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Marinobufagenin enhances cardiac contractility in mice with ouabain-sensitive α_1 Na^+-K^+-ATPase

Arshani N. Wansapura,[1] Valerie Lasko,[1] Zijian Xie,[3] Olga V. Fedorova,[4] Alexei Y. Bagrov,[4] Jerry B Lingrel,[2] and John N. Lorenz[1]

Departments of [1]Molecular and Cellular Physiology and [2]Molecular Genetics, University of Cincinnati College of Medicine, Cincinnati; [3]Department of Physiology and Pharmacology, University of Toledo College of Medicine, Toledo, Ohio; and [4]Laboratory of Cardiovascular Science, National Institute on Aging, Baltimore, Maryland

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Wansapura AN, Lasko V, Xie Z, Fedorova OV, Bagrov AY, Lingrel JB, Lorenz JN. Marinobufagenin enhances cardiac contractility in mice with ouabain-sensitive α_1 Na^+-K^+-ATPase. *Am J Physiol Heart Circ Physiol* 296: H1833–H1839, 2009. First published April 17, 2009; doi:10.1152/ajpheart.00285.2009.—Endogenous Na^+ pump inhibitors are thought to play important (patho)physiological roles and occur in two different chemical forms in the mammalian circulation: cardenolides, such as ouabain, and bufadienolides, such as marinobufagenin (MBG). Although all α Na^+-K^+-ATPase isoforms ($\alpha_{1\text{-}4}$) are sensitive to ouabain in most species, in rats and mice the ubiquitously expressed α_1 Na^+-K^+-ATPase is resistant to ouabain. We have previously shown that selective modification of the putative ouabain binding site of either the α_1 or α_2 Na^+-K^+-ATPase subunit in mice substantially alters the cardiotonic influence of exogenously applied cardenolides. To determine whether the ouabain binding site also interacts with MBG and if this interaction plays a functional role, we evaluated cardiovascular function in α_1-resistant/α_2-resistant ($\alpha_1^{R/R}\alpha_2^{R/R}$), α_1-sensitive/α_2-resistant ($\alpha_1^{S/S}\alpha_2^{R/R}$), and α_1-resistant/α_2-sensitive mice ($\alpha_1^{R/R}\alpha_2^{S/S}$, wild type). Cardiovascular indexes were evaluated in vivo by cardiac catheterization at baseline and during graded infusions of MBG. There were no differences in baseline measurements of targeted mice, indicating normal hemodynamics and cardiac function. MBG at 0.025, 0.05, and 0.1 nmol·min^{-1}·g body wt^{-1} significantly increased cardiac performance to a greater extent in $\alpha_1^{S/S}\alpha_2^{R/R}$ compared with $\alpha_1^{R/R}\alpha_2^{R/R}$ and wild-type mice. The increase in $LVdP/dt_{max}$ in $\alpha_1^{S/S}\alpha_2^{R/R}$ mice was greater at higher concentrations of MBG compared with both $\alpha_1^{R/R}\alpha_2^{R/R}$ and $\alpha_1^{R/R}\alpha_2^{S/S}$ mice ($P < 0.05$). These results suggest that MBG interacts with the ouabain binding site of the α_1 Na^+-K^+-ATPase subunit and can thereby influence cardiac inotropy.

cardiotonic steroids; sodium-potassium-adenosinetriphosphatase isoforms

THE NA^+-K^+-ATPASE is a ubiquitous transmembrane Na^+ pump and a specific receptor for cardiac glycosides such as ouabain and digoxin, which are used in the management of congestive heart failure. Cardiotonic steroids (CTS), such as ouabain and digoxin, bind to and inhibit the α-subunit of the Na^+-K^+-ATPase, of which there are four known isoforms (designated $\alpha_{1\text{-}4}$), and thereby increase intracellular Na^+ concentration. The resulting elevation in cellular Na^+ causes a secondary increase in free intracellular Ca^{2+} concentration via reduction in Na^+/Ca^{2+} exchanger activity, and the increased Ca^{2+} is responsible for the positive inotropic (therapeutic) effect. In recent years, a potential role for endogenous CTS has been explored, and it has become apparent that such compounds are elevated and may play an important role in a variety of physiological and (patho)physiological conditions. For example, elevated plasma and urine levels of endogenous CTS have been reported in chronic renal failure, congestive heart failure, essential hypertension, myocardial infarction, diabetes, and preeclampsia, with similar findings in experimental animal models (3, 5–7, 14–16, 18, 19).

All identified CTS share a common general structure, i.e., an aglycone moiety composed of the steroid nucleus, a lactone ring at position 17, and a hydroxyl group at C-14. The lactone ring at position 17 defines two chemically distinct classes of CTS, the cardenolides, such as ouabain and digoxin, and bufadienolides, such as marinobufagenin (MBG) and bufalin, consisting of five- and six-member lactone rings, respectively (8). A differential mechanism of action between these two classes of CTS compounds has been suggested (23) and attributed to *1*) differences in the steroid structures that may render differential binding characteristics; *2*) the nature of the steroid binding site, and *3*) cell and species specificity.

