Enhanced Activity of the Myocardial Na^+/H^+ Exchanger NHE-1 Contributes to Cardiac Remodeling in Atrial Natriuretic Peptide Receptor–Deficient Mice

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Background—Atrial natriuretic peptide (ANP), through its guanylyl cyclase-A (GC-A) receptor, not only is critically involved in the endocrine regulation of arterial blood pressure but also locally moderates cardiomyocyte growth. The mechanisms underlying the antihypertrophic effects of ANP remain largely uncharacterized. We examined the contribution of the Na^+/H^+ exchanger NHE-1 to cardiac remodeling in GC-A–deficient ($GC\text{-}A^{-/-}$) mice.

Methods and Results—Fluorometric measurements in isolated adult cardiomyocytes demonstrated that cardiac hypertrophy in $GC\text{-}A^{-/-}$ mice was associated with enhanced NHE-1 activity, alkalinization of intracellular pH, and increased Ca^{2+} levels. Chronic treatment of $GC\text{-}A^{-/-}$ mice with the NHE-1 inhibitor cariporide normalized cardiomyocyte pH and Ca^{2+} levels and regressed cardiac hypertrophy and fibrosis, despite persistent arterial hypertension. To characterize the molecular pathways driving cardiac hypertrophy in $GC\text{-}A^{-/-}$ mice, we evaluated the activity of 4 prohypertrophic signaling pathways: the mitogen-activated protein kinases (MAPK), the serine-threonine kinase Akt, calcineurin, and Ca^{2+}/calmodulin-dependent kinase II (CaMKII). The results demonstrate that all 4 pathways were activated in $GC\text{-}A^{-/-}$ mice, but only CaMKII and Akt activity regressed during reversal of the hypertrophic phenotype by cariporide treatment. In contrast, the MAPK and calcineurin/NFAT signaling pathways remained activated during regression of hypertrophy.

Conclusions—On the basis of these results, we conclude that the ANP/GC-A system moderates the cardiac growth response to pressure overload by preventing excessive activation of NHE-1 and subsequent increases in cardiomyocyte intracellular pH, Ca^{2+}, and CaMKII as well as Akt activity. **(*Circulation*. 2005;112:2307-2317.)**

Key Words: hypertension ■ natriuretic peptides ■ hypertrophy ■ calcineurin ■ sodium-hydrogen antiporter

Cardiac atrial natriuretic peptide (ANP) lowers blood pressure and stimulates diuresis and natriuresis by a combination of direct and indirect effects that involve vasodilation, inhibition of renal Na^+ reabsorption, and inhibition of the sympathetic as well as renin-angiotensin-aldosterone systems.[1] Actions of ANP are mediated by a membrane-bound guanylyl cyclase receptor, GC-A, which mediates increases in intracellular cyclic GMP levels in response to ANP binding.[1] Mice with global disruption of the GC-A gene ($GC\text{-}A^{-/-}$ mice) not only have increased systemic arterial blood pressure but also display a marked cardiac hypertrophy that is disproportionate to their increased blood pressure and partly resistant to antihypertensive medication.[2,3] Extending these studies, our own recent observations in mice with conditional, cardiomyocyte-restricted deletion of GC-A (CM GC-A KO mice) demonstrated that ANP, through GC-A/cGMP signaling, exerts important local, auto/paracrine functions to moderate cardiomyocyte growth.[4] The intracellular signaling pathways mediating the local antihypertrophic effects of ANP/GC-A are unknown. Because patients with essential hypertension and/or cardiac hypertrophy/insufficiency exhibit diminished systemic and cardiac responsiveness to ANP,[1,5] clarification of this issue is important from a physiological and also pathophysiological perspective.

To address this question, genetically altered mice with either global ($GC\text{-}A^{-/-}$) or conditional, cardiomyocyte-restricted deletion of GC-A (CM GC-A KO) were used.[2,4] In the present study, we demonstrate that cardiac hypertrophy in

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GC-A–deficient mice is concomitant to increased myocardial activity of the Na^+/H^+ exchanger NHE-1, leading to intracellular alkalinization, increased cytosolic Ca^{2+}-levels, and enhanced activity of the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and the serine-threonine kinase Akt. Pharmacological inhibition of NHE-1 reversed cardiac remodeling and these biochemical changes, despite persistent arterial hypertension. Cardiac activity of the phosphatase calcineurin and the mitogen-activated protein kinases (MAPK) ERK1/2 and p38 was also increased in GC-$A^{-/-}$ mice, but the activity of these signaling pathways was dissociated from the changes in cytosolic pH and Ca^{2+} and from cardiac hypertrophy. Our results suggest that the ANP/GC-A system moderates the cardiac growth response to pressure overload partly by preventing excessive stimulation of NHE-1 and subsequent increases in cardiomyocyte pH, Ca^{2+}, and CaMKII, as well as Akt activity.

