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14. AlF also transforms Golgi membranes into an array of coated buds and vesicles after 30 min of treatment, which could disrupt their continuity (P. Peters, L. Yuan, R. Klausner, J. Lippincott-Schwartz, unpublished electron microscope observations).
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17. An additional argument that vesicular traffic does not play a major role in the observed movement of the GFP chimeras during FLIP is that vesicles traveling through the cytoplasm should have an equal probability of fusing with acceptor membranes in stacks that are equidistant from a donor membrane. We found, however, that some stacks show very little loss of fluorescence during FLIP compared with others, even though they are equidistant from the zone of photobleaching. Moreover, there appears to be little or no interstack communication after microtubule depolymerization, when Golgi stacks reversibly scatter throughout the cytoplasm. These results are difficult to explain by vesicle traffic but are easily explained by differences in lateral continuities between Golgi stacks.
18. FLIP experiments at 37°C with cells expressing Man II–GFP and KDELR-GFP also showed loss of fluorescence throughout the Golgi complex, suggesting that these molecules diffuse rapidly between Golgi stacks. In cells expressing GFP chimeras in the Golgi, FLIP of a region of the cytoplasm that did not contain Golgi, but presumably did contain ER, did not result in significant loss of Golgi fluorescence over this time frame, suggesting that Golgi membranes are not in direct continuity with the ER.
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24. As one example, for models that assume the existence of functionally discrete Golgi cisternae or subcompartments [J. E. Rothman and L. Orci, *Nature* **355**, 409 (1992)], our findings would imply that mechanisms exist for ensuring that membrane continuities between adjacent stacks only form between homologous membranes (that is, cis to cis and trans to trans). Otherwise, Golgi cisternae within a Golgi stack could not remain completely separate and distinct from each other.
25. The microscope system described in (*15*) was used in the quantitative FPR experiments. The FPR beam was imaged into the sample as a stripe 2 μm wide. Because the stripe extended across the entire width of the Golgi or ER and bleached through the whole depth, diffusion was into and out of a line bounded on its sides, and not on its end. Hence, recovery of fluorescence was due to one-dimensional diffusion. The imposition of one-dimensional geometry on a complicated membrane as well as the mathematics for this case are covered in C.-L. Wey, M. A. Edidin, R. A. Cone, *Biophys. J.* **33**, 225 (1981). Briefly, a tortuous diffusion path reduces the apparent *D*, so our measurements in that case would be an underestimation.
26. Cells were transfected with GFP chimera cDNAs by $CaPO_4$ precipitation. Fluorescent cells were imaged at 37°C in buffered medium with a Zeiss LSM 410 confocal microscope system having a 100× Zeiss planapo objective (NA 1.4). The GFP molecule was excited with the 488 line of a krypton-argon laser and imaged with a 515–540 bandpass filter. Images were transferred to a Macintosh computer for editing and were printed with a Fujix Pictrography 3000 Digital Printer.
27. We thank R. Klausner, E. Siggia, J. Bonifacino, J. Zimmerberg, J. Donaldson, J. Presley, J. Ellenberg, and K. Zaal for valuable comments and suggestions, and M. Chalfie, K. Moremen, M. Fukuda, R. Poljak, and V. Hsu for generous gifts of reagents. M.E. is supported by grant R37 AI14584. Quicktime movies are available at http://www.uchc.edu/hterasaki/flip.html.

22 March 1996; accepted 20 May 1996

Central Hypotensive Effects of the α_{2a}-Adrenergic Receptor Subtype

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α_2-Adrenergic receptors (α_2ARs) present in the brainstem decrease blood pressure and are targets for clinically effective antihypertensive drugs. The existence of three α_2AR subtypes, the lack of subtype-specific ligands, and the cross-reactivity of α_2AR agonists with imidazoline receptors has precluded an understanding of the role of individual α_2AR subtypes in the hypotensive response. Gene targeting was used to introduce a point mutation into the α_{2a}AR subtype in the mouse genome. The hypotensive response to α_2AR agonists was lost in the mutant mice, demonstrating that the α_{2a}AR subtype plays a principal role in this response.

α_2ARs located in the rostral ventrolateral medulla respond to norepinephrine and epinephrine to decrease sympathetic outflow and reduce arterial blood pressure (*1*). This hypotensive effect has been the rationale for the use of clonidine, an α_2AR agonist, in the treatment of hypertension (*1*). There is controversy, however, concerning whether agents such as clonidine, which contain an imidazole moiety, elicit their hypotensive effects by interacting with α_2ARs or with a separate so-called imidazoline receptor population (*2*). Endogenous agonists of the putative imidazoline receptor population have been described (*3*). We explored the role of α_{2a}AR, one of three α_2AR subtypes (*4*), in eliciting a hypotensive effect because brainstem localization of α_{2a}AR mRNA suggested that the α_{2a}AR subtype might participate in this response (*5*).