In most species, including humans, all α Na^+-K^+-ATPase isoforms are sensitive to ouabain (25). In mice and rats, however, the α_1 Na^+-K^+-ATPase has relatively low affinity for, and is resistant to, the inhibitory effects of ouabain and other cardiac glycosides (17). It is currently understood that the effects of cardenolides such as ouabain and digoxin are largely mediated through a specific binding site on the first extracellular loop of the catalytic Na^+-K^+-ATPase α-subunit (20–22), and these binding properties have been used to generate mutant mice with altered sensitivity to ouabain to explore ligand binding site interactions. Our previous studies using these mice have shown that exogenously applied ouabain, and other cardenolides, can enhance cardiac contractility in mice that express a sensitive α_1 or α_2 Na^+-K^+-ATPase, but not in mice in which both the α_1 and α_2 Na^+-K^+-ATPase are resistant to ouabain. A remaining question is whether the mutation of the ouabain binding site also alters the sensitivity and binding of the other class of CTS, namely bufadienolides.

The primary objective of this study, therefore, was to determine whether the ouabain binding site also interacts with bufadienolides and whether this interaction can have a functional role in the murine heart. We analyzed the enzyme-inhibitory and cardiotonic effects of MBG in mice with selective ouabain sensitivity or resistance of the α_1 and α_2 Na^+-K^+-ATPase isoforms: *1*) wild-type mice expressing ouabain-resistant α_1 and ouabain-sensitive α_2 Na^+-K^+-ATPase subunits ($\alpha_1^{R/R}\alpha_2^{S/S}$), *2*) single-mutant mice with ouabain-resistant α_1- and α_2-sub-

Address for reprint requests and other correspondence: J. N. Lorenz, Dept. of Molecular and Cellular Physiology, College of Medicine, Univ. of Cincinnati, 231 Albert Sabin Way, P.O. Box 670576, Cincinnati, OH 45267-0576 (e-mail: john.lorenz@uc.edu).

units ($\alpha_1^{R/R}\alpha_2^{R/R}$), and *3*) double-mutant mice with ouabain-sensitive α_1- and ouabain-resistant α_2-subunits ($\alpha_1^{S/S}\alpha_2^{R/R}$).

MATERIALS AND METHODS

Animal models. Animals were obtained from established colonies at the University of Cincinnati and were on a mixed 129SvJ and Black Swiss background. The colony of single-mutant mice expressing ouabain-resistant α_2 Na^+-K^+-ATPase subunits ($\alpha_1^{R/R}\alpha_2^{R/R}$) was maintained by mating heterozygous male and female animals ($\alpha_1^{R/R}\alpha_2^{S/R} \times \alpha_1^{R/R}\alpha_2^{S/R}$) (9). The colony of double-mutant mice expressing ouabain-sensitive α_1 Na^+-K^+-ATPase subunits and ouabain-resistant α_2 Na^+-K^+-ATPase subunits ($\alpha_1^{S/S}\alpha_2^{R/R}$) were maintained by mating homozygous double-mutant animals (10). Breeding pairs were periodically backcrossed to a parallel subcolony of wild-type mice to sustain a consistent genetic background between mutant and wild-type mice. Wild-type animals used in these studies were obtained from both colonies. Genotypes were confirmed by PCR analysis of DNA from tail biopsies, as described (9, 10). Experiments were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Cincinnati.

Determination of α_1 Na^+-K^+-ATPase activity. Kidney samples from $\alpha_1^{S/S}\alpha_2^{R/R}$ and $\alpha_1^{R/R}\alpha_2^{S/S}$ mice were ground in liquid nitrogen, and microsomes were prepared as described previously (27). ATPase activity was assayed by the determination of the initial rate of release of P_i from ATP. In a final volume of 1 ml, the reaction mixture for the assay contained 2 mM [γ-^{32}P]ATP, 3 mM $MgCl_2$, 100 mM NaCl, 5 mM KCl, 1 mM EGTA, 5 mM $NaNO_3$, 50 mM Tris·HCl (pH 7.4), the indicated amount of enzyme source, and ouabain or MBG when indicated. Azide was included in the mixture to inhibit mitochondrial ATPase, and EGTA was included to inhibit Ca^{2+}-dependent ATPase. For each enzyme sample, the assay was done in the presence of increasing concentrations of ouabain or MBG and in its absence, the difference between the two being the Na^+-K^+-ATPase activity. Assays were conducted for the indicated periods at 37°C. Each reaction was terminated by the addition of 1 ml of 8% perchloric acid. Released $^{32}P_i$ was converted to phosphomolybdate, extracted into 2-methylpropanol, and counted.

Analysis of cardiovascular performance in intact mice. Adult mice, 10–12 wk of age from $\alpha_1^{S/S}\alpha_2^{R/R}$ ($n = 6$), wild-type $\alpha_1^{R/R}\alpha_2^{S/S}$ ($n = 7$), and $\alpha_1^{R/R}/\alpha_2^{R/R}$ ($n = 7$) genotypes were used for assessment of cardiac function. Mice were anesthetized with an intraperitoneal injection of ketamine (50 μg/g body wt) and Inactin (thiobutabarbital, 100 μg/g body wt; Sigma). A tracheotomy was performed, and body temperature was monitored and maintained with a feedback-controlled heating table. The right femoral artery and vein were cannulated with polyethylene tubing for the measurement of blood pressure and for infusion of experimental agents. A high-fidelity, 1.4-Fr Millar Mikro-Tip transducer (model SPR-671; Millar Instruments, Houston, TX) was inserted in the right carotid artery and advanced into the left ventricle (LV) to monitor cardiac performance. Experimental solutions were delivered as a constant infusion (0.1 μl·min^{-1}·g body wt^{-1}).