Methods

ANP Receptor–Deficient Mice

Mice with global deletion of GC-A (GC-$A^{-/-}$) and corresponding wild-type (WT) mice were provided by Dr D.L. Garbers (University of Texas Southwestern Medical Center, Dallas).[2] Mice with conditional, cardiomyocyte-restricted deletion of GC-A (CM GC-A KO mice) and control littermates (floxed GC-A mice, with normal GC-A expression levels) were generated and genotyped as described.[4]

Measurement of Cardiomyocyte pH_i, $Ca^{2+}{}_i$ Transients, and Single-Cell Contractility

Ventricular cardiomyocytes were isolated by collagenase digestion.[6] Intracellular pH (pH_i) as well as $Ca^{2+}{}_i$ transients, together with simultaneously recorded cell length of electrically paced (0.5 Hz) myocytes, were determined in parallel experiments. To rule out the contribution of HCO_3^--dependent transport mechanisms, the experiments were carried out in HCO_3^--free Tyrode buffer containing (in mmol/L) 140 NaCl, 5.8 KCl, 0.5 KH_2PO_4, 0.4 Na_2HPO_4, 0.9 $MgSO_4$, 11.1 glucose, 2.5 $CaCl_2$, and 10.0 HEPES, pH 7.3. To evaluate whether the observed genotype-dependent alterations in pH_i persist when HCO_3^--dependent transporters are active, a second series of experiments was carried out in Krebs buffer with the following composition (mmol/L): 104 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.17 $MgSO_4$, 1.8 KH_2PO_4, 25 $NaHCO_3$, 10 glucose, pH 7.3. From each animal, 2 to 3 cardiomyocytes were analyzed. For pH_i measurements, the cells were transferred to a superfusion chamber, mounted on an inverted microscope (Axiovert 135, Zeiss), and incubated with the membrane-permeable fluorescent dye BCECF-AM (2 μmol/L, dissolved in 0.1 g/L Pluronic F-127 in cell culture medium).[7] Loaded cardiomyocytes were excited at 488 and 436 nm, and emitted fluorescence was detected at 520 to 560 nm.[7] The ratio of fluorescence (488/436) was calculated. Calibration of the BCECF fluorescence signal was done in separate experiments with the protonophore carbonyl cyanide m-chloro-phenylhydrazone (CCCP, 1 μmol/L), and external pH values were titrated between 6.5 and 8.0 as described.[7] In this range, the dependence of the fluorescence ratio on external pH was linear. Intracellular Ca^{2+} transients and single-cell contractility (by video edge detection) of Indo-1–loaded cardiomyocytes were analyzed as described.[6] After obtention of basal recordings during 10 minutes, the acute effects of the NHE-1 inhibitor cariporide, the CaMKII inhibitory drug KN-93 (Alexis), or its inactive analog KN-92 (Sigma) were tested (all 10 μmol/L, superfusion of cardiomyocytes during 5 minutes). In additional experiments, caffeine (10 mmol/L) was used to evaluate sarcoplasmic reticulum (SR) Ca^{2+} loading. Caffeine activates the SR Ca^{2+} release channel (ryanodine receptor) and thereby elicits a whole-cell Ca^{2+} transient that is proportional to the extent of SR Ca^{2+} storage.

Animal Treatment Protocol

GC-$A^{-/-}$ and corresponding WT mice were fed with standard animal chow containing 6000 ppm cariporide[8] or control chow beginning at 6 weeks of age for 5 months (20 male litter mates per group). With this diet, a mean plasma concentration of 2.5±0.3 μmol/L cariporide was achieved.[8] After this period, the mice were subjected to the physiological, biochemical, and histological studies described below. All investigations were approved by the local animal care committee and conform to the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1996).