We used gene targeting to mutate the α_{2a}AR gene to express an $Asp^{79}\rightarrow Asn$ (D79N) α_{2a}AR in mice. The D79N mutation substitutes asparagine for the aspartate residue at position 79, which is predicted to lie within the second transmembrane span of α_{2a}AR and is highly conserved among heterotrimeric GTP-binding protein (G protein)–coupled receptors (*6*). In AtT20 anterior pituitary cells, the D79N α_{2a}AR is selectively uncoupled from activation of K^+ currents, but remains coupled to inhibition of voltage-gated Ca^{2+} channels and of adenosine 3′,5′-monophosphate (cAMP) production characteristic of the wild-type receptor (*7*). We created a mouse line with this D79N α_{2a}AR to explore both the role of the α_{2a}AR subtype in cardiovascular and other physiological functions and the role of various signal-transduction pathways in α_{2a}AR effects. We now report the cardiovascular functions of this mutant D79N α_{2a}AR.

The substitution of the mutant for the wild-type α_{2a}AR gene in the mouse genome (*8*) was documented by Southern (DNA) analysis of diagnostic restriction digests in offspring of heterozygous intercrosses (Fig. 1A) and by DNA sequencing (Fig. 1B). The density of α_{2a}AR, assessed through use of the ^{3}H-labeled α_2AR antagonist RX 821002, was significantly reduced (80%) in mice homozygous for the D79N α_{2a}AR compared with wild-type mice (Fig. 2A). This reduction in density was not caused by changes in the amount of mRNA encoding D79N α_{2a}AR (Fig. 2B). These findings indicate that, in vivo, the D79N α_{2a}AR is improperly processed or stabilized in target cells. α_{2a}AR binding properties in mutant animals, however, showed appropriate α_{2a}AR selectivity and the absence of allosteric

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regulation by Na^+, which is characteristic of the D79N α_{2a}AR (*9*). α_2AR agonists were not less potent at the D79N α_{2a}AR (*10*); thus, functional losses in the mutant mice did not result from an inability of the D79N α_{2a}AR to bind agonist.

α_2AR agonists evoke a centrally mediated hypotensive response secondary to a transient hypertensive response mediated by α_2AR-elicited contraction of the peripheral vasculature (*1*). Infusion of UK 14,304 or dexmedetomidine into the carotid artery of conscious, unrestrained wild-type mice resulted in a transient pressor response followed by an extended hypotensive response (Fig. 3A). The hypotensive response was essentially ablated in the D79N α_{2a}AR mice, independent of the agonist studied or the site of agonist infusion (carotid or femoral artery) (Fig. 3A, upper panels, and Fig. 3B). Because these agonists are imidazoline analogs, the nearly complete loss of the hypotensive response to these agents in the D79N mice reveals a principal role of the α_{2a}AR subtype in regulating blood pressure in response not only to native catecholamines but also to imidazoline-based α_2AR agonists. The similar baseline blood pressure and heart rate of wild-type and D79N mice (Fig. 3A) suggest that mechanisms independent of the α_{2a}AR, or compensatory changes in response to the D79N mutation, establish basal cardiovascular set points.

The α_{2b}AR subtype appears to have a dominant role in eliciting the immediate hypertensive response to α_2AR agonists because targeted deletion of the α_{2b}AR but not the α_{2c}AR subtype eliminates the transient increase in blood pressure after infusion of dexmedetomidine into the carotid artery (*11*). Our finding that the hypertensive response in D79N mice is absent after femoral administration of UK 14,304 (Fig. 3B) suggests that the contribution of the α_{2a}AR subtype to peripheral vasoconstriction varies in different vascular compartments.

