline. The differences obtained led to further studies with α_2-adrenoceptor agonist

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and antagonist. Some of these results have been presented in abstract form (Vandeputte & Docherty, 2002).

Methods

Female wild-type and $\alpha_{2A/D}$-adrenoceptor knockout C57 Black mice (18–24 g) were obtained from Jackson Laboratories (Bar Harbor, Maine, U.S.A.) and the aorta was used as outlined below.

Mouse aorta

Aortic rings, 2–3 mm in length, were gently rubbed to remove the endothelium and mounted in a small vessel myograph with 40 μM tungsten wires (Mulvany & Halpern, 1977). Data were recorded on a dual channel electronic display recorder (Myo-Interface Model 400A) and analogue acquisition system (MacPacq. MP100, Biopac Systems). Vessels were allowed to equilibrate at 37°C in Krebs-Henseleit solution (95% O_2/5% CO_2) of the following composition (mM): NaCl 119, $NaHCO_3$ 25, glucose 11.1, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.0, EDTA 0.03 and ascorbic acid 0.28. Propanolol (3 μM) was also present. The vessel was set to a tension generated at 0.9 times the diameter of the vessel at 100 mmHg transmural pressure (Mulvany & Halpern, 1977). Arteries were allowed to equilibrate for 30 min under this passive tension.

In a first set of experiments, tissues were contracted with noradrenaline administered cumulatively in 0.5 log unit increments beginning with 1 nM up to 300 μM. At the plateau of contraction, acetylcholine (10 μM) was added to test the absence of endothelium, and the tissues were then washed. After washing for another 90 min, the concentration–response curve to noradrenaline was repeated. The bath was changed every 15 min.

In experiments with glibenclamide or yohimbine, tissues were contracted with noradrenaline administered cumulatively in 0.5 log unit increments beginning with 1 nM up to 10 μM. At the plateau of contraction, acetylcholine (10 μM) was added to test the absence of endothelium, and the tissues were then washed. After wash-out, arteries were allowed to relax for 60 min during which the bath was changed every 15 min. Tissues were then contracted with noradrenaline (10 μM) until the plateau of contraction was reached and glibenclamide (1 μM), yohimbine (0.1 to 1 μM) or vehicle was added to the bath for 10 min per concentration.

In the last set of experiments, arteries were challenged once with KCl (80 mM) and washed. Following a 30 min washout, arteries were challenged with a single concentration of noradrenaline (10 μM), at the plateau of contraction, acetylcholine (10 μM) was added to test the absence of endothelium, and the tissues were then washed. After washing-out for another 60 min, during which the bath was changed every 15 min, tissues were contracted with prostaglandin F2α ($PGF_{2\alpha}$, 10 μM) and, when the plateau of contraction was reached, xylazine (10 nM to 1 μM) was added cumulatively to the bath.

Drugs

Acetylcholine chloride; glibenclamide; (−)-noradrenaline bitartrate; (±)-propranolol hydrochloride; $PGF_{2\alpha}$ and yohimbine hydrochloride were purchased from Sigma, Ireland; xylazine was a gift from Bayer.

Drugs were dissolved in distilled water, except for glibenclamide which was first dissolved in 100% DMSO to give a stock solution of 10 mM, and $PGF_{2\alpha}$ which was first dissolved in 100% ethanol to give a stock solution of 10 mM. Final percentage of DMSO and ethanol in organ bath was 0.01 and 0.1% respectively.

Statistics

Values are mean ± s.e.mean from n experiments. Agonist pD_2 ($-\log EC_{50}$) values as well as maximal contraction in presence or absence of antagonist were compared with the effects of vehicle, by one-way ANOVA followed by the Student's t-test for unpaired data or the Bonferonni test. Statistical and graphical analysis was carried out using Instat for Macintosh and GraphPad Prism for PC. Data used to plot the dose response curves are the mean contraction induced at each concentration of the drug, and hence, the maximum response shown graphically differs from the maximum calculated from individual tissue maxima.

