Abnormal Regulation of the Sympathetic Nervous System in α_{2A}-Adrenergic Receptor Knockout Mice

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This paper is available online at http://www.molpharm.org

ABSTRACT

α_2-Adrenergic receptors (ARs) play a key role in regulating neurotransmitter release in the central and peripheral sympathetic nervous systems. To date, three subtypes of α_2-ARs have been cloned (α_{2A}, α_{2B}, and α_{2C}). Here we describe the physiological consequences of disrupting the gene for the α_{2A}-AR. Mice lacking functional α_{2A} subtypes were compared with wild-type (WT) mice, with animals lacking the α_{2B} or α_{2C} subtypes, and with mice carrying a point mutation in the α_{2A}-AR gene (α_{2A}D79N). Deletion of the α_{2A} subtype led to an increase in sympathetic activity with resting tachycardia (knockout, 581 ± 21 min^{-1}; WT, 395 ± 21 min^{-1}), depletion of cardiac tissue norepinephrine concentration (knockout, 676 ± 31 pg/mg protein; WT, 1178 ± 98 pg/mg protein), and down-regulation of cardiac β-ARs (B_{max}: knockout, 23 ± 1 fmol/mg protein; WT, 31 ± 2 fmol/mg protein). The hypotensive effect of α_2 agonists was completely absent in α_{2A}-deficient mice. Presynaptic α_2-AR function was tested in two isolated vas deferens preparations. The nonsubtype-selective α_2 agonist dexmedetomidine completely blocked the contractile response to electrical stimulation in vas deferens from α_{2B}-AR knockout, α_{2C}-AR knockout, α_{2A}D79N mutant, and WT mice. The maximal inhibition of vas deferens contraction by the α_2 agonist in α_{2A}-AR knockout mice was only $42 \pm 9\%$. [^{3}H]Norepinephrine release studies performed in vas deferens confirmed these findings. The results indicate that the α_{2A}-AR is a major presynaptic receptor subtype regulating norepinephrine release from sympathetic nerves; however, the residual α_2-mediated effect in the α_{2A}-AR knockout mice suggests that a second α_2 subtype (α_{2B} or α_{2C}) also functions as a presynaptic autoreceptor to inhibit transmitter release.

α_2-Adrenergic receptors (ARs) play a prominent role in the regulation of the sympathetic nervous system (Ruffolo et al., 1991). Activation of α_2-ARs in the brainstem leads to a reduction in sympathetic tone, with a resultant decrease in heart rate and blood pressure. This effect is augmented by stimulation of α_2-ARs on sympathetic nerve terminals. These presynaptic α_2-ARs serve as autoreceptors regulating catecholamine release. There are three α_2-AR subtypes (α_{2A}, α_{2B}, and α_{2C}), and studies using gene-targeting strategies indicate independent functions for each (Link et al., 1996; MacMillan et al., 1996; Sallinen et al., 1997). Resting blood pressure and heart rate were not significantly altered by disruption of either the α_{2B} or α_{2C} gene, indicating that neither receptor is necessary for normal sympathetic regulation (Link et al., 1996). Intra-arterial administration of clonidine-like α_2 agonists produced a biphasic blood pressure response in wild-type (WT) mice so an initial brief pressor effect was followed by a sustained fall in arterial blood pressure (Link et al., 1996; MacMillan et al., 1996). This is a characteristic cardiovascular response pattern of clonidine-like drugs in other mammals (Hoefke and Kobinger, 1966). The vasopressor response to α_2 agonists was absent in the α_{2B} knockout (KO) mice, indicating that the α_{2B}-AR mediates vasoconstriction in some vascular beds. The response to α_2 agonist was not altered in the α_{2C}-deficient mice. Recent studies indicate that the α_{2C}-AR plays a role in several aspects of behavior (Sallinen et al., 1998).

Much has been learned about the function of the α_{2A}-AR from mice with a targeted mutation of the α_{2A}-AR in the second transmembrane at position 79 (α_{2A}D79N) (MacMillan et al., 1996). In cultured cell lines, the α_{2A}D79N mutant receptor failed to activate K$^+$ currents but exhibited normal inhibition of voltage-gated calcium channels and cAMP production (Surprenant et al., 1992). The α_{2A}D79N mice were developed to study the physiological importance of K$^+$ current regulation by the α_{2A}-AR. Surprisingly, targeted mutation of the α_{2A}-AR gene reduced expression of the α_{2A}D79N mutant receptor by 80% as determined by radioligand binding assays in brain (MacMillan et al., 1996). α_{2A}D79N mu-

ABBREVIATIONS: AR, adrenergic receptor; KO, knockout; ES, embryonic stem; KAC, potassium acetate; CYP, iodocyanopindolol.

tant mice had normal resting heart rate and blood pressure. The initial hypertensive response to an α_2 agonist was similar in α_{2A}D79N and WT mice; however, the hypotensive response to α_2 agonists was absent, demonstrating that the α_{2A}-AR mediates this brainstem response.

We now report the physiological effects of disrupting the α_{2A}-AR gene and describe differences between these mice and the α_{2A}D79N mice. Cardiovascular studies show that unlike the α_{2A}D79N mice, α_{2A}KO mice have a resting tachycardia. This difference can be attributed to the loss of presynaptic autoregulation in α_{2A}KO mice, which is preserved in α_{2A}D79N mutant mice.