The α_{2a}AR subtype appears to have a critical role in the hypotensive response to α_2AR agonists, despite data implicating a role for independent imidazoline binding sites in this response (*2*). We do not know whether the loss of function observed in the D79N mice resulted from selective (*7*) or generalized (*12*) uncoupling of the mutant receptor from its signal transduction pathways, from the re-

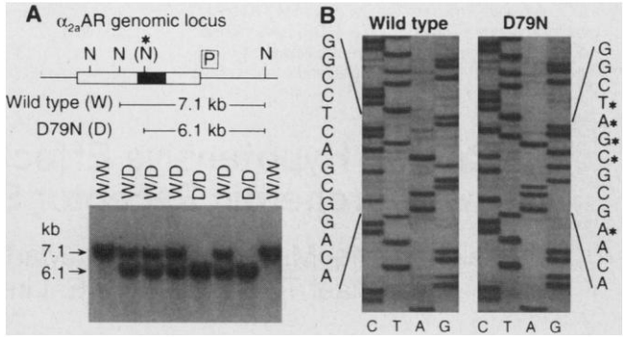

Fig. 1. Production of D79N α_{2a}AR mice through use of gene targeting (*18*). (**A**) α_{2a}AR genomic locus and representative Southern blot showing genotyping of offspring from wild-type α_{2a}AR/D79N α_{2a}AR heterozygote intercrosses. The open rectangle represents DNA included in the targeting vector, the black box indicates the α_{2a}AR coding region, and the asterisk denotes the D79N mutation. Genomic DNA was isolated from tail biopsies, digested with Nhe I (N), and hybridized to the external probe (P). D79N homozygous mutant mice were produced at the expected Mendelian ratio. (**B**) Representative sequencing gel confirming D79N mutation of the α_{2a}AR locus (*19*). Asterisks indicate bases altered in the original targeting vector and detected in the D79N mouse DNA.

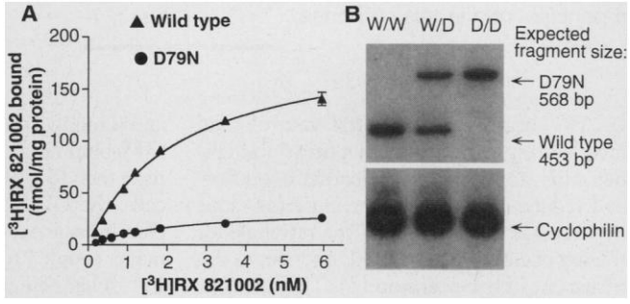

Fig. 2. Characterization of D79N α_{2a}AR binding and expression. (**A**) Saturation binding of [^{3}H]RX 821002 to α_{2a}AR in isolated brain membranes from wild-type and D79N mice (*13*). Assay included 1 μM prazosin to block contributions to the binding due to α_{2b}AR and α_{2c}AR subtypes (*20*). A nonlinear regression fit of the data to a hyperbola characteristic of binding to a single site yielded the following values for B_{max} (fmol/mg): wild-type, 208.6 ± 24.8; D79N, 39.8 ± 2.7; and for K_d (nM): wild-type, 2.5 ± 0.1; D79N, 2.4 ± 0.1. Values are averages (mean ± SEM) from three experiments. The D79N B_{max} was significantly different from wild-type B_{max} ($P < 0.01$, unpaired Student's *t* test). Mice heterozygous for the D79N mutation exhibited an α_{2a}AR density halfway between those of the wild-type and homozygous D79N mice (*21*). (**B**) Ribonuclease protection analysis representative of three independent preparations of total brain RNA isolated from wild-type (W/W), heterozygous (W/D), and D79N mice (D/D) (*22*). Protection of cyclophilin mRNA was used to normalize RNA loading in each lane, and quantitation confirmed that amounts of wild-type and D79N RNA were not different in male or female mice.