Hemodynamic Measurements and Tissue Harvesting

Arterial blood pressure measurements were taken in conscious mice by tail cuff (Softron), before and after cariporide treatment.[9] After 5 months of treatment, 12 mice of each study group (cariporide-treated versus untreated GC-$A^{-/-}$ and WT mice) were subjected to Doppler echocardiography. After euthanasia, the individual cardiac chambers were dissected, halved, and were either frozen in liquid nitrogen or fixed in 4% buffered formaldehyde.

Doppler Echocardiography Studies

Left ventricular M-mode and Doppler flow measurements were carried out during sedation with diazepam (17.5 mg/kg body weight IP).[10] Measurements were conducted by a digital Doppler echocardiography system (Philips Sonos 5500) equipped with a 15-MHz linear transducer for 2-dimensional and M-mode imaging and a 12-MHz sector scanner for Doppler measurements.[10]

Histology and Morphometric Analyses

The mean cardiomyocyte diameters and interstitial collagen fractions were evaluated on periodic acid Schiff (PAS) or picrosirius red stained left ventricular sections as described.[4,9]

Northern Blot Analysis

Left ventricular total RNA was subjected to Northern blot as described.[4,9] Mouse ANP, α-skeletal actin, and GAPDH cDNA probes[4,9] as well as a DNA fragment encompassing the 5′exon 4-splice variant of murine myocyte-enriched calcineurin interacting protein 1 (MCIP1)[11] were ^{32}P-dCTP labeled. Signals were visualized in a PhosphorImager and quantified by ImageQuant software.[4,9]

Western Blot Analyses

Left ventricular proteins were solubilized in SDS–sample buffer and separated by 10% PAGE. The primary antibodies were against NHE-1 (Chemicon Int Inc), the Na^+/Ca^{2+} exchanger NCX-1 (Affinity Bioreagents), phosphorylated/unphosphorylated MAPKs, Akt, phospho-Akt (all Cell Signaling Technology), CaMKII (BD Transduction Laboratories), autophosphorylated (active) CaMKII (Santa Cruz), and $Threonin_{17}$-phosphorylated as well as total phospholamban (PLB) (Badrilla). Either calsequestrin[6] or GAPDH (Trevigen Inc) were used for loading controls. The blots were developed with the use of the ECL detection system (Amersham-Pharmacia), and results were quantified by densitometry (ImageQuant).[6] To analyze the activation status of endogenous calcineurin, the relative phosphorylation status of its endogenous transcriptional effector, nuclear factor of activated T cells (NFAT), was determined by immunoprecipitation and Western blotting, as previously described.[11]

Immunocytochemical Staining of CaMKII

The intracellular distribution of autophosphorylated CaMKII was visualized on paraffin-embedded left ventricles. The slides were incubated overnight with primary antibody (1:1500) at 4°C and then with a secondary antibody (goat anti-rabbit, Dianova) labeled with biotin, streptavidin POX (1:250, 1 hour at room temperature). The enzyme reaction was developed with DAB and the slide was counterstained with hematoxylin. The number of pCAMKII-positive cardiomyocyte nuclei was scored by measuring in total ≈100 cardiomyocyte nuclei per specimen (8 hearts per group) and then

calculating the ratio between pCAMKII-positive (brown) and total number of cardiomyocyte nuclei in the corresponding section.[4,9] The evaluations were conducted by 2 independent observers under blinded conditions, using a computer-assisted image analysis system.[4,9]

Quantitative Real-Time RT-PCR

Total RNA (Trizol; 1 μg) isolated from tissue was reverse-transcribed with 200 U of Superscript reverse transcriptase (Gibco) according to standard procedures. Primers were designed against mouse connective tissue growth factor (ctgf), mouse dystrophia myotonica kinase, B15 (dm15), and mouse ribosomal L7 (L7), using Beacon Designer primer design software (Premier Biosoft Int). The specific melting point of the amplicons was analyzed by using the iCycler Dissociation curve software (Qiagen). Quantitative RT-PCR was performed on an iCycler (Qiagen), using the SYBR Green PCR master mix (Qiagen). Relative mRNA copy numbers were calculated by using the derivative $\Delta\Delta$Ct, and the expression of each target gene was normalized to the expression of the control gene (L7).[12]

Statistics

Results are presented as mean±SEM. Group data were compared by means of 1-way or 2-way ANOVA (with genotype and treatment as categories) followed by the multiple-comparison Bonferroni *t* test to assess differences between groups. The paired Student *t* test was applied to evaluate the acute effects of cariporide and KN-93 on $[Ca^{2+}]_i$. The significance level was set at $P<0.05$.

