The α2 Isoform of Na,K-ATPase Mediates Ouabain-induced Cardiac Inotropy in Mice*

Inhibition of Na,K-ATPase activity by cardiac glycosides is believed to be the major mechanism by which this class of drugs increases heart contractility. However, direct evidence demonstrating this is lacking. Furthermore it is unknown which specific α isoform of Na,K-ATPase is responsible for the effect of cardiac glycosides. Several studies also suggest that cardiac glycosides, such as ouabain, function by mechanisms other than inhibition of the Na,K-ATPase. To determine whether Na,K-ATPase, specifically the α2 Na,K-ATPase isozone, mediates ouabain-induced cardiac inotropy, we developed animals expressing a ouabain-insensitive α2 isoform of the Na,K-ATPase using Cre-Lox technology and analyzed cardiac contractility after administration of ouabain. The homozygous knock-in animals were born in normal Mendelian ratio and developed normally to adulthood. Analysis of their cardiovascular function demonstrated normal heart function. Cardiac contractility analysis in isolated hearts and in intact animals demonstrated that ouabain-induced cardiac inotropy occurred in hearts from wild type but not from the targeted animals. These results clearly demonstrate that the Na,K-ATPase and specifically the α2 Na,K-ATPase isozone mediates ouabain-induced cardiac contractility in mice.

Na,K-ATPase is a transmembrane protein that transports three Na⁺ out of the cell and two K⁺ in, utilizing ATP hydrolysis as the driving force. The electrochemical gradient, established by the Na,K-ATPase, is essential for maintenance of cell-specific functions, such as muscle contraction and nerve transmission. The ion gradient also drives many plasma membrane transport processes, including Na⁺/H⁺ exchange and uptake of nutrients, such as glucose, in kidney and gut via Na⁺/cotransporters. Na,K-ATPase is composed of two subunits: the catalytic α subunit, which binds translocating cations and ATP, and the β subunit, which modulates cation affinity and is necessary for proper folding and translocation of the Na,K-ATPase to the plasma membrane (1–4). There are four isoforms of the α subunit (α1, α2, α3, and α4), each exhibiting unique tissue distribution and developmental expression suggestive of differential and tissue-specific functional roles (5–12).

Cardiac glycoside sensitivity is conferred by the α subunit of the Na,K-ATPase, and this class of drugs is used to treat congestive heart failure. Inhibition of Na,K-ATPase activity by cardiac glycosides is believed to be a major mechanism by which these drugs exert their positive cardiac inotropic effect (13–16). Inhibition of the Na,K-ATPase raises intracellular Na⁺, which in turns increases intracellular Ca²⁺ concentrations. The increase in intracellular Ca²⁺ produces a positive inotropic response of the heart. Two isoforms, α1 and α2, are expressed in rodent heart, and three isoforms, α1, α2, and α3, are expressed in human heart. However, it is unknown which specific α isoform mediates this cardiac glycoside positive inotropic effect in either species. It has been suggested that inhibition of the high affinity α2 isoform followed by inhibition of the low affinity α1 isoform by the cardiac glycoside digoxin results in increased contractility of rat hearts (17). It has also been suggested that differences in cardiac glycoside sensitivity of Na,K-ATPase isozymes determine their therapeutic and toxic effects such that the high affinity Na,K-ATPase mediates the positive inotropic effects, whereas the low affinity Na,K-ATPase is responsible for toxic effects of cardiac glycosides (18).

Analysis of mice expressing genetically reduced levels of α1 and α2 isoforms demonstrated differential cardiac function of these isoforms, indicating that they may differentially regulate the cardiac glycoside-induced positive inotropy (9). The approximate 50% reduction in α2 isoform expression resulted in an increase in contractility of the heart, whereas decreased cardiac contractility was observed in mice lacking one copy of the α1 isoform gene. As reduction of the α2 isoform mimicked the cardiac glycoside-induced positive inotropy, it was suggested that the α2 isoform might play a role in this effect, whereas inhibition of the α1 isoform may be responsible for toxic effects of cardiac glycosides. Recently it has been suggested that both α1 and α2 isoforms regulate cardiac contractility and cardiac glycoside-induced positive inotropy (19). However, evidence also exists implicating only the α1 isoform in regulation of cardiac contractility (20). Therefore, further studies are necessary to determine the functional role of different isoforms in the regulation of cardiac contractility and the cardiac glycoside effects.

Several studies suggest that cardiac glycosides may function by mechanisms other than inhibition of Na,K-ATPase. Low concentrations of digitoxin have been shown to activate the sarcoplasmic reticulum ryanodine receptor (RyR), suggesting its involvement in positive cardiac inotropy by digitoxin (21, 22).
obtained by heterozygous mating. Genotyping was performed by allele-desired mutation, in exon 4 was analyzed in targeted ES cells by PCR above. The presence of the BstBI site, and therefore the presence of the second electroporation with the Cre-recombinase-encoding vector. Following ultracentrifugation the pellet was resuspended in 1 mM EDTA buffer and stored at −80 °C. Protein concentration was determined by BCA protein assay kit (Pierce).