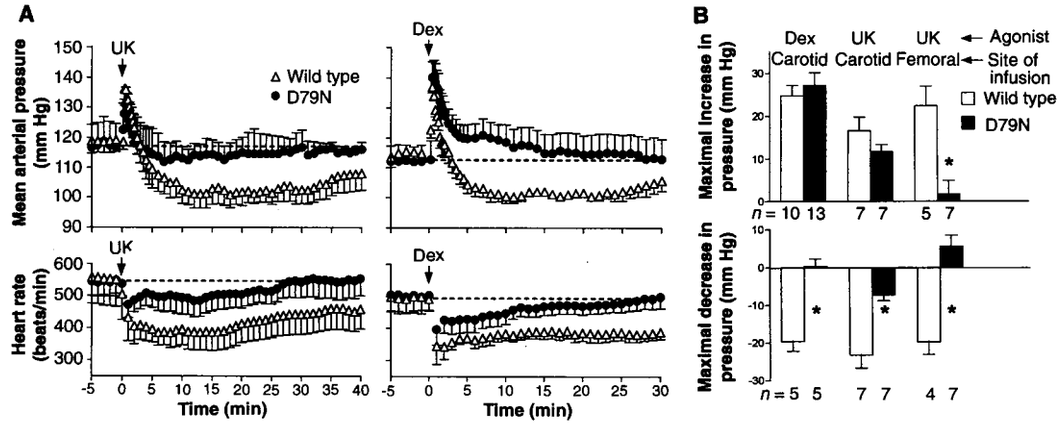

Fig. 3. Hemodynamic measurements for wild-type and D79N α_{2a}AR mice (*23*). (**A**) Mean blood pressure (upper panels) and heart rate (lower panels) for bolus injections of UK 14,304 (UK, 100 μg per kilogram of body weight) and dexmedetomidine (Dex, 5 μg/kg) into a carotid arterial catheter at time 0. Baseline blood pressure and heart rate are shown as dotted lines. Data are shown as the mean ± SEM (n = 5). (**B**) Maximal increase and decrease in mean blood pressure (±SEM) after administration of Dex and UK in the carotid artery and after injection of UK (218 μg/kg, cumulative dose) into a femoral arterial catheter. D79N values significantly different from those of wild type ($P < 0.01$, unpaired Student's *t* test) are indicated by an asterisk.

duction in receptor density (Fig. 2), or from a combination of these effects. Our results and those of Link *et al.* (*11*) indicate that subtype-selective ligands might provide a therapeutic advantage in the treatment of hypertension.

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10. Agonist competition for [^{3}H]RX 821002 was performed (*13*). Median effective concentration (EC_{50}) values in the absence of Na^+ were as follows for epinephrine: wild-type, 1.6 μM; D79N, 0.5 μM; and for dexmedetomidine: wild-type, 37.5 nM; D79N, 3.6 nM. The potency of agonists was reduced by the inclusion of Na^+ in the binding incubations for the wild-type α_{2a}AR but not for the D79N α_{2a}AR (*9*).
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13. Mouse brains (mice were about 2 months old) were homogenized in hypotonic lysis buffer (*14*) and membranes were resuspended so that final assay conditions were 25 mM glycylglycine, 40 mM Hepes (pH 8), 5 mM EGTA, 5 mM EDTA, 5 mM $MgCl_2$, 100 μM 5′-guanylylimidodiphosphate, and 1 μM prazosin. Membranes (1 to 1.5 mg of protein per incubation, as estimated with the Bradford assay) were incubated with various concentrations of [^{3}H]RX 821002 and 100 mM NaCl or 100 mM *N*-methyl-D-glucamine (pH 8) at 25°C for 60 min. The maximum binding capacity (B_{max}) and dissociation constant (K_d) values reported in the figure were in the presence of Na^+. In the absence of Na^+, values were as follows for B_{max} (fmol of [^{3}H]RX 821002 bound per milligram of protein): wild-type, 210.8 ± 13.6; D79N, 43.2 ± 2.3; and for K_d (nM): wild-type, 2.9 ± 0.3; D79N, 2.8 ± 0.3. Competition studies were done with 6 nM [^{3}H]RX 821002. Nonspecific binding was defined as binding not displaced by 10 μM phentolamine (*14*). Nonlinear regression fit of the data was done with GraphPad Prism software.
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18. To construct the targeting vector, we subcloned a 6-kb Eco RI to Hind III fragment containing the 1.35-kb α_{2a}AR coding region from a 129/Sv genomic phage clone into pSP71 (Promega). Oligonucleotide-directed mutagenesis in M13mp18 was used to alter the codon at position 79 and to introduce an Nhe I site. The entire mutant fragment was sequenced with Sequenase 2.0 (USB). PGKtk and PGKneo constructs were from B. L. M. Hogan (Vanderbilt University). Hit-and-run gene targeting in mouse embryonic stem (ES) cells was done essentially as described (*8*). D3H mouse ES cells were from H. E. Ruley (Vanderbilt University). [^{32}P]-Labeled DNA probes used for Southern analysis were as follows: 3′ external region, a 0.8-kb Hind III to Sal I fragment; *neo* coding region, a 0.8-kb Eco RI to Xba I fragment derived from Pol2neobpA (*15*); and α_{2a}AR coding region, a 0.79-kb Bgl II to Xmn I fragment derived from the α_{2a}AR coding region. Correctly targeted ES cell clones were injected into C57BL/6 blastocysts; chimeras were bred with C57BL/6 mice to generate heterozygous mice (*8*). Wild-type and D79N breeding pairs were established from offspring of the heterozygous pairs. The B6,129 hybrid offspring of these breeding pairs were used in the studies described and are designated B6,129-*Adra2a*^{tm1Lel}.
19. A region of the α_{2a}AR coding sequence [base pairs (bp) 113 to 385] from wild-type and D79N mouse tail DNA was amplified by polymerase chain reaction (PCR) and subcloned into pBluescriptII SK(+). Multiple transformants were sequenced with Sequenase 2.0 (USB).
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22. The probe for ribonuclease (RNase) protection (*16*), bp 113 to 680 of the D79N α_{2a}AR coding region, was amplified by PCR and subcloned into pBluescriptII KS(+). Total brain RNA (2 μg), isolated from 2-month-old mice, was used in the protection assays. Single-stranded RNA was digested with RNase A (5 μg/ml); wild-type RNA was also digested with RNase A at the site of the mutation, resulting in the smaller expected wild-type fragment. The cyclophilin probe, bp 40 to 146 of rat cyclophilin (*17*), was from R. B. Emeson (Vanderbilt University). Probe excess was confirmed in incubations containing 4 and 8 μg of total RNA. PhosphorImager analysis (Molecular Dynamics) was used to quantitate RNA.
23. For carotid catheterization, male mice (2 to 3 months old) were anesthetized with halothane (1.75% v/v in O_2), and a PE-10 polyvinyl catheter tubing was inserted into the left common carotid artery (*11*). Mice recovered for 18 to 24 hours before the catheter was connected to a Gould amplifier, by a Gould-Statham pressure transducer, for hemodynamic measurements. Systolic, diastolic, and mean arterial blood pressures, and heart rate were recorded on a microcomputer equipped with DataFlow software (Crystal Biotech). α_2AR agonists were injected directly into the arterial catheter in a 10-μl bolus. For the femoral catheter experiments, a Microrenathane (0.064 cm outer diameter, 0.030 cm inner diameter cannula; Braintree Scientific) was inserted into the femoral artery of anesthetized male mice (2 to 3 months old). Mice recovered for 4 to 6 hours before arterial blood pressure was recorded on a Grass Model 7 polygraph by means of a Cobe blood pressure transducer. Heart rate was calculated from blood pressure tracings. UK 14,304 was injected into the arterial catheter; each sequential dose of UK 14,304 was injected after the blood pressure of wild-type mice had returned to baseline or after a comparable time (about 5 min) for D79N mice. Vehicle injection had no effect on the recorded cardiovascular responses.