Results

Enhanced Activity of NHE-1 in GC-A–Deficient Cardiomyocytes Led to Alkaline Intracellular pH_i and Increased Ca^{2+} Transients

In vitro studies have shown that ANP may regulate cardiomyocyte pH in an acute fashion by cyclic GMP–mediated inhibition of NHE-1, the main Na^+/H^+ exchanger in the heart.[13] We therefore postulated that a chronic loss of function of cardiomyocyte GC-A may lead to activation of NHE-1, and the subsequent rise in $[Na^+]_i$ then provokes an increase in $[Ca^{2+}]_i$ mediated by reverse-mode Na^+-Ca^{2+} exchange (NCX).[14] To test this hypothesis, intracellular pH and Ca^{2+} transients were first compared in isolated ventricular cardiomyocytes obtained from CM GC-A KO mice as well as respective control littermates with normal GC-A expression levels. As shown in Figure 1A, steady-state pH_i of cardiomyocytes perfused with HCO_3^--free buffer was markedly more alkaline in GC-A–deficient as compared with control cardiomyocytes. In GC-A–deficient cardiomyocytes superfused with a phosphate- and bicarbonate-containing buffer, the alkalinization was still present. However, the absolute pH_i values were lower (8.0±0.2, n=13), as to be expected for a more physiological buffering capacity. Concomitantly, cardiomyocytes from CM GC-A KO mice had markedly increased baseline systolic $Ca^{2+}{}_i$ levels (Indo-1 ratio: 1.68±0.016 versus 1.59±0.013, $P<0.05$), as well as increased peak amplitudes of $Ca^{2+}{}_i$ transients as compared with control mice (Figure 1, B and C). Diastolic $Ca^{2+}{}_i$ levels were not different between genotypes (Indo-1 ratio: 1.47±0.012 versus 1.46±0.013). Similar changes in pH_i and $Ca^{2+}{}_i$ transients were observed in cardiomyocytes obtained from mice with global, systemic deletion of GC-A (GC-$A^{-/-}$ mice, see below and Figure 4).

To further verify whether increased activity of NHE-1 was involved in these changes, we assessed the acute effects of 10 μmol/L cariporide on cytoplasmic-free Ca^{2+} transients. Notably, cariporide had no effect on $Ca^{2+}{}_i$ transients of control cardiomyocytes but provoked significant decreases in systolic $Ca^{2+}{}_i$ levels and peak amplitudes of $Ca^{2+}{}_i$ transients in GC-A–deficient cardiomyocytes (Figure 1, B and C). Diastolic $Ca^{2+}{}_i$ levels were not affected by cariporide. Taken together, these results demonstrate a clear link between enhanced NHE-1 activity, alkaline pH_i, and increased amplitude of $Ca^{2+}{}_i$ transients in GC-A–deficient cardiomyocytes.

Cariporide Regressed Cardiac Enlargement in Hypertensive GC-$A^{-/-}$ Mice

To evaluate whether increased NHE-1 activity and the subsequent changes in pH_i and $Ca^{2+}{}_i$ contribute to the hypertrophic response of GC-A–deficient cardiomyocytes in vivo, we studied the effect of chronic oral treatment with cariporide on the cardiovascular phenotype of mice with global GC-A deletion. We performed our study in this genetic model rather than CM GC-A KO mice because the hypertrophic changes are much more pronounced. In agreement with previous studies by our own and other groups,[2,3,9] GC-$A^{-/-}$ mice showed marked chronic arterial hypertension and global cardiac enlargement (Table 1). As previously reported, this cardiac phenotype is apparent from the first day of life.[9] Treatment of GC-$A^{-/-}$ mice with cariporide during 5 months (from 6 weeks to ≈28 weeks of age) did not affect the hypertensive phenotype but significantly reversed both right and left ventricular enlargement (Table 1). In WT mice, the NHE-1 inhibitor had no effect on blood pressure or cardiac weights (Table 1).

Transthoracic Doppler echocardiography corroborated the regression of cardiac enlargement by cariporide (Table 1). It also showed that despite cardiac hypertrophy in GC-$A^{-/-}$ mice, left ventricular contractile function was preserved. This is indicated by the fractional shortening (FS) of the left ventricular wall, ejection fraction (EF), and velocity of circumferential fiber shortening (Vcf), parameters that all were similar between GC-$A^{-/-}$ and WT mice and were not affected by cariporide treatment (Table 1).