Protocol 1. To examine the effects of MBG on cardiac performance, MBG was infused in three doses of 0.025, 0.05, and 0.1 nmol·min^{-1}·g body wt^{-1}. Each dose was administered over a 15-min period. For comparison of peak responses, separate animals were treated with a bolus infusion of ouabain at 1 nmol/g body wt. In preliminary experiments, we found that this dose of ouabain produced maximal inotropic responses and that higher doses were toxic. Hemodynamic variables were collected and analyzed using a PowerLab system (AD Instruments, Colorado Springs, CO). Average values for heart rate, arterial pressure, and indexes of left ventricular function were determined for each animal from at least 50 consecutive beats during peak response. Maximum dP/d*t* (dP/dt_{max}) and dP/d*t* at 40 mmHg of developed pressure (dP/dt_{40}) were calculated from the first derivative of the LV pressure waveforms. At the end of cardiac function assessment, 0.5 ml of blood was collected from the femoral artery. In addition, blood samples were collected from separate groups of untreated mice from each genotype. Plasma samples were stored at −80°C for determination of plasma MBG levels.

Protocol 2. To address the possibility of MBG interaction with cardiac β-adrenergic receptors, we assessed cardiovascular function in $\alpha_1^{S/S}\alpha_2^{R/R}$ ($n = 6$) and $\alpha_1^{R/R}\alpha_2^{R/R}$ ($n = 6$) mice in response to incremental doses of β-adrenergic agonist dobutamine at doses of 2, 8, and 32 ng·min^{-1}·g body wt^{-1} under control conditions and during infusion of MBG at 0.05 nmol·min^{-1}·g body wt^{-1}.

Determination of plasma MBG levels. MBG immunoassay was performed as described in previous studies with modifications (14). The assay is based on competition between immobilized antigen (MBG-glycoside-thyroglobulin) and steroid within the sample for a limited number of binding sites on polyclonal rabbit MBG antibody against MBG-glycoside-BSA (antiserum aMBG-P, 1:40,000). Secondary (goat anti-rabbit) antibody (200 ng/1 ml) labeled with europium was obtained from Perkin Elmer. Cross-reactivity of the MBG antibody was as follows (%): 100 MBG, 0.1 ouabain, 0.1 digoxin, 3.0 digitoxin, 1.0 bufalin, 1.0 cinobufagin, <0.1 prednisone, <0.1 spironolactone, <1.0 proscillaridin, and <0.1 progesterone.

MBG was isolated from toad (*Bufo marinus*) venom as described previously (4). The isolated MBG was >98% pure based on high-performance liquid chromatography and mass spectroscopy analysis.

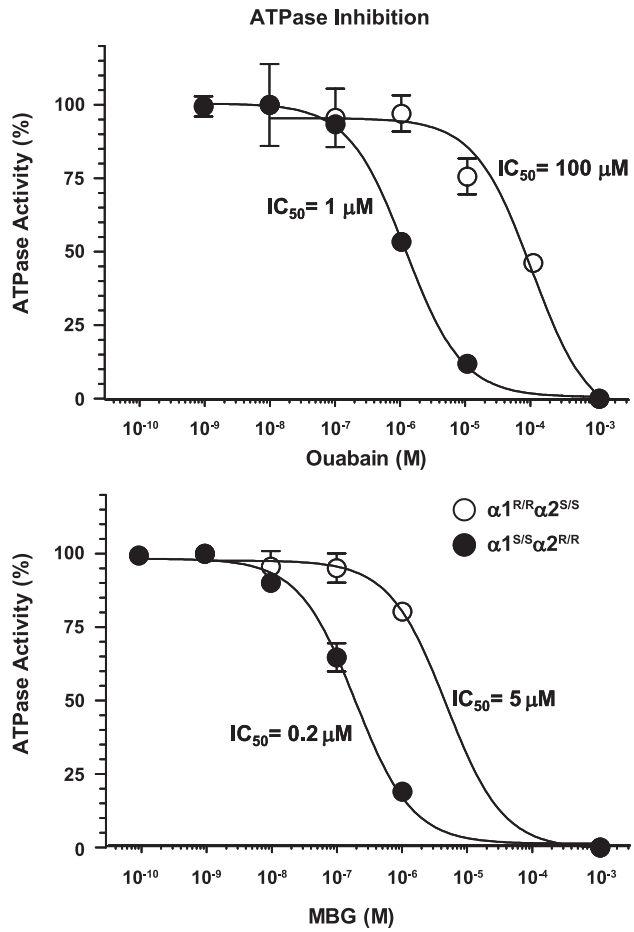

Fig. 1. Concentration-response curves of ouabain- (*top*)- and marinobufagenin (MBG; *bottom*)-induced inhibition of α_1 Na^+-K^+-ATPase activity in microsomal kidney fractions from α_1-resistant/α_2-sensitive ($\alpha_1^{R/R}\alpha_2^{S/S}$; ○) and α_1-sensitive/α_2-resistant ($\alpha_1^{S/S}\alpha_2^{R/R}$; ●) mice. IC_{50} values, as indicated, were calculated from the sigmoidal (Boltzman) fit of the normalized data. Individual values are expressed as means ± SE of three separate measurements.