**Crude Microsomal Preparations**—Crude microsomal preparations were used for [3H]ouabain binding assays and were prepared as described previously (26), omitting NaI treatment. Briefly, for each preparation, tissues were pooled from five animals and homogenized on ice with two 30-s bursts of a Polytron homogenizer in 2–7 ml, depending on the tissue, of 1 mM imidazole, 1 mM EDTA buffer, pH 7.4. Following Polytron homogenization, tissues were further homogenized and stored at −80 °C. Protein concentration was determined by BCA protein assay kit (Pierce).

**Western Blot Analysis**—Western blot analysis was performed via standard methods (9). In brief, protein samples were incubated for 30 min at 37 °C in loading buffer (50 mM Tris (pH 6.9), 5% SDS, 1% β-mercaptoethanol, 10% glycerol, and 0.05% (w/v) bromphenol blue) and separated by electrophoresis in 10% polyacrylamide gels. The separated proteins were transferred to polyvinylidene difluoride membranes and blocked in 1% nonfat dry milk in TBST (5 mM Tris, pH 7.4, 150 mM NaCl, 0.01% Tween 20) for 1 h at room temperature. The blots were incubated in TBST containing specific monoclonal antibody (αEF, University of Iowa Developmental Studies Hybrid Bank), α2 isoform-specific monoclonal Mcb2 antibody (Dr. K. Swedner), α3 isoform-specific monoclonal MA3-915 antibody (Affinity Bioreagents), and RETTY polyclonal antibody, which recognized the C-terminal RETTY sequence of all α isoforms (Dr. Jack Ryte) at 4 °C overnight. After incubation with peroxidase-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch), immunoreactivity was visualized following treatment with the ECL™ Western blotting detection reagents (Amersham Biosciences) by using the Kodak BioMax MR x-ray film. Equal protein loading for each Western blot analysis was confirmed following initial immunoreactivity visualization by reprobing the membranes with glyceraldehyde-3-phosphate dehydrogenase specific rabbit monoclonal 6C5 antibody (Advanced ImmunoChemical Inc.). In brief, polyvinylidene difluoride membranes were washed in TBST for 1 h at room temperature followed by their incubation in anti-glyceraldehyde-3-phosphate dehydrogenase antibody for 1 h. Peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) was used as secondary antibody. Immunoreactive visualization was performed as described above.

**Analysis of Ouabain Binding by Na,K-ATPase in Microsomal Protein Preparations from Wild Type (α2/wt) and Homozygous Knock-in Mice (α2<sup>−/−</sup>)**—Total [3H]ouabain binding was determined as described previously (9). Briefly 100–500 μg of crude membrane preparations were incubated at 37 °C for 6 h in 100 mM Tris-HCl (pH 7.4), 1 mM Tris-PO4, and either 0 or 30 μM unlabeled ouabain. Following incubation the crude membrane preparations were filtered through 1.2-μm glass microfiber Whatman GF/C filter paper and washed five times with 7 ml of water using a Brandel cell harvester. The filtered-associated radioactivity was determined by liquid scintillation counting. Nonspecific [3H]ouabain for each microsomal preparation was determined by using an excess of unlabeled ouabain (1 μM) and was subtracted from total binding obtained using 30 μM ouabain. Subtraction of nonspecific binding from total binding obtained in kidney samples from both genotypes and in heart and skeletal muscle samples from targeted animals resulted in zero value.

**Analysis of Cardiovascular Performance in Intact Closed Chest Animals**—Adult male and female mice were anesthetized with an intraperitoneal injection of 50 μg ketamine/g of body weight and 100 μg xylazine/tarbitulabital/g of body weight (Inactin, Research Biochemical International, Natick, MA). Polyethylene tubing (0.4 mm in diameter) was inserted into the right carotid artery and connected to a low compliance pressure transducer (COBE Cardiovascular, Arvada, CO) for measurement of mean arterial pressure. A high fidelity, 1.8-French Millar Mikro-Tip transducer (Model SPR-612, Millar Instruments, Houston, TX) was inserted into the right carotid artery and advanced into the left ventricle to monitor cardiac performance. The femoral vein was cannulated for infusion of dobutamine or ouabain. Mean arterial pressure (MAP) and intraventricular pressure from the COBE transducer and the Millar transducer were analyzed using a MacLab/4 data acquisition system connected to a Macintosh 7100/80 computer for each dobutamine dose. Average values for heart