Results

Noradrenaline-induced maximal contractions (first concentration response curves) of wild-type ($n=35$) and α_2-adrenoceptor knockout ($n=26$) mice aortas were 0.62 ± 0.07 and 0.89 ± 0.11 mN respectively (response was significantly greater in vessels from knockout mice, $P<0.05$) with pD_2 values of 7.54 ± 0.15 and 7.45 ± 0.14 (non-significant). The addition of acetylcholine (10 μM) at the plateau of noradrenaline-induced contraction induced a small relaxation of 20 ± 4% (vehicle, 7 ± 6%, $n=5$, no significant differences) and 22 ± 4% (vehicle, -3 ± 8%, $n=4$, $P<0.05$) in wild-type and knockout mice respectively. In comparison, the acetylcholine-induced relaxation in endothelium-intact arteries is 63 ± 9% ($n=3$; $P<0.05$ *versus* endothelium-denuded arteries).

Noradrenaline-induced maximal contractions (second concentration response curve) of wild-type ($n=12$) and α_2-adrenoceptor knockout ($n=5$) mice aortas were 0.78 ± 0.14 and 1.36 ± 0.24 mN respectively (response was significantly greater in vessels from knockout mice, $P<0.05$) with pD_2 values of 6.87 ± 0.14 and 6.75 ± 0.30 (non-significant) (Figure 1).

In the following set of experiments, the second addition of noradrenaline (10 μM) to the bath induced a contraction of 0.65 ± 0.07 and 0.94 ± 0.10 mN in aortas from wild-type ($n=27$) and knockout mice ($n=22$) respectively ($P<0.05$). Increasing cumulative concentrations of yohimbine (0.1–1 μM) added at the plateau of the noradrenaline (10 μM)-induced contraction, potentiated in a dose-dependent manner the vasoconstriction in wild-type ($n=6$) but not in knockout ($n=5$) aortas (Figure 2). The maximal potentiation induced by yohimbine (1 μM) was 36 ± 10% of the initial noradrenaline-induced increase in tension in wild-type (to 136 ± 10% of control) and 0 ± 12% in knockout mice (to 100 ± 12% of control, $P<0.05$).

KCl (80 mM) induced increases in tension of 1.06 ± 0.22 and 1.19 ± 0.23 mN ($n=6$ per group) and $PGF_{2\alpha}$ (10 μM) induced increases in tension of 1.51 ± 0.21 and

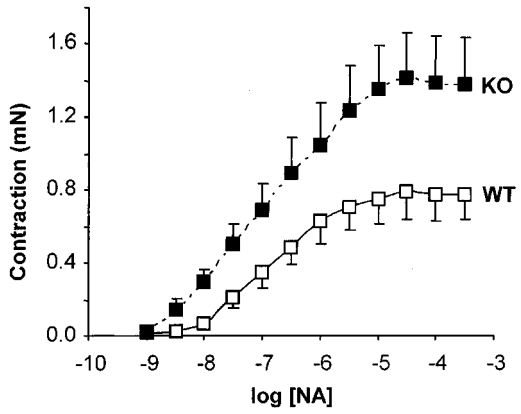

Figure 1 Noradrenaline (NA, 10^{-9} to 3×10^{-4} M)-induced contraction (second dose response curve) of aorta from wild-type (WT, $n=12$) and α_2-adrenoceptor knockout (KO, $n=5$) mice. Vertical bars indicate s.e.mean.