Materials and Methods

Gene Targeting. The murine α_{2A}-AR gene (4 kb) and flanking regions (5′ arm, 3 kb; 3′ arm, 6 kb) was subcloned into pBluescript II SK(−). A 1672-bp neomycin resistance cassette (*neo*) containing the PGK promoter, neomycin resistance gene, and bovine growth hormone poly(A) sequence was inserted into a unique *Bgl*II site within the α_{2A}-AR gene. The *neo* sequence was inserted in the opposite orientation relative to the α_{2A}-AR gene, resulting in a premature termination codon within the third transmembrane domain. A herpes simplex virus thymidine kinase cassette (*hsv-tk*) was inserted downstream of the 6-kb 3′ flanking region. R1 embryonic stem (ES) cells were electroporated with 40 μg of linearized targeting construct and placed under selection with G418 and ganciclovir. Resistant ES cell colonies were screened, and 37 of 132 were correctly targeted. Targeted ES cells clones were aggregated with eight cell FVB/N mouse embryos and cocultured overnight. The resulting blastocysts were transferred to pseudopregnant mice. Twenty-two male chimeric mice were generated and distinguished by agouti coat color carried by the R1 ES cell genome. Three of these chimeric mice transmitted the R1 ES cell genome with the disrupted α_{2A}-AR locus to offspring. The generation of α_{2B}KO mice, α_{2C}KO mice, and α_{2A}D79N mice has been described previously (Link et al., 1995, 1996; MacMillan et al., 1996).

Saturation and Competition Binding. Brain membranes were prepared by homogenizing whole brain in lysis buffer (10 mM Tris · HCl, 5 mM EDTA, pH 7.4), followed by centrifugation at 10,000g. The pellet was washed in Tris · HCl buffer (75 mM Tris · HCl, 12.5 mM MgCl$_2$, 1 mM EDTA, pH 7.4), followed by centrifugation at 10,000g. The pellet was resuspended in potassium acetate (KAC) binding buffer (50 mM KAC, pH 7.4), and protein concentration was determined. For α_2-AR saturation binding experiments, 180 to 250 μg of homogenate protein was used in a 500-μl reaction containing 1 to 10 nM [³H]RX81002, with or without 1 μM atipamezole and KAC binding buffer. For α_2-AR competition binding experiments, 200 to 250 μg of homogenate protein was used in a 500-μl reaction containing 1 nM [³H]RX81002, 1 to 1000 nM yohimbine, with or without 1 μM atipamezole, and KAC binding buffer. All binding assay mixtures were incubated at room temperature for 1 h. For β-AR saturation binding, heart homogenates were prepared by Polytron homogenization of whole heart in lysis buffer, followed by centrifugation at 10,000g. The pellet was washed, and 50 to 100 μg of homogenate protein was used in a 500-μl reaction containing 1 to 300 pM [¹²⁵I]iodocyanopindolol ([¹²⁵I]CYP), with or without 1 μM (*dl*)-propranolol, in Tris · HCl binding buffer. The binding assay mixtures were incubated for 2 h. Binding reactions were terminated by filtration using a Brandel cell harvester. Membrane-bound [³H]RX821002 was determined by scintillation counting, and [¹²⁵I]CYP was determined by gamma emission. The results were analyzed with a nonlinear least-squares curve-fitting technique (Prism; GraphPAD, San Diego, CA).

In Vivo Cardiovascular Physiology. Studies were performed on eight WT and eight α_{2A}-AR KO adult mice (10–20 weeks old) that were generated from α_{2A}-AR heterozygote breeding. The mice were anesthetized with isoflurane (1–3%), and a polyethylene (PE10) catheter that had been stretched (0.5 mm o.d.) was inserted into the left internal carotid artery. The catheter was tunneled s.c. to exit at the base of the neck and placed within a s.c. pouch. After 24 h of recovery, the catheter was removed from the s.c. pouch, flushed with saline, and connected to a Spectramed DTX Plus pressure transducer. Heart rate and mean aortic blood pressure were recorded with a Gould eight-channel recorder and digitized on the Crystal Biotech Dataflow system (Hopkinton, MA). Baseline hemodynamics were continuously recorded for 1 h after the animal was placed in the study cage. To examine the role of vagal tone on baseline heart rate, atropine sulfate (1 mg/kg i.a.) was administered, and hemodynamics were recorded. Similarly, to examine the role of sympathetic tone on baseline heart rate, hemodynamics were recorded after propranolol administration (3 mg/kg i.a.). The next day, hemodynamic responses to dexmedetomidine (5 μg/kg i.a.) were recorded.

Tissue Norepinephrine Levels. Tissue norepinephrine concentrations were measured from the supernatants of whole heart and kidney homogenates. Tissue samples were homogenized on ice in 0.1 M sodium phosphate (pH 7.4), and a small sample was removed for protein determination. Perchloric acid was added (0.6 M final concentration), and samples were centrifuged at 10,000g for 3 h at 4°C. The resulting supernatants were analyzed by HPLC.