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**EXPERIMENTAL PROCEDURES**

**Generation of Mice Expressing the Cardiac Glycoside-insensitive α2 Na,K-ATPase Isozyme**—The L111R and N122D amino acid substitution were introduced into the α2 isoform by two base pair mutations in exon 4 using PCR site-directed mutagenesis as shown in Fig. 1. The whole exon 4 was used as template for PCR site-directed mutagenesis and was excised from an 8.6-kb subclone of the α2 isoform gene. Furthermore two silent base pair substitutions were made to introduce a BstBI site. The amplified fragment containing the desired mutations was ligated back into the 8.6-kb subclone. The LoxP-neomycin-LoxP cassette was cloned into intron 3 at a site 750 bp upstream of exon 4. The thymidine kinase gene was inserted downstream of the cloning sequence. The presence of the desired mutation in the targeting vector was verified by both sequence analysis and BstBI restriction digest. The Duffy ES cell line was transfected with the targeting vector by electroporation, and successful homologous recombination was confirmed by Southern blot analysis. The 5′ probe was derived from intron 3, whereas the 3′ probe was derived from intron 4 of the α2 isoform gene. The presence of the BstBI site and desired mutation in exon 4 was analyzed in successfully targeted ES cells by PCR. The assumption was made that the existence of a BstBI site in exon 4 indicates the presence of the desired mutation as sequence analysis of the targeting vector demonstrated the successful introduction of this site along with the desired RD mutation. The exon 4 sequence was amplified by PCR using P1 (5′-AGCTCAAGGACATCGCGTC-3′) and P2 (5′-CTCTAAGACCACTTGGC-3′) primers, and the amplification products were treated with BstBI restriction enzyme. Successfully targeted ES cells were subjected to the second electroporation with the Cre-recombinase-encoding vector. Following the transfection, ES clones were duplicated, and one set was grown in medium supplemented with 250 μg/ml G418. Genomic DNA was extracted from the neomycin-sensitive ES cells, digested with PstI, and analyzed by Southern blot using the 3′ and 5′ probes described above. The presence of the BstBI site and therefore the presence of the desired mutation, in exon 4 was analyzed in targeted ES cells by PCR as described above. Chimeric mice were generated via blastocyst injection of the ES cell clone 39 and were bred to Black Swiss mice for two generations to establish a hybrid line. Homozygous knock-in mice were obtained by heterozygous mating. Genotyping was performed by allele-specific PCR across the LoxP targeting cassette. The presence of the BstBI site and therefore the presence of the desired mutation, in exon 4 was analyzed in targeted ES cells by PCR as described above. Whole Tissue Preparation—Whole tissue homogenates were used for Western blot analysis. For each tissue preparation, tissues were pooled and homogenized on ice with two 30-s bursts of a Polytron homogenizer in 2–7 ml, depending on the tissue, of 1 mM imidazole, 1 mM EDTA buffer, pH 7.4. Following Polytron homogenization, tissues were further homogenized and stored at −80 °C. Protein concentration was determined by BCA protein assay kit (Pierce).
rate, MAP, and systolic left ventricular pressure were measured from the pressure waveforms and were determined for each animal from at least 50 consecutive beats during the final 30 s of each 3-min dosage period. Maximum $\frac{dP}{dt}$ and $\frac{dP}{dt}$ at 40 mm Hg ($dP/dt_{max}$) for each dobutamine and ouabain infusion were calculated from the first derivative of the pressure waveforms.

**Work-performing (Anterograde) Mouse Heart Preparation**—Cardiac contractile parameters were also obtained using a work-performing heart apparatus as described previously (27). In brief, wild type and targeted mice were anesthetized, and the hearts were quickly removed and placed in a dish containing heparinized, oxygenated Krebs-Henseleit solution prewarmed at 37.7 °C. The aorta was isolated, and the mouse heart was suspended via its ascending aorta on a 20-gauge cannula allowing for retrograde perfusion of the coronary arteries. Perfusion with 37.7 °C oxygenated Krebs-Henseleit solution occurred from heated storage cylinders positioned above the heart to produce a constant pressure of 60 mm Hg, which was maintained via an overflow. A PE50 catheter was inserted through the pulmonary vein opening of the left atrium past the mitral valve into the left ventricle. The catheter was pushed through the apical wall of the left ventricle until the flanged tip of the catheter rested against the ventricular wall. The atria, ventricles, and valves remained intact. The pulmonary vein was cannulated, and the perfusion was switched to anterograde. The left ventricular pressure and its derivatives, $+\frac{dP}{dt}$ and $-\frac{dP}{dt}$, were recorded via a COBE pressure transducer and data acquisition system. Heart rate, aortic pressure, and temperature were also recorded on a Grass polygraph and computerized. All cardiac measurements were taken with mean aortic pressure fixed at 50 mm Hg and venous return fixed at 5 ml/min (left ventricular minute work = 250 mm Hg × ml/min). Coronary flow was measured with a Transonic flowmeter (Model T206). Temperature was continuously monitored via a Physi-temp probe (Thermalert Model TH-8). Hearts were perfused with Krebs-Henseleit solution containing 2.0 mM Ca²⁺ (standard) or 1.5 mM Ca²⁺ (low), and base-line functional parameters were obtained at the different Ca²⁺ concentrations.

The effects of the cardiac glycoside ouabain on the functional parameters of the hearts were assessed at 1.5 mM Ca²⁺. Increasing concentrations ($1 \times 10^{-5}$ to $8 \times 10^{-6}$ M) of ouabain, to produce a cumulative concentration-response curve, were infused and mixed into the perfusate via catheters inserted into the aortic tubing 5 cm above the heart. The hearts were exposed to each concentration until maximal effect was reached (usually a period of 5 min). The final concentration of ouabain delivered to the heart was calculated based on stock concentration, infusion rate, and cardiac output.