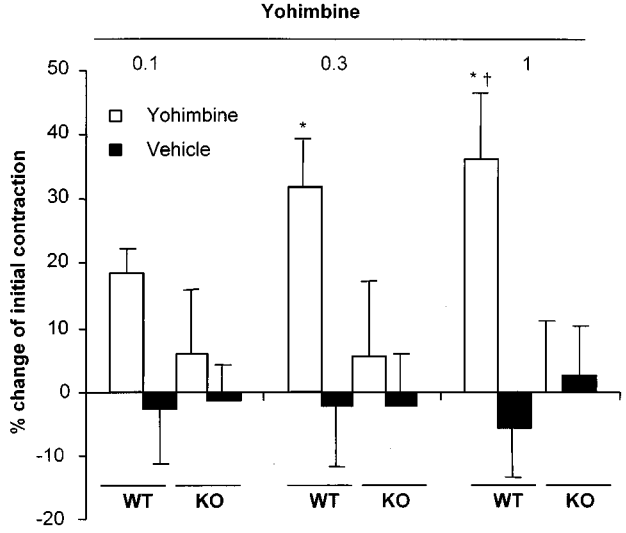

Figure 2 Effect of the α_2-adrenoceptor antagonist yohimbine (0.1–1 μM) on noradrenaline (10 μM)-induced vasoconstriction of aorta from wild-type (WT) and α_2-adrenoceptor knockout (KO) mice. *$P<0.05$ *versus* Vehicle; †$P<0.05$ *versus* KO. Vertical bars indicate s.e.mean. $n=4-6$ per group.

1.41 ± 0.33 mN ($n=6-7$ per group) in wild-type and knockout mice respectively (no significant differences). Increasing concentrations of xylazine (10 nM to 1 μM) added at the plateau of $PGF_{2\alpha}$-induced contraction produced a relaxation in wild-type but not in knockout mice ($n=6$ per group, Figure 3). In this last protocol, the addition of acetylcholine (10 μM) at the plateau of noradrenaline (10 μM)-induced contraction ($n=6-7$ per group) produced $11\pm4\%$ and $16\pm4\%$ relaxation in wild-type and knockout mice respectively (non-significant *versus* vehicle).

Glibenclamide (1 μM) had no effect on either strain of mice. In wild-type mouse aorta ($n=5$), increases in tension were 18 ± 9 and $17\pm6\%$ of the initial contraction induced by noradrenaline (10 μM) in presence of vehicle or glibenclamide (1 μM) respectively (to 118 ± 9 and $117\pm6\%$ respectively, $P>0.05$). In knockout mice ($n=3$), these changes in tension were -8 ± 5 and $-1\pm1\%$ in presence of vehicle and glibenclamide (1 μM) respectively (to 92 ± 5 and $99\pm1\%$ respectively, $P>0.05$).

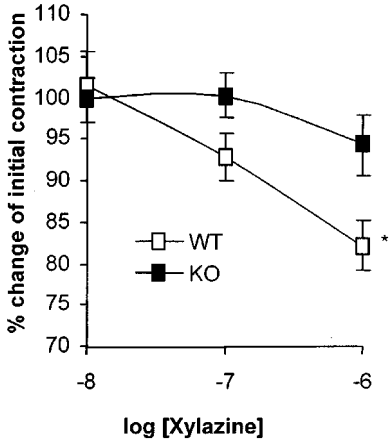

Figure 3 Effect of the α_2-adrenoceptor agonist xylazine (10 nM–1 μM) on $PGF_{2\alpha}$ (10 μM)-induced vasoconstriction of aorta from wild-type (WT) and α_2-adrenoceptor knockout (KO) mice. *$P<0.05$ *versus* KO. Vertical bars indicate s.e.mean. $n=6$ per group.

Discussion

This study of vascular contractions in aorta from wild-type and $\alpha_{2A/D}$-knockout mice did not start as simply a piece of serendipity, but the final outcome was largely unexpected.