Vas Deferens Contractions. Vasa deferentia were isolated after sacrifice by cervical dislocation. The tissue was suspended on a force transducer and placed in an organ bath filled with physiological buffer solution consisting of 118 mM NaCl, 4.7 mM KCl, 3.0 mM CaCl$_2$, 1.22 mM KH$_2$PO$_4$, 25 mM NaHCO$_3$, and 10 mM glucose, oxygenated with a mixture of 95% O$_2$/5% CO$_2$. The samples were allowed to equilibrate for 30 min, and then 200 mg of resting tension was applied. Electrical field stimulation was applied to produce nerve terminal depolarization and neurotransmitter release (two electrical pulses every 10 s: 30 V, 0.9-ms width, 100-ms interval). The force of muscle contraction was digitized and displayed on a MacLab data analysis package. After contractions had equilibrated, dexmedetomidine was added to the organ chamber in a cumulative fashion every 1.5 min without changing the bath solution. In a separate group of animals, yohimbine was administered to the organ baths, also in a cumulative manner every 1.5 min without changing the medium.
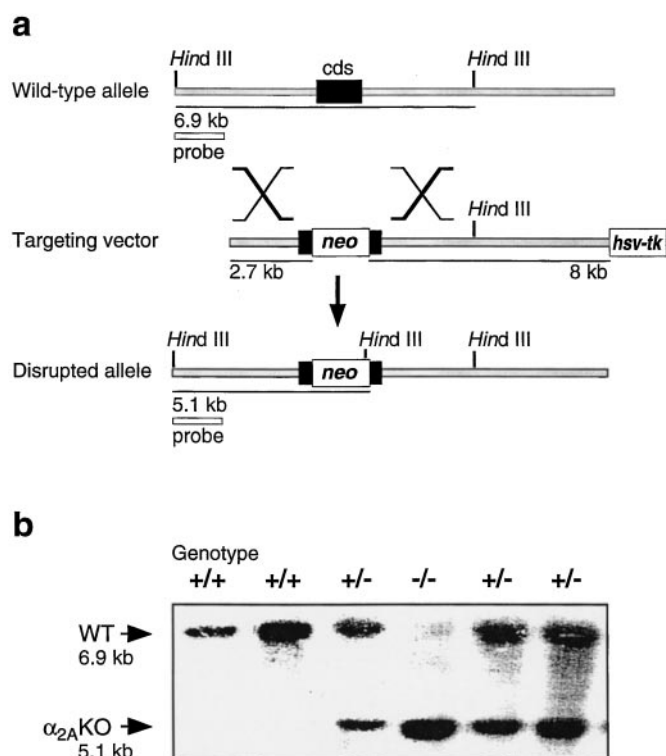
[³H]Norepinephrine Release. The release of [³H]norepinephrine from mouse vas deferens was determined as described previously for other mouse tissues (Limberger et al., 1995; Wahl et al., 1996), with minor modifications. Briefly, small pieces of the vas deferens were incubated with [³H]norepinephrine (0.1 μM) in physiological buffer (Wahl et al. 1996) for 30 min. Tissue pieces were then superfused with [³H]norepinephrine-free medium containing 1 μM desipramine at a rate of 1.2 ml/min. Transmitter release was elicited by rectangular pulses of 1-ms width and 47-V/cm voltage, giving a current strength of 80 mA. There were six stimulation periods in each experiment, applied at intervals of 18 min (S$_1$ to S$_6$). Each period consisted of one train of 20 pulses at 50 Hz. Medetomidine was added at cumulatively increasing concentrations 12 min before S$_2$ to S$_6$. At the end of experiments, tissues were solubilized, and tritium was determined in superfusate samples and tissues. Electrically evoked overflow of total tritiated compounds was calculated as the difference between total tritium outflow and estimated basal outflow and was expressed as a percentage of tissue tritium at the time of stimulation (Wahl et al., 1996). A logistic curve was fitted to the concentration-inhibition data of medetomidine (Trendelenburg et al., 1993). The electrically evoked overflow of total tritium reflects exocytotic release of [³H]norepinephrine (Taube et al., 1977) and is termed thus in this report. Medetomidine (racemic) was used in these experiments because of the limited availability of the dextroisomer dexmedetomidine. Both compounds are nonselective α_2 agonists.

Statistical Analysis. All results are expressed as mean ± S.E.M. Baseline hemodynamics and tissue catecholamine levels were ana-

lyzed by independent t test. Responses to dexmedetomidine were analyzed by two-way ANOVA for repetitive measures. The Mann-Whitney test was used for statistical comparison between experimental groups in the catecholamine release studies.
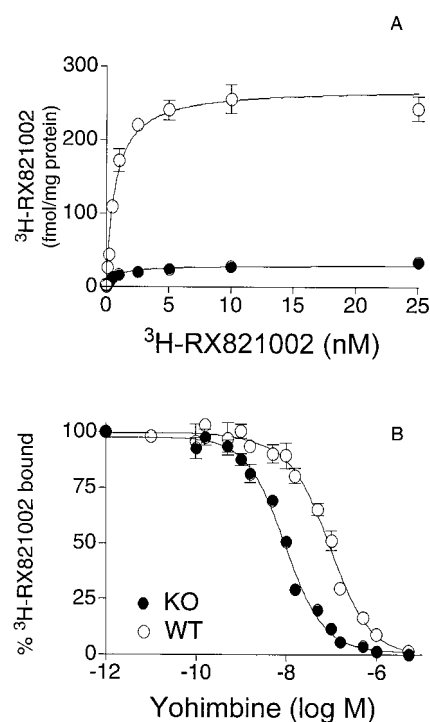
Results

Generation of α_{2A}-AR-Deficient Mice. To disrupt the α_{2A}-AR gene (*Adra2a*) in embryonic stem cells, a targeting vector was constructed that interrupted the coding region of the α_{2A}-AR by insertion of a neomycin resistance cassette (*neo*; Fig. 1a). The disrupted α_{2A} gene could be distinguished from the WT allele as a 5.1-kb *Hin*dIII fragment by a 5′ Southern probe that lies outside the region of homology with the targeting vector (Fig. 1a). From the correctly targeted stem cell clones, three germline transmitting chimeras were generated by morula aggregation. One hundred thirty-one F_2 mice were generated from heterozygous α_{2A}-AR (FVB/N, 129/SV) intercrosses and genotyped to determine whether disruption of the α_{2A}-AR gene had a significant impact on development or viability before weaning. The distribution of WT, heterozygous, and homozygous (α_{2A}KO) mice did not deviate significantly from that predicted by Mendelian genetics, indicating that there was no significant increase in mortality rates in α_{2A}-AR-deficient mice (data not shown). KO mice could not be distinguished from WT or heterozygous littermates by appearance, body weight, fertility, or viability.

α_{2A}-AR Expression in Brain. Saturation binding with [³H]RX821002, a nonsubtype-selective α_2 antagonist, was performed on membranes prepared from brains of α_{2A}KO and WT mice (Fig. 2A). α_{2A}KO mice showed a 90% reduction in specific [³H]RX821002 binding (B_{max}: α_{2A}KO 29 ± 2 fmol/mg protein; WT, 281 ± 27 fmol/mg protein). The residual α_2-AR binding in α_{2A}KO mice was anticipated based on previous reports suggesting that ~10% of the α_2-AR in the brain is of the α_{2C} subtype (Ordway et al., 1993). To confirm that the residual binding in the KO mice was not of the α_{2A}-AR subtype, competition assays were performed with yohimbine (Fig. 2B). Yohimbine, an α_2 antagonist, has an unusually low affinity for the rodent α_{2A}-AR subtype (Link et al., 1992). In the WT mice, yohimbine inhibited binding of [³H]RX821002 in a concentration-dependent manner displaying a small population (4 ± 6%) of high-affinity receptors (K_i = 1 nM) and large population of low-affinity receptors (K_i = 37 ± 3 nM). In KO mice, only high-affinity sites were present (K_i = 4.0 ± 0.3 nM), confirming that the residual α_2-AR in the KO mice is not of the α_{2A}-AR subtype.