**Statistical Analysis**—Two-way analysis of variance for time and treatment with repeated measurements was performed, and posthoc test of Fisher’s least significant difference was obtained.

### RESULTS

**Generation of Mice Expressing a Cardiac Glycoside-insensitive $\alpha_2$ Na,K-ATPase Isozyme**—The ouabain sensitivity of the mouse $\alpha_2$ isoform was reduced by introducing L111R and N122D amino acid substitutions in the first extracellular domain of this isoform (Fig. 1). The Arg-111 and Asp-122 residues are naturally present in the low affinity rodent $\alpha_1$ isoforms and were shown previously to confer cardiac glycoside insensitivity of the rodent $\alpha_1$ isoforms. Also our laboratory has previously demonstrated that these substitutions in the sheep $\alpha_1$ isoform reduced its ouabain affinity by 1000-fold without altering its enzymatic activity (28, 29). In addition, a BstBI restriction enzyme site was introduced as a silent mutation for easy identification of the targeted allele.

The ouabain-insensitive $\alpha_2$ isoform mice were generated by the Cre-loxP gene targeting strategy as depicted in Fig. 2. The strategy consists of two sequential ES cell transfections. The first transfection introduces the targeting sequence into one of the wild type alleles by homologous recombination. Successful homologous recombination results in heterozygous ES cell clones having a targeted allele containing the loxP-flanked neomycin resistance cassette and the desired L111R and N122D mutations. The second transfection introduces Cre-recombinase, which excises the neomycin resistance-conferring cassette from the targeted allele by recombination of two loxP sites. Successful recombination generates heterozygous ES cells containing the desired mutation in exon 4 and one loxP site in intron 3 within the targeted allele.

Following the first ES cell transfection, two successfully targeted ES cell clones, 5A and 10A, were identified by Southern blot analysis following the Fst1 restriction digestion of their genomic DNA (Fig. 2B, upper panel). The 7-kb fragment, which was detected in all screened ES cell clones, corresponded to the wild type allele. The 3.5-kb fragment corresponded to the targeted allele and results from the additional Fst1 site within the neomycin resistance cassette of the targeted allele. The introduced BstBI site was retained in ES cell clones 5A and 10A, and therefore, it was assumed that L111R and N122D amino acid substitutions were also retained (data not shown). Cre-recombinase transfection of clone 5A resulted in successful excision of the neomycin resistance cassette in one ES cell clone, clone 39 (Fig. 2B, lower panel). As excision of the neomycin cassette resulted in elimination of the additional Fst1 site, both targeted and wild type alleles are detected as 7-kb fragments by Southern blot analysis of the Fst1-digested genomic DNA. The intensity of the 7-kb fragment is higher in clone 39 than in the original 5A clone, indicating that it is composed of two 7-kb fragments, one corresponding to the wild type allele and the other corresponding to the targeted allele. ES cell clone 39 retained a BstBI site and therefore the desired L111R and N122D amino acid substitution in exon 4 (data not shown). Chimeric animals were generated via blastocyst injection of ES cell clone 39 and were crossed to Black Swiss females to yield heterozygous $\alpha_2^{WR}$ animals. Heterozygous mating was used to generate wild type ($\alpha_2^{W/W}$), heterozygous ($\alpha_2^{W/R}$), and homozygous ($\alpha_2^{R/R}$) offspring that were born in normal Mendelian ratios. The genotypes of the animals was identified by the PCR amplification of their genomic DNA, which identifies the wild type allele as a 307-bp fragment and the targeted allele as a 342-bp fragment (Fig. 2C). The $\alpha_2^{WR}$ animals retained the desired mutation as shown by the sequence histogram of their genomic DNA (Fig. 2D).

The $\alpha_2^{WR}$ Mice Express the $\alpha_2$ Isoform of the Na,K-ATPase with Reduced Ouabain Affinity and Normal Expression and Tissue Distribution—Introduction of the L111R and N122D

### Table: a2 Na,K-ATPase Isoform and Ouabain-induced Cardiac Inotropy

| Mouse a2 Gene | CTG | GCC | GCG | ATG | GAG | GAC | GAA | CCA | TCC | AAT | GAT | AAT | GTG |
|---------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Reverse PCR Primer | G   | CCT | TCG | AAT | GAT | CAT | CAT | G   |
| Nucleotide Substitution | G   | T   | G   | G   |
| Silent Mutation | T   | TCG | AA  |
| Mouse a2 Protein Sequence | L   | A   | A   | M   | E   | E   | D   | E   | P   | S   | N   | D   | N   |