Cariporide Reversed Cardiomyocyte Hypertrophy and Expression of Hypertrophy Markers

Morphometric analyses of left ventricular sections demonstrated that the cardiomyocyte diameters of untreated GC-$A^{-/-}$ mice were significantly enlarged as compared with WT mice (Figure 2, A and B). This was accompanied by increased ventricular interstitial collagen deposition (Figure 2, C and D). As shown, cardiac remodeling was completely reversed by cariporide treatment (Figure 2, A through D). Global cardiac hypertrophy in GC-$A^{-/-}$ mice was associated with increased left ventricular mRNA expression of ANP (by 2.0±0.07-fold versus WT) and α-sk-actin (by 1.5±0.1-fold) (Figure 3). Cariporide treatment fully reversed increased α-sk-actin and partially reversed increased ANP mRNA levels to the levels in WT mice (Figure 3). Taken together, these data demonstrate that chronic inhibition of NHE-1 significantly regressed cardiac hypertrophy of GC-$A^{-/-}$ mice, despite persistent arterial hypertension.

Chronic Treatment With Cariporide Normalized Intracellular pH and Ca^{2+}_i Transients in GC-$A^{-/-}$ Cardiomyocytes

Next we studied the effect of oral cariporide treatment on cardiomyocyte pH_i and Ca^{2+}_i transients. As shown, chronic oral cariporide treatment totally reversed enhanced pH_i (Figure 4A) and increased Ca^{2+}_i transients of GC-$A^{-/-}$ cardiomyocytes (Figure 4B) to the values found in WT. In contrast, in WT mice the inhibitor had no effect on these parameters. Because free cytosolic Ca^{2+}_i transients mainly result from SR Ca^{2+}_i uptake and release, we investigated whether increased amplitudes of Ca^{2+}_i transients in GC-$A^{-/-}$ cardiomyocytes are related to increased SR Ca^{2+} storage. Notably, the peak amplitude of the caffeine-triggered Ca^{2+}_i transients was increased by ≈2-fold in GC-$A^{-/-}$ as compared with WT cardiomyocytes, indicating a higher SR Ca^{2+} load in the former (Figure 4C). Chronic oral treatment of GC-$A^{-/-}$ mice with cariporide fully reversed these changes. In contrast, in WT mice cariporide treatment had no effect on caffeine-induced Ca^{2+} release (Figure 4C). We conclude that increased activity and/or expression of NHE-1 in GC-$A^{-/-}$ cardiomyocytes and the subsequent rise in $[Na^+]_i$ provokes increased influx of extracellular Ca^{2+} through reverse-mode Na^+/Ca^{2+} exchange (NCX) and ultimately increases SR Ca^{2+} load.

TABLE 1. Effect of Oral Cariporide Treatment on Arterial Blood Pressure, Cardiac Morphology, and Contractile Function of WT and GC-$A^{-/-}$ Mice

	WT	WT Cariporide	GC-$A^{-/-}$	GC-$A^{-/-}$ Cariporide
SBP, mm Hg	101±1.3	103±1.2	132±1.1*	133.2±0.6‡
DBP, mm Hg	74±1.3	73±1.5	81.5±0.6*	80.6±1.6‡
Body weight, g	30.5±0.9	31.3±0.4	31.0±0.6	32.6±1.3
HW/BW, mg/g	5.5±0.2	5.7±0.3	7.5±0.4*	6.3±0.1†
LVW/BW, mg/g	4.1±0.1	3.9±0.2	5.3±0.2*	4.3±0.1†‡
RVW/BW, mg/g	0.9±0.1	0.9±0.5	1.4±0.1**	1.1±0.1†‡
FS, %	44±2	NT	45±2	45±1
Vcf, circ/s	7.5±0.4	NT	7.8±0.4	7.9±0.4
EF, %	76±2	NT	77±2	77±2
IVSd, mm	0.89±0.02	NT	1.21±0.02*	1.04±0.02*†
PWd, mm	0.79±0.01	NT	1.0±0.02*	0.94±0.02*†
LVEDD, mm	3.54±0.11	NT	3.61±0.12	3.69±0.07
LVEDS, mm	1.98±0.09	NT	1.98±0.09	2.02±0.08
LVW, mg	105±5	NT	160±8*	139±4*†
LVW/BW, mg/g	3.44±0.2	NT	5.16±0.2*	4.26±0.2*†

Systolic (SBP) and diastolic blood pressure (DBP) were estimated by tail-cuff plethysmography. The heart weight (HW), left ventricular (LVW), and right ventricular weight (RVW) to body wt (BW) ratios were obtained by necropsy. Cardiac functional parameters were measured by Doppler echocardiography during sedation with diazepam.