Cardiovascular Physiology. Mean aortic blood pressure and heart rate were recorded 24 h after the insertion of a left carotid artery catheter and while the mice were quietly resting (Fig. 3). Heart rate was significantly higher in α_{2A}KO mice (KO, 581 ± 21 min⁻¹; WT, 395 ± 21 min⁻¹). Mean aortic blood pressure was not significantly different (KO, 131 ± 8 mm Hg; WT, 128 ± 5 mm Hg). Atropine was then administered to determine whether the observed tachycardia in α_{2A}KO mice

Fig. 1. Generation of mice lacking a functional α_{2A}-AR (*Adra2a*) gene. a, targeting vector for disruption of the *Adra2a* gene in mouse embryonic stem cells by homologous recombination. Insertion of the neomycin resistance gene (*neo*) disrupts the coding sequence (cds) of the α_{2A}-AR. hsv-tk, herpes simplex virus thymidine kinase gene. b, Southern blot analysis of progeny from crosses of heterozygous mice carrying the mutant α_{2A} allele. Using the external probe depicted in a, the WT allele can be detected as a 6.9-kb band. The disrupted allele (KO) is detected as a 5.1-kb band after *Hin*dIII digestion of genomic DNA.

Fig. 2. Characterization of α_2-AR radioligand binding in brain membranes from α_{2A}KO and WT mice. A, saturation binding of the α_2-AR ligand [³H]RX821002 in WT (○) and α_{2A}KO (●) membranes. Maximal specific binding of [³H]RX821002 is reduced by 90% in α_2-deficient membranes compared with WT membranes. Nonspecific binding was determined in the presence of 1 μM atipamezole. B, competition of [³H]RX821002 binding to brain membranes by yohimbine. Each point represents the mean ± S.E.M. derived from three mice. Yohimbine displaced specifically bound [³H]RX821002 with higher affinity in α_{2A}-deficient membranes than in membranes from WT mice.
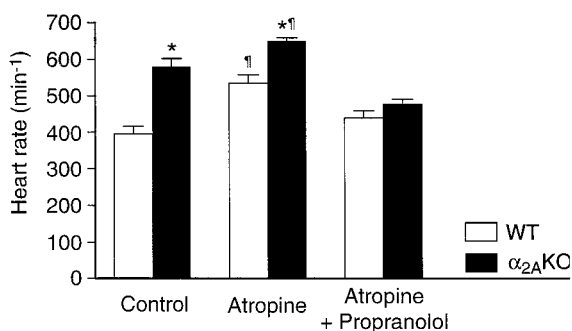
was due to a reduction in vagal tone (Fig. 3). Atropine significantly increased heart rate in both WT and α_{2A}KO mice. This increase was greater in WT mice, although heart rate remained significantly higher in KO mice. Propranolol was then administered to block sympathetic influence on heart rate. Propranolol produced a greater reduction in heart rate in KO mice so that heart rates were no longer significantly different between genotypes (KO, 479 ± 13 min^{-1}; WT, 437 ± 23 min^{-1}). In a separate group of WT and α_{2A}KO mice, propranolol was administered without pretreatment with atropine. This was done to determine whether propranolol alone could normalize the heart rate. The reduction in heart rate was greater in KO mice, so the heart rates were not significantly different after β-blockade (before propranolol: KO, 520 ± 46 min^{-1}; WT, 409 ± 29 min^{-1}; after propranolol: KO, 370 ± 32 min^{-1}; WT, 365 ± 44 min^{-1}, $n = 3$).

The hemodynamic response to infusion of the nonsubtype-selective α_2 agonist dexmedetomidine in WT and α_{2A}KO mice is shown in Fig. 4. Dexmedetomidine produced a biphasic blood pressure response in WT mice. The initial pressor response was greater in α_{2A}KO mice (maximum increase: KO, 20 ± 4 mm Hg; WT, 10 ± 4 mm Hg). However, dexmedetomidine failed to reduce blood pressure in α_{2A}KO mice (maximum decrease: KO, 0 ± 2 mm Hg; WT, 25 ± 4 mm Hg). Administration of the α_2 agonist caused a marked decrease in heart rate in both WT and α_{2A}KO mice (Fig. 4b); however, the maximal bradycardic response to dexmedetomidine was reduced in α_{2A}KO compared with WT mice.

Tissue Norepinephrine Levels. The increased heart rate in α_{2A}KO mice suggested that norepinephrine release from sympathetic terminals was enhanced in vivo. Because reliable determinations of plasma catecholamines are difficult to obtain in mice, tissue norepinephrine concentrations were determined in WT and α_{2A}KO heart and kidney (Fig. 5a). The concentrations of norepinephrine in heart were significantly reduced in α_{2A}KO compared with WT mice, suggesting that the sympathetic catecholamine stores are depleted due to enhanced transmitter release. Similarly, norepinephrine concentration were reduced in the kidney, but this achieved only borderline significance ($p < .1$).
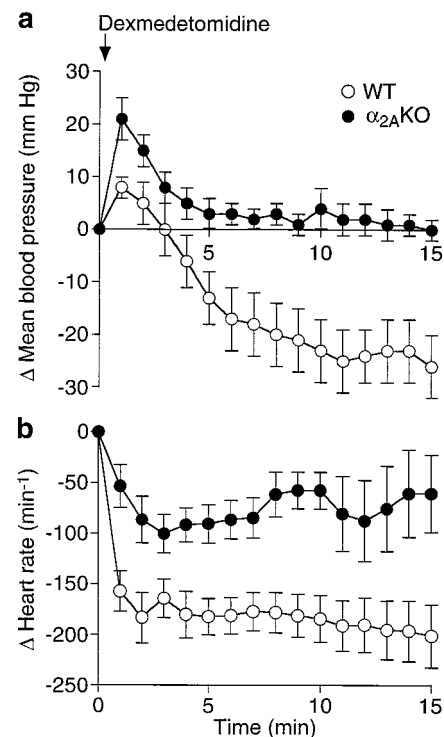
Cardiac β-AR Down-Regulation. The results presented above suggest that loss of presynaptic autoregulation in sympathetic nerves of α_{2A}-AR KO mice leads to baseline tachy-