24. Supported by National Institutes of Health grants HL43671 (to L.E.L.), HL38120 (to M.T.P.), and HL48638 (Project 4) (to B. K. Kobilka in support of L.H.); by a Vanderbilt University Graduate Fellowship (to L.B.M.); and by an Established Investigator Award from the National Association for Research on Schizophrenia and Depression (to L.E.L.).

4 March 1996; accepted 30 May 1996

Cardiovascular Regulation in Mice Lacking α_2-Adrenergic Receptor Subtypes b and c

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α_2-Adrenergic receptors (α_2ARs) are essential components of the neural circuitry regulating cardiovascular function. The role of specific α_2AR subtypes (α_{2a}, α_{2b}, and α_{2c}) was characterized with hemodynamic measurements obtained from strains of genetically engineered mice deficient in either α_{2b} or α_{2c} receptors. Stimulation of α_{2b} receptors in vascular smooth muscle produced hypertension and counteracted the clinically beneficial hypotensive effect of stimulating α_{2a} receptors in the central nervous system. There were no hemodynamic effects produced by disruption of the α_{2c} subtype. These results provide evidence for the clinical efficacy of more subtype-selective α_2AR drugs.

α_2ARs have a prominent role in the cardiovascular system and influence vascular tone at multiple points in a complex reflex arc. α_2ARs located in the brain stem are targets for antihypertensive therapy because stimulation of these receptors produces a long-lasting drop in systemic blood pressure. Paradoxically, stimulation of α_2ARs on arterial smooth muscle cells increases blood pressure by increasing vascular resistance. Three subtypes of α_2AR (α_{2a}, α_{2b}, and α_{2c}) have been isolated and share a high degree of structural similarity (50 to 60% identity). All these receptors couple to the inhibitory heterotrimeric GTP-binding protein (G_i) and inhibit adenyl cyclase. The three α_2ARs differ, however, in their patterns of tissue expression (*1*). Little is known about the role of the three α_2AR subtypes in cardiovascular physiology. Studies have been hampered both by the lack of subtype-

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