IVS and PW indicate end-diastolic interventricular septum and posterior wall thickness; LVEDD and LVESD, left ventricular end-diastolic and end-systolic diameter; and NT, not tested. LV mass and parameters of contractility were calculated as described.[10]

Two-way ANOVA results showed a significant genotype–treatment interaction for HW/BW, LVW/BW, and RVW/BW ($P<0.05$).

*$P<0.01$ compared with WT; †$P<0.01$ compared with untreated GC-$A^{-/-}$ mice; ‡$P<0.05$ compared with cariporide-treated WT mice (all n=12).

Cardiac Expression Levels of NHE-1 and NCX Were Not Altered in GC-$A^{-/-}$ Mice

As shown in Figure 5, the left ventricular expression levels of NHE-1 were not different between GC-$A^{-/-}$ and WT mice, indicating that enhanced activity (not expression) of the exchanger is responsible for increased cardiomyocyte pH_i in the former. Notably, chronic treatment of GC-$A^{-/-}$ mice with cariporide provoked a drastic reduction in left ventricular NHE-1 levels, by almost 50% (Figure 5). In contrast, NHE-1 levels in WT hearts were not affected by cariporide treatment. As also shown, left ventricular NCX-1 expression levels were not different between genotypes and were not affected by cariporide treatment.

Cardiac Hypertrophy in GC-$A^{-/-}$ Mice Was Accompanied by Activation of ERK-1/2 and p38 MAPK as Well as Akt

To further characterize the molecular pathways that ultimately drive cardiac hypertrophy in GC-$A^{-/-}$ mice, we examined the expression and phosphorylation of the ERK-1/2, JNK, and p38 terminal branches of the MAPK superfamily as well as of Akt. As shown in Figure 6 (A and B), the cardiac expression levels of total MAPKs and Akt were not different between genotypes and were not affected by cariporide treatment. However, we observed a significant increase in the cardiac levels of the dually phosphorylated (Thr202/Tyr204), active forms of ERK-1/2 as well as of phosphorylated p38 and Akt in GC-$A^{-/-}$ mice. Treatment of GC-$A^{-/-}$ mice with cariporide did not reverse these changes in ERK-1/2 and p38 phosphorylation (Figure 6A) but totally reversed the enhanced phosphorylation of Akt (Figure 6B).

Cardiac Hypertrophy in GC-$A^{-/-}$ Mice Was Accompanied by Activation of Ca^{2+}/Calmodulin-Dependent Pathways Such as Calcineurin and CaMKII

Because increases in cytoplasmic Ca^{2+} can induce cardiomyocyte hypertrophy through Ca^{2+}/calmodulin-mediated activation of CaMKII and/or calcineurin signaling, we assessed the activity of these pathways in GC-$A^{-/-}$ hearts. Unfortunately, the traditional biochemical calcineurin and CaMK activity assays rely on homogenized extracts in the presence of exogenously supplied Ca^{2+} and calmodulin and therefore may approximate total calcineurin or CaMK availability rather than the specific endogenous activity of these enzymes.[15] Instead, we analyzed the endogenous phosphorylation status of respective specific downstream targets, namely the transcription factor NFAT (which is specifically dephosphorylated by calcineurin[15]) and the regulatory SR protein phospholamban (specifically phosphorylated by CaMKII at position threonin-17[16,17]).