cardia and depletion of catecholamine stores. To investigate the effect of the loss of presynaptic α_{2A}-AR function on postsynaptic ARs, we measured the density of cardiac β-ARs using the nonselective β antagonist [^{125}I]CYP. It has previously been shown that chronic agonist infusion leads to down-regulation of cardiac β-AR (Chang et al., 1982; Nanoff et al., 1989). Saturation binding studies revealed a significant reduction in cardiac β-AR density in α_{2A}KO mice compared with WT mice (Fig. 5b).

Presynaptic α_2-Autoreceptor Function. A vas deferens contraction assay was used to examine α_2-AR regulation of sympathetic transmitter release. Electrical stimulation of the vas deferens suspended in an organ bath leads to release of norepinephrine and ATP from intramural sympathetic nerve endings and subsequent contraction due to activation of α_1-ARs and P$_2$-purinergic receptors. Although the smooth muscle of the mouse vas deferens contains α_2-ARs, they play only a minor role in mediating neurogenic contractions (Bültmann et al., 1991). Stimulation of presynaptic α_2-AR on the sympathetic nerve terminals, however, markedly inhibits transmitter release and therefore the neurogenic contraction. Concentration-inhibition curves for the nonselective α_2 agonist dexmedetomidine in vas deferens isolated from the α_{2B}-AR KO, α_{2C}-AR KO, α_{2A}D79N mutant, and WT mice are shown in Fig. 6. Dexmedetomidine at sufficient concentrations completely blocked the contractile response to electrical stimulation in vas deferens from all of these mice in a similar manner. The data indicate that disruption of the α_{2B}-AR and

Fig. 3. Heart rate regulation in unrestrained, conscious WT and α_{2A}KO mice. Baseline heart rate was significantly elevated in α_{2A}KO mice compared with WT mice (*$p < .001$, KO versus WT). Injection of atropine led to an increase in heart rate in both groups of animals (¶$p < .001$, atropine versus control), but α_{2A}KO mice were still tachycardic compared with WT mice (*$p < .001$, KO versus WT). Subsequent injection of the β antagonist propranolol abolished the difference in heart rate between WT and α_{2A}KO mice.

Fig. 4. Hemodynamic effects of the α_2 agonist dexmedetomidine on mean arterial blood pressure (a) and heart rate (b) in unrestrained, conscious WT and α_{2A}KO mice. At time 0, a bolus of dexmedetomidine (5 μg/kg) was administered through the arterial catheter. In α_{2A}KO mice, the initial hypertensive effect of the α_2 agonist was greater than that in WT mice. However, the hypotensive effect of the α_2 agonist was completely abolished in α_{2A}KO mice. The bradycardic response to dexmedetomidine was significantly blunted in α_{2A}KO mice compared with WT mice. Data are derived from eight animals per group (mean ± S.E.M.).

α_{2C}-AR genes or mutation of the α_{2A}-AR (D79N) does not alter presynaptic function in sympathetic nerves. In contrast, the inhibition by dexmedetomidine in the α_{2A}KO mice (Fig. 7a), although not abolished, was markedly impaired. The maximal inhibition of vas deferens contraction by the α_2 agonist in α_{2A}KO mice was only $42 \pm 9\%$ compared with nearly complete inhibition in the WT mice. The concentration-inhibition curve of dexmedetomidine was also shifted to the right in α_{2A}KO mice (EC$_{50}$: α_{2A}KO, 4.2 ± 1.6 nM; WT, 0.7 ± 0.2 nM).

In vasa deferentia from a separate group of α_{2A}KO and WT mice, increasing concentrations of yohimbine were added to block presynaptic α_2-ARs and thereby disinhibit transmitter release (Fig. 7b). In this way, the extent of autoinhibition could be quantified. The α_2 antagonist increased the contractile response in both WT and KO mice (Fig. 7b). This increase was significantly greater in WT mice (maximum increase: WT, $185 \pm 40\%$ over baseline; KO, $52 \pm 8\%$ over baseline). The vas deferens from KO mice was more sensitive to yohimbine than the vas deferens from WT mice (EC$_{50}$: α_{2A}KO, 28 ± 9 nM; WT, 215 ± 45 nM; Fig. 7c).

Contraction responses to phenylephrine in unstimulated vas deferens were similar in WT and α_{2A}KO mice, indicating no difference in postsynaptic α_1-AR function (Fig. 7d). Similarly, dexmedetomidine did not alter the response to phenylephrine, confirming its specific activity at presynaptic receptors.