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**Introduction of the L111R and N122D substitutions in mouse a2 isoform of Na,K-ATPase.** Two base pair mutations were introduced into exon 4 by PCR amplification, resulting in the L111R and N122D amino acid substitutions. Two silent base pair mutations were also introduced to form a unique BstBI site.
amino acid substitutions in the α2 isoform reduced its ouabain affinity in the α2βR mice as demonstrated by [3H]ouabain binding (Fig. 3A). As expected, no [3H]ouabain binding was detected in kidney from each genotype due to expression of only the low affinity α1 isoform. Total [3H]ouabain binding in wild type heart and skeletal muscle microsomal preparations was attributed to the high affinity α2βR isoform. No [3H]ouabain binding was detected in microsomal preparations from the same tissues of the α2βR/R mice. This demonstrated a successful reduction in the ouabain affinity of the α2β isoform carrying the L111R and Q122D substitutions. The total [3H]ouabain binding in brain tissue from the α2βR/R animals was not fully abolished due to expression of high affinity α3 isoform of the Na,K-ATPase. However, it was reduced by 21%, which correlates well with the amount of the α2 isoform expressed in brain.

We also analyzed the protein expression of the α2 isoform in tissues from α2βR mice to determine whether the desired amino acid substitutions altered its expression and tissue distribution. Western blot analysis of tissue preparations from the α2βRWT and α2βR mice indicated normal protein expression and tissue distribution of the α2 isoform carrying the L111R and N122D amino acid substitutions (Fig. 3B). The α2 isoform of Na,K-ATPase was expressed in heart, brain, and skeletal muscle in both wild type and targeted animals. It is important to point out that an anomalous migration pattern of the α2 isoform was observed in heart and skeletal muscle from both genotypes and resulted from protein overload in these samples.

The expression and tissue distribution of the α1 and α3 isoforms in α2βR mice were analyzed to determine whether any compensatory mechanisms in expression of these two α isoforms resulted from modification of the α2 isoform gene. Western blot analysis showed normal protein expression and tissue distribution of both the α1 and the α3 isoforms in α2βR mice (Fig. 3B). The α1 isoform was expressed in kidney, heart, brain, and skeletal muscle, and protein expression levels in these tissues were qualitatively indistinguishable between the two genotypes. The α3 isoform retained its brain-specific expression in targeted animals, and its protein expression level was indistinguishable from that in wild type (Fig. 3B). Furthermore total Na,K-ATPase protein levels, as detected by KETTY antibody that recognizes all the α isoforms, remained normal in tissues of α2βR animals compared with wild type (Fig. 3B).

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*Fig. 2. Generation of mice expressing the cardiac glycoside-insensitive α2 Na,K-ATPase (α2βR). A, Cre-LoxP targeting strategy. Top, a restriction map of the region in one of the wild type alleles that would be involved in homologous recombination. Middle, targeting vector and a schematic of the targeted allele following successful homologous recombination. Bottom, targeted allele following successful excision of the neomycin cassette by Cre-recombinase. The 5′ and 3′ probes were used for screening of targeted ES cells. Primers P1 and P2 were used in PCR amplification of exon 4 for verification of BstBI site presence. Primers P3 and P4 were used for genotyping of the offspring animals. B, Southern blot analysis of targeted ES cells following the first transfection (upper panel) and second Cre-recombinase transfection (lower panel). C, genotype analysis of offspring following the heterozygous α2βR matings. Primers P3 and P4 amplification of the genomic DNA generated the wild type 307-bp and targeted 342-bp fragments. D, sequence histogram of the partial exon 4 sequence from the α2βR animals. Introduced L111R and N122D amino acid substitutions and a BstBI site were retained in genomic DNA of the α2βR animals. WT, wild type; TK, thymidine kinase; EX, exon.

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a P. F. James and J. B. Lingrel, unpublished data.
There was no observable difference in glyceraldehyde-3-phosphate dehydrogenase expression in each Western blot analysis, indicating relatively equal protein loading (data not shown). Thus, amino acid substitutions reduced ouabain affinity of the α2 isoform but did not alter its normal protein expression or tissue distribution in the α2<sup>R/R</sup> mice. In addition, normal expression and tissue distribution of both α1 and α3 isoforms were not altered in the α2<sup>R/R</sup> mice. The presence of one LoxP site within intron 3 of the α2 isoform gene did not alter its normal expression.

Since the α2 isoform carrying the L111R and N122D substitutions has ouabain affinity similar to the α1 isoform, ouabain-inhibiting Na,K-ATPase activity cannot be distinguished between two isoforms. Therefore, the enzymatic activity of the α2 isoform carrying the mutations could not be measured in tissues from α2<sup>R/R</sup> mice. However, total ouabain-inhibiting Na,K-ATPase activity was indistinguishable between the two genotypes.