Indeed, we have found that there were differences between wild-type and $\alpha_{2A/D}$-adrenoceptors knockout mice. The maximum contraction to noradrenaline, but not to KCl or $PGF_{2\alpha}$, was significantly increased in aorta from knockout without change in potency. This suggested that an $\alpha_{2A/D}$-adrenoceptor acts in wild-type to diminish contractions to noradrenaline. However, comparison between two groups of animal is fraught with difficulties when only baseline responses to agonists are examined. It was thus necessary to test whether α_2-adrenoceptor blockade could eliminate the differences. In $\alpha_{2/AD}$-adrenoceptor knockout mice, the α_2-adrenoceptor antagonist yohimbine, over the concentration range 0.1–1.0 μM, had no effect on the sustained contraction to noradrenaline. Higher concentrations of yohimbine tended to inhibit contractions to noradrenaline, which by analogy with the rat aorta (in which the pA_2 of yohimbine against noradrenaline at α_1-adrenoceptors is approximately 6.5), this action in mouse aorta is by α_1-adrenoceptor antagonism. In contrast, yohimbine 1 μM significantly increased contractions to noradrenaline in wild-type mice by approximately 40%. Hence, we have evidence that yohimbine blocks an inhibitory influence in aorta of wild-type but not $\alpha_{2A/D}$-knockout mouse, reducing the difference between wild-type and $\alpha_{2A/D}$-adrenoceptor knockout mice in contractions to noradrenaline. In order to confirm this hypothesis, we have also tested the impact of a α_2-adrenoceptor agonist, xylazine, on the $PGF_{2\alpha}$-mediated vasoconstriction. Xylazine relaxed $PGF_{2\alpha}$-induced contraction, and this effect reached significance for 1 μM xylazine. In contrast, we were unable to observe such an effect in aorta from $\alpha_{2A/D}$-adrenoceptor knockout mice.

It could be argued that the increased responsiveness of $\alpha_{2A/D}$-adrenoceptor knockout mice could be due to a vascular

remodelling which might be induced by the increased blood pressure of those animals (Duka *et al*., 2000). However, KCl- and $PGF_{2\alpha}$- induced tone were not different between the two strains. This suggests that the altered responsiveness of the aortas from $\alpha_{2A/D}$-adrenoceptors knockout mice is not dependent on a structural modification such an increase in smooth muscle cell content but is specific to the presence or absence of a α_2-adrenoceptors. Furthermore, we have shown that an α_2-adrenoceptor antagonist can eliminate this difference, ruling out vascular remodelling.

Having established that an $\alpha_{2A/D}$-adrenoceptor acts as an inhibitory modulator of contractions in mouse aorta, we wished to establish the mode of action. Since the vessels were functionally endothelium-denuded, as gauged by lack of effect of acetylcholine, we can exclude a role for endothelial mediators. It has been reported that the K^+ channel blocker glibenclamide prevents an α_2-adrenoceptor mediated hyperpolarization in rat aorta, suggesting that α_2-adrenoceptors are linked to opening of ATP-sensitive K^+ channels to diminish α_1-adrenoceptors mediated responses (Fauaz *et al*., 2000). However, since glibenclamide had no consistent effect in aorta from wild-type mouse, we are unable to confirm that the above finding also occurs in mouse. This may suggest the involvement of another K^+ channel or another mechanism, in the inhibitory effects of α_2-adrenoceptor stimulation in mouse aorta.

In conclusion, we have established that a $\alpha_{2A/D}$-adrenoceptor mediates inhibition of contraction in aorta from wild-type mouse and this effect is absent in aorta from $\alpha_{2A/D}$-adrenoceptor knockout mouse.

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References

ABOUD, R., SHAFII, M. & DOCHERTY, J.R. (1993). Investigation of the subtypes of α_1-adrenoceptor mediating contractions of rat aorta, vas deferens and spleen. *Br. J. Pharmacol.,* **109,** 80 – 87.

CARRIER, G.O. & WHITE, R.E. (1985). Enhancement of alpha-1 and alpha-2 adrenergic agonist-induced vasoconstriction by removal of endothelium in rat aorta. *J Pharmacol Exp Ther.,* **232,** 682 – 687.

DUKA, I., GAVRAS, I., JOHNS, C., HANDY, D.E. & GAVRAS, H. (2000). Role of the postsynaptic α_2-adrenergic receptor subtypes in catecholamine-induced vasoconstriction. *Gen. Pharmacol.,* **24,** 101 – 106.

FAUAZ, G., FERES, T., BORGES, A.C.R. & PAIVA, T.B. (2000). Alpha-2 adrenoceptors are present in rat aorta smooth muscle cells, and their action is mediated by ATP-sensitive K^+ channels. *Br. J. Pharmacol.,* **131,** 788 – 794.