To more directly investigate the autoreceptor role of the α_{2A} subtype, [^{3}H]norepinephrine release was measured in small pieces of the vas deferens from WT and α_{2A}-AR-deficient animals. Electrical stimulation by single trains of 20 pulses at 50 Hz elicited release of [^{3}H]norepinephrine, which was smaller in the WT tissue ($0.17 \pm 0.01\%$ of tissue tritium; $n = 16$) than in the KO tissue ($0.30 \pm 0.03\%$ of tissue tritium; $n = 16$). Under the stimulation conditions used, 1 μM rauwolscine increased [^{3}H]norepinephrine release by $38 \pm 7\%$ in WT mice ($p < .01$) and only tended to increase release ($15 \pm 6\%$; $p > .05$) in α_{2A}KO mice. Thus, little autoinhibition of release is observed because the single-pulse trains are too short for significant autoinhibition to develop (Marshall, 1983; Singer, 1988; Limberger et al., 1995; Wahl et al., 1996). In WT vas deferens, the α_2 agonist medetomidine caused concentration-dependent inhibition of the release of [^{3}H]norepinephrine with an IC$_{50}$ value of 0.44 ± 0.04 nM and by maximally $90.1 \pm 0.5\%$ (Fig. 8). In mice lacking the α_{2A}-AR, the effect of medetomidine, although not abolished, was reduced: medetomidine caused inhibition with an IC$_{50}$ value of 0.76 ± 0.24 nM and a maximum of $74.7 \pm 2.2\%$ (Fig. 8). The maximal inhibition was thus decreased by 17%.

Discussion

Disruption of α_{2A}-AR Gene. α_{2A}-AR gene disruption was confirmed by ligand-binding experiments in whole brain. Disruption of the α_{2A}-AR gene resulted in a 90% reduction in total α_{2A}-AR binding in the mouse brain. The 10% residual binding is similar to previous estimates of the extent of α_{2C}-AR expression in brain (Ordway et al., 1993). We found that the residual α_2-AR binding in α_{2A}KO mice had high affinity for yohimbine. This finding is consistent with the higher affinity of yohimbine for the rodent α_{2B} and α_{2C} subtypes than for the α_{2A} subtype (Link et al., 1992).
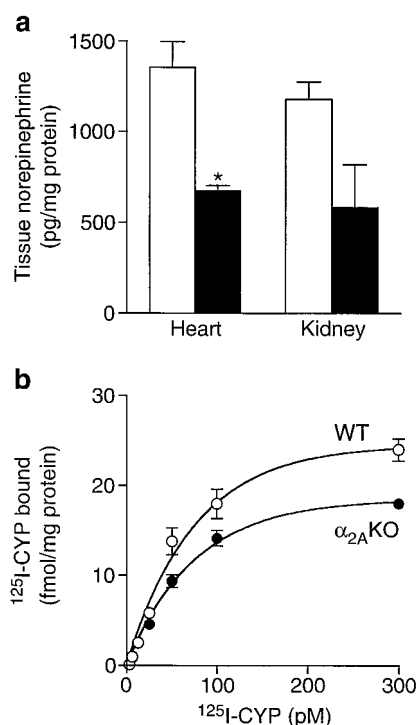
Fig. 5. Tissue norepinephrine levels and cardiac β-AR density are decreased in α_{2A}KO mice. a, norepinephrine concentrations in heart and kidney of α_{2A}KO mice are decreased compared with WT mice (*$p < .01$). Open columns, WT; solid columns, α_{2A}KO. b, enhanced release of norepinephrine from sympathetic terminals leads to down-regulation of cardiac β-ARs. Saturation isotherms for the β-AR ligand [^{125}I]CYP revealed a reduction in β-AR density in heart membranes from α_{2A}KO compared with WT mice. Data are derived from five or six animals per group (mean $\pm$ S.E.M.).
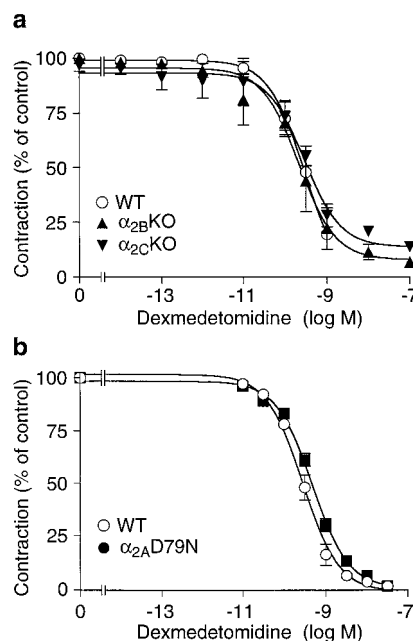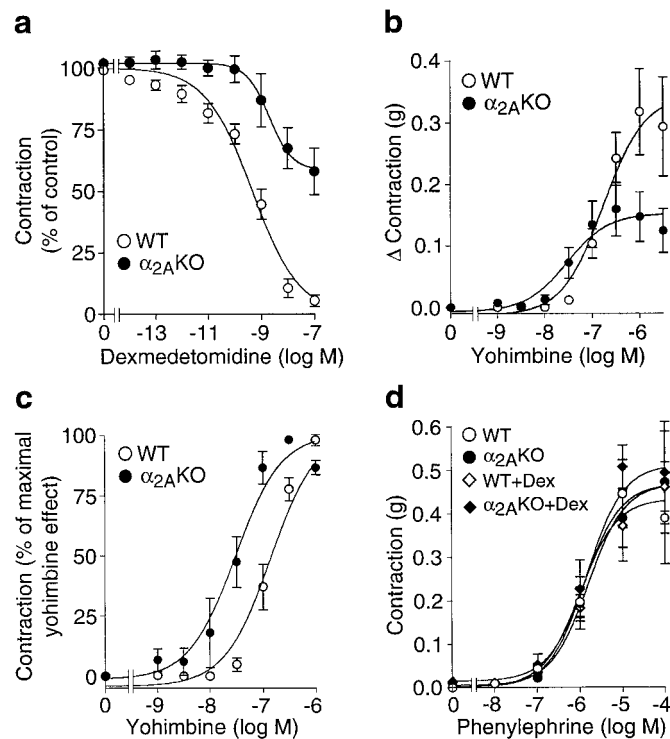