Table 1. *Cardiovascular indexes in anesthetized mice*

	$\alpha_1^{R/R}\ \alpha_2^{S/S}$ (n = 7)	$\alpha_1^{R/R}/\alpha_2^{R/R}$ (n = 7)	$\alpha_1^{S/S}\alpha_2^{R/R}$ (n = 6)
Heart rate, beats/min	406±19	404±28	403±18
MAP, mmHg	82±4	84±4	87±2
SBP, mmHg	99±6	100±4	105±3
Systolic LVP, mmHg	111±9	108±6	108±3
dP/dt_{max}, mmHg/s	8,269±484	8,265±450	7,558±320
dP/dt_{40}, mmHg/s	7,071±322	7,171±200	7,467±449

Values are expressed as means ± SE; n, no. of mice. $\alpha_1^{R/R}\alpha_2^{S/S}$, α_1-resistant/α_2-sensitive mice; $\alpha_1^{R/R}\alpha_2^{R/R}$, α_1-resistant/α_2-resistant; $\alpha_1^{S/S}\alpha_2^{R/R}$, α_1-sensitive/α_2-resistant; MAP, mean arterial pressure; SBP, systolic blood pressure; LVP, left ventricular pressure; dP/dt_{max}, average maximum value of first derivative of intraventricular pressure; dP/dt_{40}, average dP/dt at 40 mmHg of developed pressure. No significant differences were detected between the genotypes.

Data analysis. Data in Figs. 1–5 and Table 1 are reported as means ± SE. Data were analyzed using a mixed two-way ANOVA with repeated measures on the second factor, using SigmaStat and SuperANOVA software. Post hoc analysis was performed by interaction-comparisons between two groups and using the Holm-Sidak test for comparisons between individual means where appropriate. P values <0.05 were accepted as statistically different.

RESULTS

Inhibition of Na^+-K^+-ATPase activity by ouabain and MBG. To determine whether mutation of the α_1 Na^+-K^+-ATPase ouabain binding site altered the sensitivity of this subunit to MBG, as well as to ouabain, we examined enzymatic activity of the α_1-isoform in kidney membrane preparations from $\alpha_1^{S/S}\alpha_2^{R/R}$ and $\alpha_1^{R/R}\alpha_2^{S/S}$ (wild-type) mice in the presence of increasing concentrations of ouabain and MBG (Fig. 1). Kidney tissue is one of the highest expressing tissues of Na-K ATPase, and since renal tubular cells express only the α_1-isoform, unlike cardiac tissue which express both α_1- and α_2-isoforms, almost all of the measured ATPase activity in renal tissue can be attributed to the α_1-isoform. The data show that mutation of the α_1-isoform results in a leftward shift in both the ouabain and MBG dose-response curves, indicating greater sensitivity to both steroids compared with the wild-type α_1-isoform. These data also show that the affinity for MBG is markedly higher than for ouabain in both α_1-sensitive mice (MBG IC_{50} = 0.2 μM vs. ouabain IC_{50} = 1 μM) and α_1-resistant mice (MBG IC_{50} = 5 μM vs. ouabain IC_{50} = 100 μM). Together these data suggest that both known classes of CTS, the cardenolides and bufadienolides, interact primarily with the same binding site of the α Na^+-K^+-ATPase subunit.