**Cardiovascular Performance in Intact Closed Chest α2<sup>R/R</sup> Animals Is Normal**—To determine whether the L111R and N122D substitutions in the α2 Na,K-ATPase isozone altered the physiological hemodynamics and cardiovascular performance of the α2<sup>R/R</sup> mice, we analyzed the heart rate, left ventricular blood pressure, MAP, systolic blood pressure, maximal rate of contraction (dP/dt<sub>max</sub>), minimal rate of contraction (dP/dt<sub>min</sub>), and rate of contraction at 40 mm Hg (dP/dt<sub>40</sub>) under basal conditions and after administration of dobutamine, a β adrenergic agonist (Fig. 4). There were no significant differences in heart rate (Fig. 4A), left ventricular blood pressure (Fig. 4B), MAP (Fig. 4C), and systolic blood pressure (Fig. 4D) of the α2<sup>R/R</sup> and the α2<sup>W/W</sup> mice under basal conditions and after β adrenergic stimulation. These data demonstrate that expression of the ouabain-insensitive α2 isoform does not compromise normal physiological hemodynamics in α2<sup>R/R</sup> mice.

Analysis of cardiac function in intact animals demonstrated that there was also no significant difference in dP/dt<sub>max</sub> (Fig. 4E), dP/dt<sub>min</sub> (data not shown), and dP/dt<sub>40</sub> (Fig. 4F) in the α2<sup>R/R</sup> and the α2<sup>W/W</sup> mice under basal conditions and after β adrenergic stimulation. This demonstrates that α2<sup>R/R</sup> animals have normal heart function.

**Low Doses of Ouabain Do Not Increase Contractility of the Isolated α2<sup>R/R</sup> Hearts**—The effect of ouabain on contractility in isolated work-performing α2<sup>R/R</sup> hearts was analyzed to determine whether reduced ouabain affinity of the α2 isoform altered ouabain-induced cardiac positive inotropy. Isolated work-performing hearts from wild type and α2<sup>R/R</sup> mice were treated with increasing concentrations of ouabain, and the maximum rate of heart contraction (+dP/dt) and relaxation (−dP/dt) were measured (Fig. 5, A and B). The initial increase in cardiac function of wild type hearts, as demonstrated by the increase in maximum rate of contraction (+dP/dt) (Fig. 5A) and relaxation (−dP/dt) (Fig. 5B), was observed at 4 × 10<sup>−5</sup> M ouabain and gradually increased with increasing concentrations of ouabain. This positive cardiac inotropic effect of ouabain was similar to that shown previously (30, 31). We observed only a monophasic response of cardiac contractility to increasing concentrations of ouabain as a biphasic curve is obtained at concentrations of ouabain higher than 10<sup>−5</sup> M. In contrast to wild type, cardiac contractility in α2<sup>R/R</sup> hearts did not increase with increasing concentrations of ouabain (Fig. 5). Doses of ouabain that increased cardiac function in wild type hearts did not affect rate of contraction (Fig. 5A) or rate of relaxation (Fig. 5B) of α2<sup>R/R</sup> hearts. We also analyzed the total change in +dP/dt and −dP/dt of individual wild type and α2<sup>R/R</sup> hearts from basal conditions following perfusion of the final ouabain dose (Fig. 5, C and D). Ouabain increased heart contractility in wild type...
and slightly decreased total cardiac contractility in α2R/R hearts. Thus, the different effects of ouabain on hearts from the two genotypes clearly demonstrate that the increase in heart contractility by low doses of ouabain is mediated through the α2 Na,K-ATPase isoform.

Low Doses of Ouabain Increase Contractility in the Intact α2W/W Animals but Not in the α2R/R Animals—Cardiac contractility as a function of ouabain was also analyzed in intact anesthetized animals. Measurements of the maximal (dP/dt\(_{\text{max}}\)) and minimal rate (dP/dt\(_{\text{min}}\)) of contraction and relaxation and rate of contraction at 40 mm Hg (dP/dt\(_{40}\)) under basal conditions and following infusion of ouabain were obtained. As an estimate of the acting concentrations of ouabain in heart tissue, we calculated the amount of ouabain in extracellular fluid (ECF). The ouabain concentrations in ECF are more informative with respect to pharmacological concentrations in cardiomyocytes of intact animals. Since extracellular fluid corresponds to ~25% of total body weight, ouabain infusion of 1 and 2 nmol/g of body weight corresponded to 4 × 10\(^{-6}\) and 8 × 10\(^{-6}\) M ECF ouabain, respectively, assuming that metabolism and excretion of ouabain were negligible 10 min following the infusion, which was when measurements were made.

Ouabain infusion increased dP/dt\(_{\text{max}}\) (Fig. 6A), dP/dt\(_{\text{min}}\) (data not shown), and dP/dt\(_{40}\) (Fig. 6C) in wild type α2W/W mice. The increase was apparent at ECF ouabain concentration of 4 × 10\(^{-6}\) M and became statistically significant at ECF ouabain concentration of 8 × 10\(^{-6}\) M. No significant increase in dP/dt\(_{\text{max}}\) (Fig. 6A), dP/dt\(_{\text{min}}\) (data not shown), and dP/dt\(_{40}\) (Fig. 6C) was observed in the targeted α2R/R mice. We also analyzed the change in dP/dt\(_{\text{max}}\) and dP/dt\(_{40}\) in each animal from two genotypes. Averaging values without analysis of each animal does not provide accurate insight into the effect of ouabain as there could be subtle differences in baseline among animals within same genotype group. Thus, analysis of the change in contractility following ouabain infusion for each animal allowed accurate analysis of ouabain effects on cardiac contractility in intact animals. Total change in dP/dt\(_{\text{max}}\) (Fig. 6B) and dP/dt\(_{40}\) (Fig. 6D) in α2W/W and α2R/R mice from baseline at ECF ouabain concentration of 8 × 10\(^{-6}\) M showed a drastic increase in contractility in wild type, which was significantly different from the very slight change observed in targeted animals. These data demonstrate that low doses of ouabain increase the cardiac contractility by inhibiting the α2 Na,K-ATPase isozyme in hearts of intact animals.