Fig. 6. Inhibition of electrically evoked contractions of isolated vas deferens preparations from WT and α_2-AR-deficient mice. Stimulation of presynaptic α_2-ARs by dexmedetomidine decreased the amplitude of the evoked twitch response. No difference was found for the inhibitory effect of dexmedetomidine on twitch contractions in WT and α_{2B}- or α_{2C}-deficient mice (a) or between WT and α_{2A}D79N mice (b). Data represent mean $\pm$ S.E.M. from six to eight vas deferens preparations.
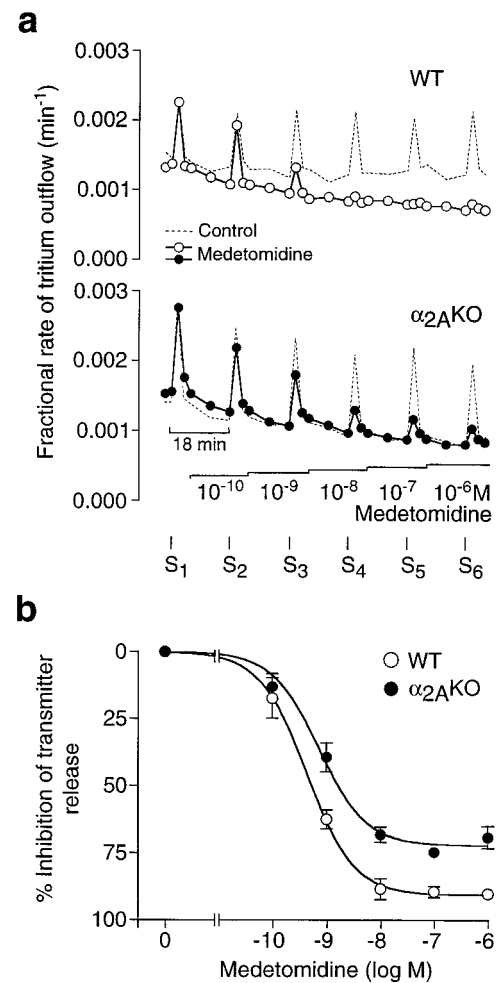
Role of α_{2A}-AR in Regulating Heart Rate and Blood Pressure. ARs form the interface between the sympathetic nervous system and the cardiovascular system. The α_2-ARs also play an important role in regulating the sympathetic nervous system both centrally, by regulating sympathetic tone, and peripherally, by regulating transmitter release from presynaptic nerve terminals. We have used strains of genetically engineered mice to investigate the regulatory functions of specific α_2-AR subtypes. Previous studies of α_{2A}D79N mice demonstrated that the α_{2A}-AR subtype mediates the hypotensive effects of nonselective α_2 agonists (MacMillan et al., 1996). Several lines of evidence suggest that the hypotensive effect of α_2 agonists could result from actions at sites within the central and/or the peripheral sympathetic nervous system (DeJonge et al., 1981; Urban et al., 1995). However, our results indicate that activation of cardiac and vascular presynaptic α_2-ARs alone is not sufficient to produce hypotension in response to α_2 agonists. The data presented in Fig. 6 demonstrate that presynaptic regulation of catecholamine release is preserved in α_{2A}D79N mice; however, these mice failed to become hypotensive in response to administered α_2 agonists (MacMillan et al., 1996).

The only significant difference that we observed between α_{2A}KO mice and α_{2A}D79N mice was in resting heart rate. The resting heart rate of α_{2A}D79N mice was not significantly

different from their WT controls (MacMillan et al., 1996), whereas α_{2A}KO mice had resting heart rates more than 180 beats/min greater than the control littermates. The tachycardia in the α_{2A}KO mice can be explained by a high basal level of sympathetic tone resulting from the loss of α_{2A}-AR-mediated inhibition of the vasomotor center combined with the loss of α_{2A}-AR-mediated inhibition of norepinephrine release from peripheral cardiac nerve terminals. The response to atropine was blunted in the α_{2A}KO mice, suggesting a state of vagal withdrawal. This finding is consistent with previous reports indicating that central α_2-ARs stimulate vagal output (Van Zwieten, 1988). However, the difference in heart rate between KO and WT mice persisted after inhibition of parasympathetic muscarinic receptors with atropine (see Fig. 3) and therefore is not principally due to a decrease in parasympathetic activity. The tachycardia observed in α_{2A}KO mice was associated with a significant depletion of the tissue

Fig. 8. Effect of medetomidine on [³H]norepinephrine release in mouse vas deferens. Pieces of vas deferens from WT or α_{2A}-AR-deficient mice were preincubated with [³H]norepinephrine, superfused, and, beginning after 54 min of superfusion, subjected to six periods of electrical stimulation 18 min apart (S_1 to S_6). Each stimulation period consisted of a train of 20 pulses delivered at 50 Hz. Increasing concentrations of medetomidine were added in a cumulative manner 12 min before S_2 to S_6. a, tritium efflux-versus-time curves from single tissue pieces. Interrupted lines, controls without medetomidine; uninterrupted lines with ○ (WT) or ● (α_{2A}KO) circles, medetomidine was added as indicated. b, concentration-response curves of medetomidine. The effect is corrected for time-matched controls. Mean ± S.E.M. from eight or nine tissue pieces.

Fig. 7. Presynaptic α_2-AR function in vas deferens from α_{2A}KO mice. a, the inhibitory effect of dexmedetomidine on electrically evoked twitch contractions was reduced by 56% in α_{2A}KO mice compared with WT mice. b, the addition of the α_2 antagonist yohimbine caused an increase in the twitch amplitude of vas deferens from WT and α_{2A}KO mice. c, the concentration-response curve for yohimbine is shifted to the left in vas deferens from α_{2A}KO compared with WT mice. d, the postsynaptic contractile effect of the α_1 agonist phenylephrine is not different in α_{2A}KO and WT vas deferens. Contractions were elicited by adding phenylephrine in a cumulative manner to nonelectrically stimulated vas deferens preparations. Contraction responses to phenylephrine were not altered in the presence of the α_2 agonist dexmedetomidine (0.1 μM Dex). Data represent mean ± S.E.M. from six to nine vas deferens preparations.

norepinephrine levels compared with normal mice. Depletion of tissue norepinephrine stores can be explained by enhanced release of norepinephrine from cardiac sympathetic nerves. Because reliable measurements of resting plasma catecholamine concentrations are difficult to obtain in mice, we examined the density of cardiac β-ARs. Chronic agonist exposure leads to pronounced down-regulation of β-ARs in several animal models (Chang et al., 1982; Nanoff et al., 1989). In α_{2A}KO mice, the level of β-ARs was decreased by 25% compared with WT mice, which is consistent with chronically increased sympathetic activity in these animals.