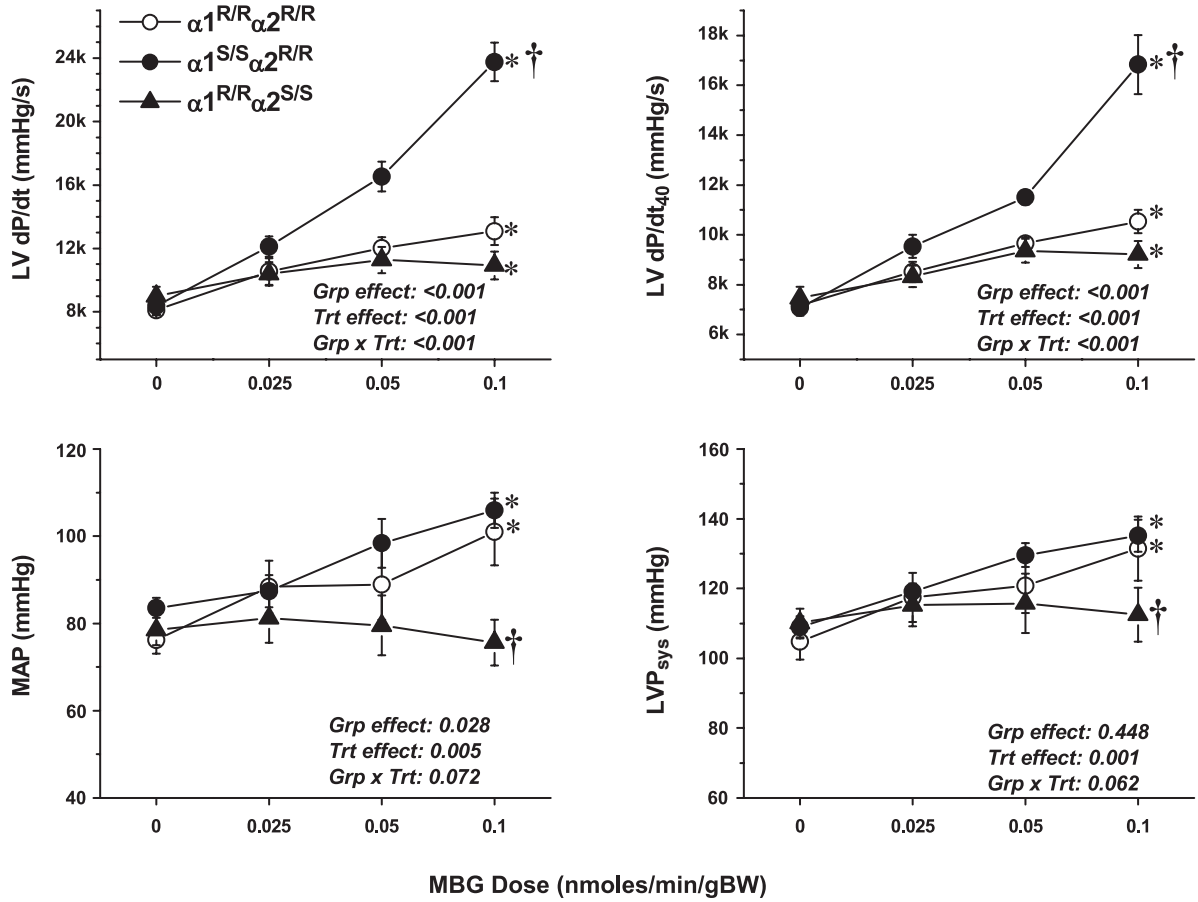

Fig. 2. Cardiac contractile and blood pressure in intact α_1-resistant/α_2-resistant ($\alpha_1^{R/R}\alpha_2^{R/R}$; n = 7), $\alpha_1^{S/S}\alpha_2^{R/R}$ (n = 6), and $\alpha_1^{R/R}\alpha_2^{S/S}$ (n = 7) mice in response to increasing doses of MBG. The effect of MBG on the maximum rate of contraction (dP/dt_{max}) and on the rate of contraction at 40 mmHg (dP/dt_{40}) are shown at *top*; mean arterial pressure (MAP) and left ventricle (LV) systolic pressure (LVP_{sys}) are shown at *bottom*. Results from the overall ANOVA are shown in each panel. Post hoc comparisons: *significant effect of MBG for individual genotypes ($P < 0.01$); †significantly different treatment effect (of MBG) compared with $\alpha_1^{R/R}\alpha_2^{R/R}$ ($P < 0.05$). Grp, group; Trt, treatment.

Left ventricular contractile response to MBG. To determine whether mutation of the α_1 Na^+-K^+-ATPase ouabain binding site altered the contractile response to exogenous MBG, we analyzed LV performance between genotypes under basal conditions and during administration of increasing doses of MBG. As shown in Table 1, there were no significant differences in baseline cardiovascular indexes of the $\alpha_1^{S/S}\alpha_2^{R/R}$, $\alpha_1^{R/R}\alpha_2^{S/S}$, and $\alpha_1^{R/R}\alpha_2^{R/R}$ mice. These data confirm that alteration of either the α_1 or α_2 ouabain binding site does not compromise normal hemodynamics and cardiac function of targeted mice. On the other hand, as shown in Fig. 2, the cardiovascular responses to exogenous MBG infusion were markedly different between the three genotypes. Although MBG increased dP/dt_{max} and dP/dt_{40} in all three groups, the increase was much greater in the $\alpha_1^{S/S}\alpha_2^{R/R}$ mice compared with $\alpha_1^{R/R}\alpha_2^{S/S}$ or $\alpha_1^{R/R}\alpha_2^{R/R}$ mice (which were not different from each other). Interestingly, we also found that both mean arterial pressure (MAP) and LV systolic pressure increased in mice with a ouabain-resistant α_2-isoform ($\alpha_1^{S/S}\alpha_2^{R/R}$ and $\alpha_1^{R/R}\alpha_2^{R/R}$) but not in the wild-type mice harboring a ouabain-sensitive α_2-isoform ($\alpha_1^{R/R}\alpha_2^{S/S}$). Although intravenous infusion of MBG also had a significant dose effect on heart rate in all groups ($P < 0.001$, data not shown) no differences were observed between genotypes. Contractile responses to 1 nmol/g body wt ouabain were also assessed in $\alpha_1^{S/S}\alpha_2^{R/R}$, $\alpha_1^{R/R}\alpha_2^{S/S}$, and $\alpha_1^{R/R}\alpha_2^{R/R}$ mice. A bolus infusion was given since the long half-life of ouabain precludes steady-state measurements at constant infusion rates. As with MBG, the $\alpha_1^{S/S}\alpha_2^{R/R}$ mutants demonstrated an enhanced inotropic response to ouabain compared with $\alpha_1^{R/R}\alpha_2^{S/S}$ and $\alpha_1^{R/R}\alpha_2^{R/R}$ mice (Fig. 3). However, in contrast to MBG, MAP increased only in the $\alpha_1^{S/S}\alpha_2^{R/R}$ mice in response to ouabain. These data indicate that mutant mice with a ouabain-sensitive α_1 Na^+-K^+-ATPase have a markedly enhanced cardiac contractile response to bufadienolides as well as to cardenolides.

To test whether MBG might influence cardiac function by interacting with β-adrenoreceptors, or through other mechanisms that may alter the response to adrenergic agonists, we compared dobutamine dose-response relationships under baseline conditions and following MBG treatment in $\alpha_1^{S/S}\alpha_2^{R/R}$ and $\alpha_1^{R/R}\alpha_2^{R/R}$ mice (Fig. 4). At baseline, dobutamine increased dP/dt_{40} similarly in the two genotypes, indicating normal β-adrenergic responsiveness. In $\alpha_1^{S/S}\alpha_2^{R/R}$ mice (Fig. 4, *right*), MBG treatment enhanced contractile performance equally at all doses of dobutamine (i.e., no treatment × dose interaction). In $\alpha_1^{R/R}\alpha_2^{R/R}$ mice, MBG had no effect at high or low doses of dobutamine. These data indicated that the inotropic effects of MBG are independent of adrenergic signaling pathways.