It is important to note that inhibition of the low sensitivity α2 isoform in α2R/R mice was probably responsible for this small, but not statistically significant, increase in contractility at ECF ouabain concentration of 8 × 10\(^{-6}\) M. As the IC\(_{50}\) for the low affinity α isoforms is 1 × 10\(^{-5}\) M ouabain, partial inhibition of the low affinity α2 isoform in α2R/R mice possibly occurred at ECF ouabain concentration of 8 × 10\(^{-6}\) M and resulted in the slight increase in cardiac contractility. This further demon-
concentrations of ouabain. Ouabain increased cardiac contraction and relaxation of the cytes. In addition, the possible presence of the high affinity isoform in peripheral nerves of the heart makes it possible for inhibition of the **(ouabain). Total change in maximum rate of contraction, \( \frac{dP}{dt} \)** in the **(wild type animals but not in targeted hearts. *, \( p < 0.01 \) versus wild type. Values are represented as mean ± S.E.**

strates the importance of the \( \alpha_2 \) isoform in ouabain-induced cardiac positive inotropy. In addition to the \( \alpha_2 \) isoform, partial inhibition of the \( \alpha_1 \) isoform could contribute to a slight increase in cardiac contractility in \( \alpha_2^{R/R} \) mice. Alternatively inhibition of the high affinity \( \alpha_3 \) isoform in peripheral nerves could have also resulted in increased sympathetic nerve activity and therefore increased cardiac contractility.

It is interesting to also point out that infusion of 4 nmol/g of body weight of ouabain, which corresponds to an ECF ouabain concentration of \( 1.6 \times 10^{-5} \) m ouabain, led to cardiac arrest in wild type animals but not in \( \alpha_2^{R/R} \) mice (data not shown). At this concentration of ouabain the \( \alpha_2 \) isoform would be fully inhibited, and only partial inhibition of the \( \alpha_1 \) isoform would have occurred. This demonstrates the importance of the \( \alpha_2 \) isoform in regulation of normal cardiac contractility.

**DISCUSSION**

The mechanism of ouabain-induced cardiac positive inotropy has been speculative and controversial. Although most studies suggest that the Na,K-ATPase mediates ouabain-induced cardiac positive inotropy, direct evidence is lacking. In addition, it is unknown whether this effect in rodents is mediated through the \( \alpha_1 \) or \( \alpha_2 \) isoforms, which are both expressed in cardiomyocytes. In addition, the possible presence of the high affinity \( \alpha_3 \) isoform in peripheral nerves of the heart makes it possible for this isoform to be involved in the ouabain effect. As alternative mechanisms of cardiac glycoside action through novel, non-Na,K-ATPase receptors have also been suggested (21–25), it is important to comprehensively analyze the functional role of Na,K-ATPase in the cardiac glycoside-induced positive inotropy. Thus, we have directly analyzed the role of Na,K-ATPase in the ouabain-induced cardiac inotropy and determined which specific \( \alpha \) isoform mediates this effect.

We generated mice expressing the \( \alpha_2 \) Na,K-ATPase isoform with reduced cardiac glycoside sensitivity and analyzed the contractility at low doses of ouabain in both isolated hearts and intact animals. Our data clearly demonstrate that the ouabain-induced positive cardiac inotropic effect is mediated through the \( \alpha_2 \) Na,K-ATPase. This represents direct evidence that this effect is mediated through inhibition of the Na,K-ATPase and specifically through the \( \alpha_2 \) Na,K-ATPase isoform. Although inhibition of the high affinity \( \alpha_3 \) isoform by ouabain may have occurred in peripheral neurons of the \( \alpha_2^{R/R} \) mice, it evidently played a negligible role in mediating the ouabain-induced cardiac positive inotropy in intact animals. We do not know whether the inhibition of the \( \alpha_3 \) isoform by ouabain in brain played a role in regulating heart contractility as ouabain does not pass readily across the blood-brain barrier.