We were somewhat surprised that baseline blood pressure was unaffected in α_{2A}KO mice. This may be due to the fact that the sympathetic nervous system mediates vasoconstriction through α_1-ARs and the α_{2B}-AR and vasodilatation through both β_1 and β_2-ARs in mice (Rohrer et al., 1998). Thus, the net effect of increasing sympathetic tone on total vascular resistance may be minimal; however, it is possible that there are differences in the distribution of blood to different vascular beds as a result of different distributions of vasoconstricting and vasodilating ARs. It is also possible that changes in other systems that regulate systemic blood pressure, such as the renin-angiotensin system, are compensating for the chronic elevation of sympathetic tone in α_{2A}KO mice. Finally, the mice examined in these studies were relatively young (less than 20 weeks old). The effects of chronic elevation of sympathetic tone may become evident as the α_{2A}KO mice age.

More Than One Presynaptic α_2-Autoreceptor. The α_{2A}-AR has been suggested to be the main presynaptic α_2-AR in mammalian tissues (Trendelenburg et al., 1993). However, previous studies indicated that at least in certain tissues such as the rat heart, a second α_2 subtype might also regulate neurotransmitter release (Limberger et al. 1992; Trendelenburg et al., 1997). Ho et al. (1998) studied the release-enhancing effect of α_2 antagonists and concluded that in addition to α_{2A}-ARs, either α_{2B}- or α_{2C}-ARs mediate presynaptic inhibition in rat heart atria.

Our experiments on the vas deferens support the view that the α_{2A} subtype is the principal presynaptic autoreceptor. No attenuation of dexmedetomidine-induced presynaptic inhibition of neurogenic contractions was observed in the α_{2B} or in the α_{2C}KO mice, whereas this inhibition was greatly reduced in the α_{2A}KO animals. Moreover, yohimbine, which blocks α_2-ARs and interrupts α_2 autoinhibition, increased neurogenic contractions much less in vasa deferentia from α_{2A}KO than from WT mice, indicating a decrease of endogenous autoinhibition. Neurogenic responses of the mouse vas deferens to sympathetic nerve stimulation are mediated by the two cotransmitters norepinephrine and ATP, and the inhibition of the responses may be due to a decrease of norepinephrine as well as of ATP release (von Kügelgen and Starke, 1991). However, in direct [^{3}H]norepinephrine release experiments, the inhibitory effect of medetomidine, the racemate of dexmedetomidine, was also reduced in the α_{2A}KO tissues.

Although the α_{2A}-AR appears to be the principal regulator of catecholamine release, two of our findings indicate that at least one other α_2-AR subtype also functions as an autoreceptor. First, presynaptic inhibition by dexmedetomidine or medetomidine was reduced but not abolished in mice that lack the α_{2A}-AR. The maximal inhibition of neurogenic contractions was reduced by 56% in the α_{2A}KO tissues, whereas

the maximal inhibition of [^{3}H]norepinephrine release was reduced by only 17%. The difference may be due to the different patterns of nerve stimulation (pairs of pulses with an interval of 100 ms in contraction experiments versus 20 pulses at 50 Hz in [^{3}H]norepinephrine experiments) or to the fact that release of norepinephrine and release of ATP are subject to differential presynaptic α_2 modulation (Driessen et al. 1993). Second, endogenous autoinhibition, as revealed by the effect of yohimbine on contractions, was attenuated but not abolished in the α_{2A}KO tissues. Although neither the α_{2B}KO nor the α_{2C}KO showed altered responses to dexmedetomidine, it is anticipated that one of these receptors is responsible for the residual response to α_2 agonists and yohimbine in α_{2A}KO mice. In support of this view, the potency of yohimbine at enhancing neurogenic contractions was increased in the α_{2A}KO tissue, in accord with its higher affinity for the α_{2B} and α_{2C} subtypes than for the α_{2A} subtype (see above). Moreover, in [^{3}H]norepinephrine release experiments on vasa deferentia from α_{2A}KO mice, α_2 antagonists shifted the concentration-inhibition curve of medetomidine to the right in a manner compatible with the α_{2B} or α_{2C} subtype (A. U. Trendelenburg, unpublished observations).

The question remains why loss of the α_{2B} or α_{2C} -AR did not interfere with presynaptic autoreceptor function. It is possible that the α_{2A} autoreceptor is functionally dominant (more abundant or more efficiently coupled), so loss of the other α_2-AR subtype cannot be detected with the assays used. It is also possible that in α_{2A}KO mice, the α_{2B}-AR or α_{2C}-AR subtype is up-regulated to compensate for the loss of the α_{2A}-AR. Of interest, α_{2A}D79N mice had normal presynaptic function in the vas deferens contraction assay. Because the α_{2A}D79N mutant receptor fails to activate K$^+$ channels but does inhibit voltage-dependent Ca^{2+} channels (see the introduction), this observation favors coupling of the α_{2A} autoreceptor to the latter transduction mechanism (Starke et al., 1989; Hille, 1994).

In conclusion, disruption of the α_{2A}-AR gene in mice has no apparent effect on viability or fertility. Examination of these mice confirms that the α_{2A}-AR is the subtype mediating the beneficial hypotensive effects of α_2 agonists on blood pressure. The α_{2A} KO mice have altered sympathetic regulation due to loss of α_{2A}-mediated inhibition of sympathetic tone in the brainstem and loss of α_{2A}-mediated inhibition of catecholamine release from sympathetic nerve terminals. Our results provide evidence that at least one other α_2 subtype also plays a contributing role in regulating catecholamine release.

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