We next sought to determine the threshold plasma concentration for the observed cardiac functional effects of MBG. As shown in Fig. 5, plasma MBG concentrations of untreated mice (baseline) were in the subnanomolar range and were similar between $\alpha_1^{S/S}\alpha_2^{R/R}$, $\alpha_1^{R/R}\alpha_2^{S/S}$, and $\alpha_1^{R/R}\alpha_2^{R/R}$ mice. Intravenous infusion of 0.025 nmol·min^{-1}·g body wt^{-1} MBG for 3 min resulted in an ~10-fold increase in the plasma MBG concentration to ~1–3 nM. This level is noteworthy, since it corresponds to the effective minimum dose that alters cardiac function in the $\alpha_1^{S/S}\alpha_2^{R/R}$ mutant mice, as shown in Fig. 5, *right*. Intravenous infusion of MBG at 0.1 nmol·min^{-1}·g body wt^{-1} for 15 min increased plasma MBG concentrations to ~50 nM.

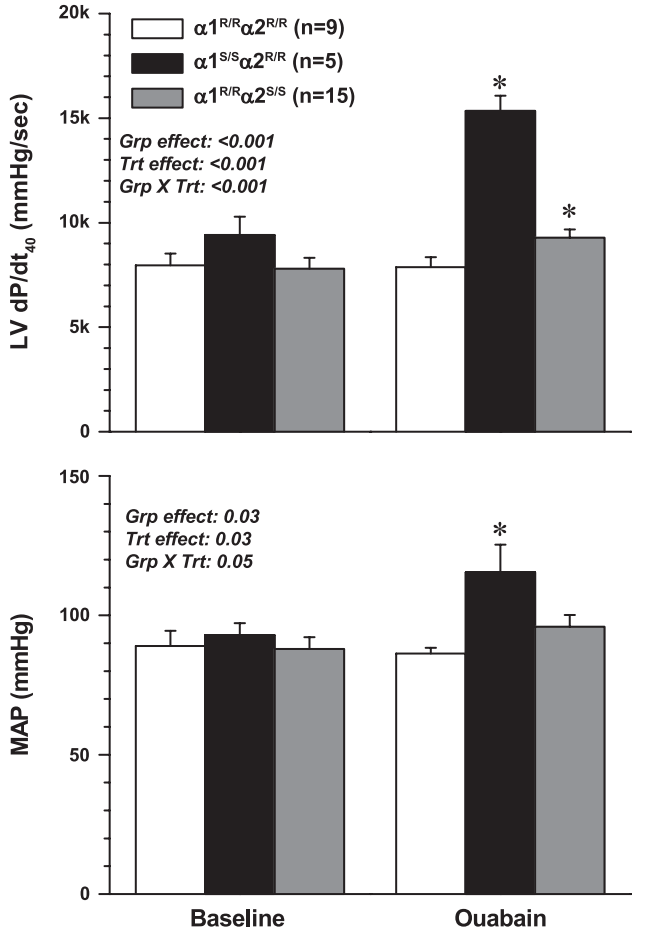

Fig. 3. Cardiovascular responses ($LVdP/dt_{40}$, MAP) in $\alpha_1^{R/R}\alpha_2^{S/S}$, $\alpha_1^{R/R}\alpha_2^{R/R}$, and $\alpha_1^{S/S}\alpha_2^{R/R}$ mice following infusion of ouabain at 1 nmol/g body wt. Results from the overall ANOVA are shown in each panel. Post hoc comparisons: $^*P < 0.05$ vs. corresponding baseline value.

DISCUSSION

We have previously demonstrated that genetic alteration of the so-called ouabain binding site of the Na^+-K^+-ATPase α-subunit results in dramatic changes in the physiological effects of cardenolides such as ouabain and digoxin (9–11). The primary goal of this study was to ascertain whether these same genetic mutations similarly influence the actions of bufadienolides such as MBG. We evaluated and compared the functional effects of MBG and ouabain in mutant mice with altered ouabain sensitivity of both α_1 and α_2 Na^+-K^+-ATPase subunits. In our first experiment, we examined the ability of ouabain and MBG to inhibit Na^+-K^+-ATPase in isolated membrane preparations from renal cortex. Compared with $\alpha_1^{R/R}\alpha_2^{S/S}$, preparations from $\alpha_1^{S/S}\alpha_2^{R/R}$ mice were 100-fold more sensitive to ouabain and 25-fold more sensitive to MBG. Furthermore, these activity data confirm that MBG has a greater affinity for the α_1 Na^+-K^+-ATPase than does ouabain regardless of whether the isoform is "sensitive" (5-fold) or "resistant" (20-fold), which is consistent with previously published data (13). In situ experiments testing the effects of MBG on heart function also showed that $\alpha_1^{S/S}\alpha_2^{R/R}$ mice were much more sensitive to MBG than either group of mice with a ouabain-resistant α_1-isoform ($\alpha_1^{R/R}\alpha_2^{R/R}$ or $\alpha_1^{R/R}\alpha_2^{S/S}$). Im-