In addition, analysis in intact animals indicated that inhibi-

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**Fig. 5. Contraction and relaxation parameters of the isolated \( \alpha_2^{W/W} \) and \( \alpha_2^{R/R} \) hearts at low doses of ouabain.** Maximal rate of contraction, \( +\frac{dP}{dt} \) (A), and maximal rate of relaxation, \( -\frac{dP}{dt} \) (B) in the \( \alpha_2^{W/W} (n = 3) \) and \( \alpha_2^{R/R} \) hearts \( (n = 3) \) were determined at increasing concentrations of ouabain. Ouabain increased cardiac contraction and relaxation of the \( \alpha_2^{W/W} \) hearts as detected by the increase in \( +\frac{dP}{dt} \) (A) and \( -\frac{dP}{dt} \) (B), respectively. No significant change in \( +\frac{dP}{dt} \) (A) and \( -\frac{dP}{dt} \) (B) was detected in the \( \alpha_2^{R/R} \) hearts with increasing concentrations of ouabain. Total change in maximum rate of contraction, \( \Delta +\frac{dP}{dt} \) (C), and total change from the base line in maximum rate of relaxation, \( \Delta -\frac{dP}{dt} \) (D), in the \( \alpha_2^{W/W} \) and \( \alpha_2^{R/R} \) hearts were determined for each animal following perfusion of \( 8 \times 10^{-5} \) m ouabain. Perfusion of \( 8 \times 10^{-5} \) m ouabain increased total \( +\frac{dP}{dt} \) (C) and \( -\frac{dP}{dt} \) (D) in wild type but not in targeted hearts. *, \( p < 0.01 \) versus wild type. Values are represented as mean ± S.E.
tion of the α1 isoform of Na,K-ATPase could have very little effect in ouabain-induced cardiac positive inotropy. The highest non-lethal dose of ouabain significantly increased cardiac contractility in wild type animals and had very little effect in α2R/R mice. If the α1 isoform played a major role in ouabain-induced positive inotropy, a significant increase in cardiac contractility would have been observed in α2R/R mice. This is in contrast to what was suggested recently (19, 20).

Additional receptors have been implicated in regulating the positive inotropic effect of ouabain. Low concentrations of digitoxin have been shown to activate RyR in sarcoplasmic reticulum (22, 23). Furthermore it has been suggested that ouabain at low concentrations is transported into the cell resulting in intracellular activation of RyR (24, 25). Although our data cannot rule out the possibility that additional intracellular receptors exist, our studies clearly show that α2 Na,K-ATPase isozyme is the cardiac glycoside receptor through which the increase in heart contractility by low doses of ouabain is mediated. Activation of RyR at low concentration of cardiac glycosides is expected as a consequence of the α2 Na,K-ATPase isozyme inhibition. Inhibition of α2 Na,K-ATPase would affect the NaCa-exchanger through alteration of the Na⁺ gradient causing a theoretical reversal of its mode and influx of Ca²⁺.

Increase in intracellular Ca²⁺ concentration would result in Ca²⁺-induced activation of RyR and the Ca²⁺-induced Ca²⁺ release from sarcoplasmic reticulum. Therefore, activation of RyR is a potential effect of the α2 isoform inhibition, thereby enabling the concentration of cytosolic Ca²⁺ to reach the necessary threshold, resulting in cardiac muscle contraction.

It is interesting to note that α2R/R animals represent a possible model for studying the role of endogenous ouabain (EO). Digitalis-like endogenous substances have been detected and isolated from human plasma (32). These endogenous cardiac glycosides have been implicated in hypertension (for a review, see Ref. 33). However, their physiological role is still unknown. As we have demonstrated that the ouabain-induced increase in heart contractility is mediated through the α2 Na,K-ATPase, it is logical to suggest that if endogenous ouabain regulates cardiac contractility it would be through the α2 Na,K-ATPase. As the α2R/R animals have normal survival rates, normal physiological hemodynamics, and normal cardiac function compared with their wild type littermates, it is likely that EO regulation of α2 Na,K-ATPase is not necessary for survival and normal function under resting conditions. This is not surprising as circulating levels of EO are very low in unchallenged animals. However, an increase in EO has been detected in conditions

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**Fig. 6. Effect of ouabain on cardiac contractility in intact α2^WW^ and α2^R/R^ animals.** The effect of ouabain on the maximum rate of contraction, dP/dt max (A), and on the maximum rate of contraction at 40 mmHg, dP/dt40 (C), were determined in α2^WW^ (n = 9) and α2^R/R^ (n = 10) mice. ΔdP/dt max (B) and ΔdP/dt40 (D) were determined following infusion of 2 nmol/g of body weight ouabain. Increasing ouabain concentrations raised the dP/dt max (A) and dP/dt40 (C) in intact α2^WW^ mice. No significant increase in the dP/dt max (A) and dP/dt40 (C) was observed in intact α2^R/R^ mice. ΔdP/dt max (B) and ΔdP/dt40 (D) were significantly larger in α2^WW^ mice than in α2^R/R^ mice. *, p < 0.01 versus wild type; #, p < 0.005 versus wild type base line (0 nmol/g of body weight ouabain). Values are represented as mean ± S.E.
such as hypertension, physical exercise, congestive heart failure, and myocardial infarction (33). Therefore, it is of interest to analyze the α2β6β5 animals under conditions known to have high circulating levels of EO. As EO may also act through the α3 isoform, it will be important to develop animals in which this isoform is also made resistant to cardiac glycosides.

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