GAVIN, K.T., COLGAN, M.P., MOORE, D., SHANIK, G. & DOCHERTY, J.R. (1997). α_{2C}-adrenoceptors mediate contractile reponses to noradrenaline in the human saphenous vein. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **355,** 406 – 411.

IWANAGA, S., SHIBATA, O., TSUDA, A., HASHIMOTO, S., MAKITA, T., CHO, S. & SUMIKAWA, K. (1998). The role of alpha1-adrenoceptors in the clonidine-induced contraction and relaxation of rat aorta. *Res. Commun. Mol. Pathol. Pharmacol.,* **102,** 137 – 147.

KENNY, B.A., CHALMERS, D.H., PHILPOTT, P.C. & NAYLOR, A.M. (1995). Characterization of an α_{1D}-adrenoceptor mediating the contractile response of rat aorta to noradrenaline. *Br. J. Pharmacol.,* **115,** 981 – 986.

MICHEL, M.C., KERKER, J., BRANCHEK, T.A. & FORRAY, C. (1993). Selective irreversible binding of chloroethylclonidine at alpha1- and alpha2-adrenoceptor subtypes. *Mol. Pharmacol.,* **44,** 1165 – 1170.

MILLAN, M.J., DEKEYNE, A., NEWMAN-TANCREDI, A., CUSSAC, D., AUDINOT, V., MILLIGAN, G., DUQUEYROIX, D., GIRARDON, S., MULLOT, J., BOUTIN, J.A., NICOLAS, J.P., RENOUARD-TRY, A., LACOSTE, J.M. & CORDI, A. (2000). S18616, a highly potent, spiroimidazoline agonist at α_2-adrenoceptors: I. Receptor profile, antinociceptive and hypothermic actions in comparison with dexmedetomidine and clonidine. *J. Pharmacol. Exp. Ther.,* **295,** 1192 – 1205.

MULVANY, M.J. & HALPERN, W. (1977). Contractile properties of small arteial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ. Res.,* **41,** 19 – 25.

MURAMATSU, I., OHMURA, T., KIGOSHI, S., HASHIMOTO, S. & OSHITA, M. (1990). Pharmacological subclassification of α_1-adrenoceptors in vascular smooth muscle. *Br. J. Pharmacol.,* **99,** 197 – 201.

NUNES, J.P. & GUIMARAES, S. (1993). Chloroethylclonidine irreversibly activates postjunctionnal alpha2-adrenoceptors in the dog saphenous vein. *Naunyn-Scmiedeberg Arch. Pharmacol.,* **348,** 264 – 268.

O'ROURKE, M., KEARNS, S. & DOCHERTY, J.R. (1995). Investigation of the actions of chloroethylclonidine in rat aorta. *Br. J. Pharmacol.,* **115,** 1399 – 1406.

PIASCIK, M.T., GUARINO, R.D., SMITH, M.S., SOLTIS, E.E., SAUSSY JR., D.L. & PEREZ, D.M. (1995). The specific contribution of the novel alpha-1D adrenoceptor to the contraction of vascular smooth muscle. *J. Pharmacol. Exp. Ther.,* **275,** 1583 – 1589.

PING, P. & FABER, J.E. (1993). Characterization of alpha-adrenoceptor gene expression in arterial and venous smooth muscle. *Am. J. Physiol.,* **265,** H1501-H1509.

ROKOSH, D.G., BAILEY, B.A., STEWART, A.F.R., KARNS, L.R., LONG, C.S. & SIMPSON, P.C. (1994). Distribution of α_{1C}-adrenergic receptor mRNA in adult rat tissues by RNase protection assay and comparison with α_{1B} and α_{1D}. *Biochem. Biophys. Res. Comm.,* **200,** 1177 – 1184.

VANDEPUTTE, C. & DOCHERTY, J.R. (2002). Investigation of α-adrenoceptor-mediated responsiveness of aorta from $\alpha_{2A/D}$-adrenoceptor knockout mice. *Br. J. Pharmacol.* (abstract, *in press*).

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