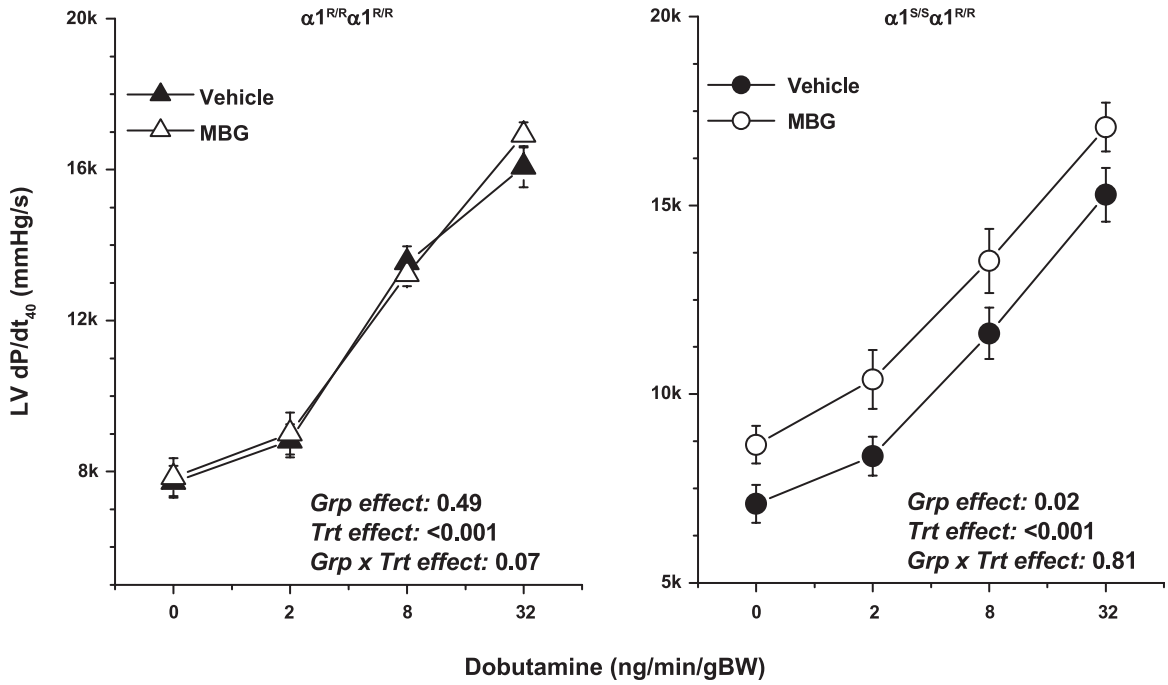

Fig. 4. Dobutamine dose-response relationships for LV dP/dt_{40} during vehicle infusion followed by 0.05 nmol·min^{-1}·g body wt^{-1} of MBG in $\alpha_1^{R/R}\alpha_2^{R/R}$ (n = 6, *left*) and $\alpha_1^{S/S}\alpha_2^{R/R}$ (n = 6, *right*) mice. Values are expressed as means ± SE. Results from the overall ANOVA are shown in each panel.

portantly, there were no differences observed in baseline function indicating that targeted mutation of the specific ouabain binding site does not alter the general properties of the sodium pump. These data provide important evidence that both classes of CTS, cardenolide and bufadienolides, bind to the same extracellular binding pocket on the Na^+-K^+-ATPase. Moreover, our results demonstrate that these mutant mice with altered Na^+-K^+-ATPase binding affinities represent a valid model for evaluating the differential effects of both cardenolides and bufadienolides. Given recent evidence that the bufadienolide MBG represents an important endogenous CTS influencing cardiovascular and renal function (1, 24), validation of these mice as relevant models was essential.

There is a high degree of homology (~87%) between the α_1- and α_2-isoforms of the Na^+-K^+-ATPase, and the sequence variability includes the first extracellular loop, which contains the so-called ouabain binding site. Two amino acid substitutions at positions 111 (Leu/Arg/Gln) and 122 (Asn/Asp) are

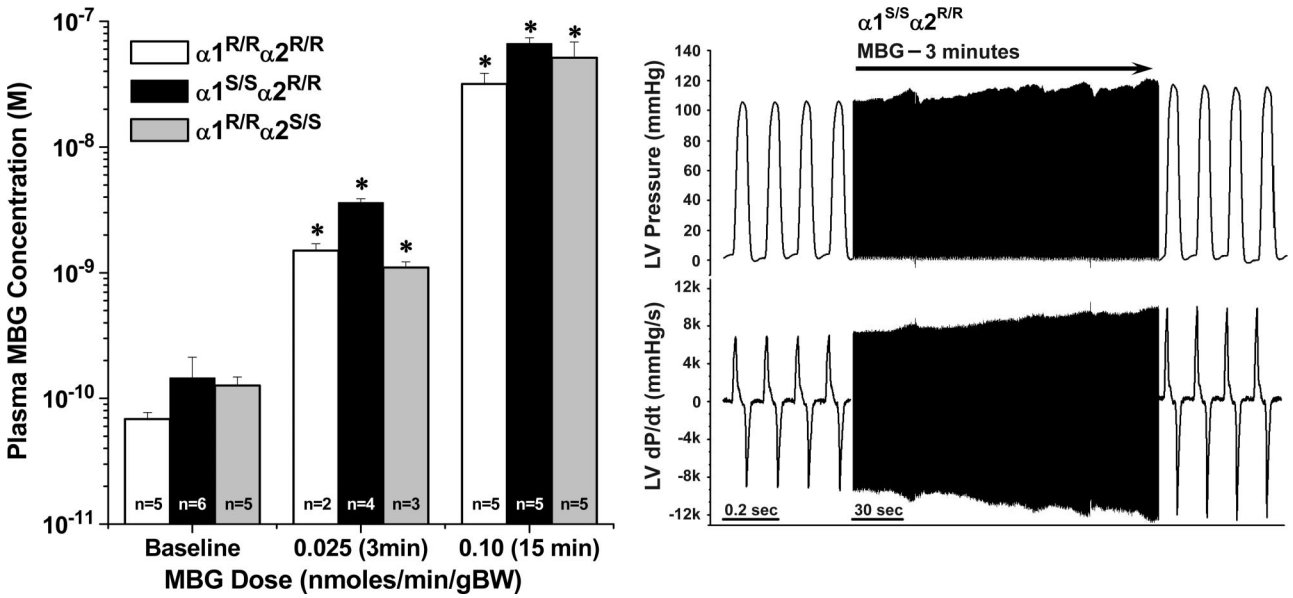

Fig. 5. Plasma MBG levels at baseline and following treatment with MBG. MBG levels were measured after 3 min of MBG at 0.025 nmol·g body wt^{-1}·min^{-1}, and after 15 min at 0.1 nmol·g body wt^{-1}·min^{-1}. An illustration of the cardiotonic effects (LV pressure and dP/dt) of the lower dose of MBG in an $\alpha_1^{S/S}\alpha_2^{R/R}$ mouse is shown on *right*. At each dose, there was no statistical difference between the genotypes. *$P < 0.001$ compared with baseline value in corresponding genotype.

sufficient to substantially alter the sensitivity of the enzyme toward commonly used cardiac glycosides, such as ouabain and digoxin (11, 21). However, to what extent the nature of the lactone ring (i.e., 5 or 6 carbon rings) influences the affinity of CTS for the Na^+-K^+-ATPase binding pocket has not been established. Our data indicate that these amino acid substitutions alter the binding properties for bufadienolides, but perhaps not to the extent of cardenolides. Thus steroid selectivity appears to depend substantially, but not exclusively, on the nature of the lactone ring.

One of the central findings of this study was to establish the concentration range at which MBG might have physiologically relevant effects. One troublesome issue often cited regarding the functional role of endogenous ouabain-like factors has been that they appear to circulate at very low levels and increase to relevant levels only in response to extreme pathological situations. Furthermore, the widespread use of rats and mice expressing a ouabain-resistant α_1 Na^+-K^+-ATPase isoform as a common experimental model has certainly contributed to the uncertainty regarding the physiological relevance of endogenous CTS. Recent data have identified MBG as a potentially important endogenous CTS that circulates normally at subnanomolar levels but can increase to ~1 nM in the rat and 10 nM in the dog in response to maneuvers such as salt loading (2, 12). Our experiments provide several indications that these levels are physiologically meaningful. First, in membrane preparations of the kidney cortex, which express the α_1-subunit almost exclusively, we found that 10 nM MBG decreased ATPase activity by ~10% in $\alpha_1^{S/S}\alpha_2^{R/R}$ (see Fig. 1). Previous studies have shown that therapeutic inotropic effects of cardiac glycosides occur when pump activity is inhibited by only 10–20% (26). In addition, we observed that infusion of MBG at 0.025 nmol·min^{-1}·g body wt^{-1} for only 3 min resulted in a 30–40% increase in LV contractile function in the $\alpha_1^{S/S}\alpha_2^{R/R}$ mice (Fig. 5). In separate experiments, we found that this level of MBG infusion increased plasma MBG levels from ~0.1 to 1–3 nM. These data provide compelling evidence that physiological concentrations of MBG can interact significantly with a Na^+-K^+-ATPase α-subunit that is "ouabain-sensitive".

It is relevant that $\alpha_1^{R/R}\alpha_2^{R/R}$ and $\alpha_1^{R/R}\alpha_2^{S/S}$ mice exhibited small, but significant, increases in cardiac contractile performance (dP/dt_{max} and dP/dt_{40}; Fig. 2). It is not clear whether this increase is due to a partial effect of MBG on the low-affinity α_1 ouabain-resistant isoform or to an effect of MBG on the α_2-isoform, which is also expressed in the heart, but at lower levels than the α_1-isoform. The effect may also be mediated by the α_3-isoform, expressed in neuronal tissue, through an alteration of sympathetic outflow to the heart. More puzzling perhaps are the disparate effects of MBG on blood pressure between the $\alpha_1^{R/R}\alpha_2^{S/S}$ and the $\alpha_1^{R/R}\alpha_2^{R/R}$ or $\alpha_1^{S/S}\alpha_2^{R/R}$ mice, also shown in Fig. 2. Both mean arterial blood pressure and LV systolic pressure were increased by MBG in mice expressing a ouabain-resistant α_2-isoform, but not in mice expressing a ouabain-sensitive α_2-isoform (i.e., wild type). It is not clear how MBG would presumably cause vasoconstriction in $\alpha_1^{R/R}\alpha_2^{R/R}$ mice but not in $\alpha_1^{R/R}\alpha_2^{S/S}$ mice, but the response seems to be unique to MBG, since ouabain infusion did not elevate blood pressure in the $\alpha_1^{R/R}\alpha_2^{R/R}$ mice (Fig. 3). A distinctive effect of MBG on vascular function would be an important finding and would require further studies to elucidate potential mechanisms.

In summary, our findings demonstrate that cardenolides and bufadienolides share a common binding site in the α_1 Na^+-K^+-ATPase and that binding of either of these classes of CTS can exert positive inotropy in the murine heart. Our data also provide compelling evidence that physiologically relevant concentrations of circulating endogenous MBG can importantly influence cardiovascular function. Importantly, these data also illustrate that mutant mice with altered "ouabain-sensitivity" of the various α-subunits of the Na^+-K^+-ATPase represent useful models for investigating the (patho)physiological effects of endogenous caredenolides and bufadienolides and the underlying molecular mechanisms that involve specific Na^+-K^+-ATPase α-subunits.

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