As shown in Figure 7A, the cardiac levels of phospho-NFATc3 were decreased by ≈50% in GC-$A^{-/-}$ as compared with WT mice, consistent with relatively higher calcineurin activity. Because activated NFAT in turn drives expression of the MCIP1 exon 4-splice isoform (MCIP1.4) through an upstream cluster of NFAT binding sites, the mRNA expression levels of MCIP1.4 were also determined. Northern blot

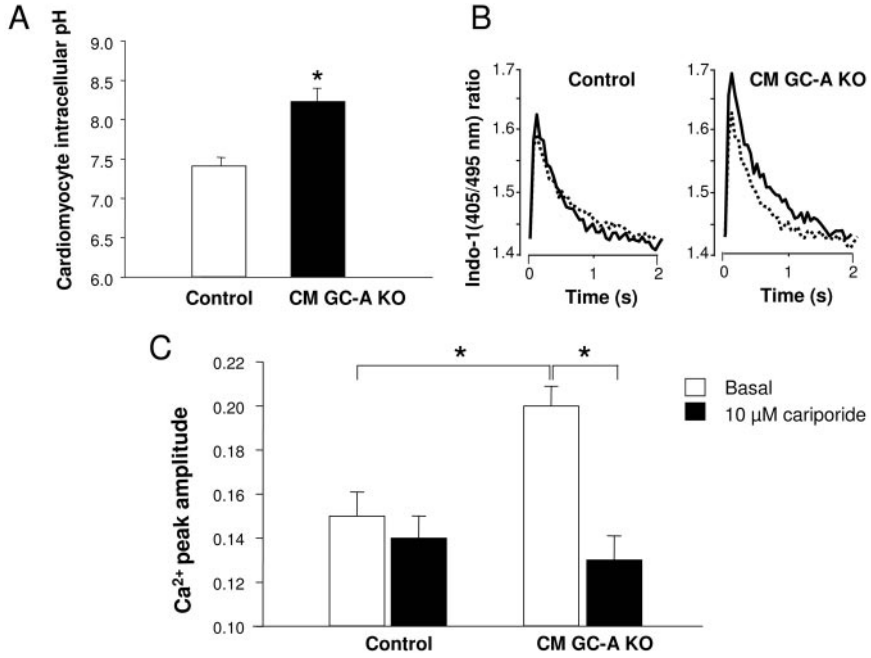

Figure 1. Intracellular pH, Ca^{2+} transients, and acute effects of cariporide on Ca^{2+}_i in electrically paced cardiomyocytes from CM GC-A KO and respective control mice. A, Resting pH_i calculated from the BCECF fluorescence ratios was markedly alkaline in GC-A–deficient as compared with control cardiomyocytes. B, Representative tracings of the Indo-1 ratio in single cardiomyocytes from control and CM GC-A KO mice under baseline conditions (continuous line) and during superfusion with 10 μmol/L cariporide (dotted line). C, On average, the peak amplitude of Ca^{2+} transients (Indo-1 $ratio_{405/495\ nm}$, systolic-diastolic) was significantly increased in GC-A–deficient (CM GC-A KO) as compared with control cardiomyocytes. Cariporide had no effect on Ca^{2+} transients in control cardiomyocytes and fully reversed increased peak amplitudes of Ca^{2+}-transients in GC-A–deficient cardiomyocytes (n=8 mice per genotype, $^*P<0.05$).

analysis indicated an ≈1.6-fold induction of MCIP1.4 in GC-$A^{-/-}$ mice, providing a second indication that cardiac calcineurin activity was elevated in vivo (Figure 7B). Intriguingly, treatment of GC-$A^{-/-}$ mice with cariporide did not reverse these changes, indicating persistent calcineurin activation despite regression of hypertrophy (Figure 7, A and B).

As shown in Figure 7C by Western blot analyses, the cardiac expression levels of total and autophosphorylated (active) CaMKII were increased in GC-$A^{-/-}$ mice. Concomitantly, the phosphorylation of PLB at threonin-17, providing an index of endogenous CaMKII activity,[17] was also significantly enhanced in GC-$A^{-/-}$ hearts (Figure 7D). Notably, chronic oral treatment of GC-$A^{-/-}$ mice with cariporide fully reversed these changes (Figure 7, C and D). The immunocytochemical stainings depicted in Figure 8 corroborated these results, showing increased expression of pCAMKII in both the cytosol and nuclei of GC-$A^{-/-}$ cardiomyocytes and reversal of these changes by cariporide treatment.

To further evaluate the activation of CaMKII, we quantified the expression of 2 MEF2 target genes (identified from microarray analyses in heart-specific MEF2A transgenic mice; Ralph van Oort and Leon de Windt, unpublished findings), as MEF2 is a direct transcriptional target of CaMKII. Accordingly, we found that ctgf and dm15 expression was increased by 14.7±5.4-fold and 11.3±8.1-fold in GC-$A^{-/-}$ hearts, compared with WT, respectively. Cariporide

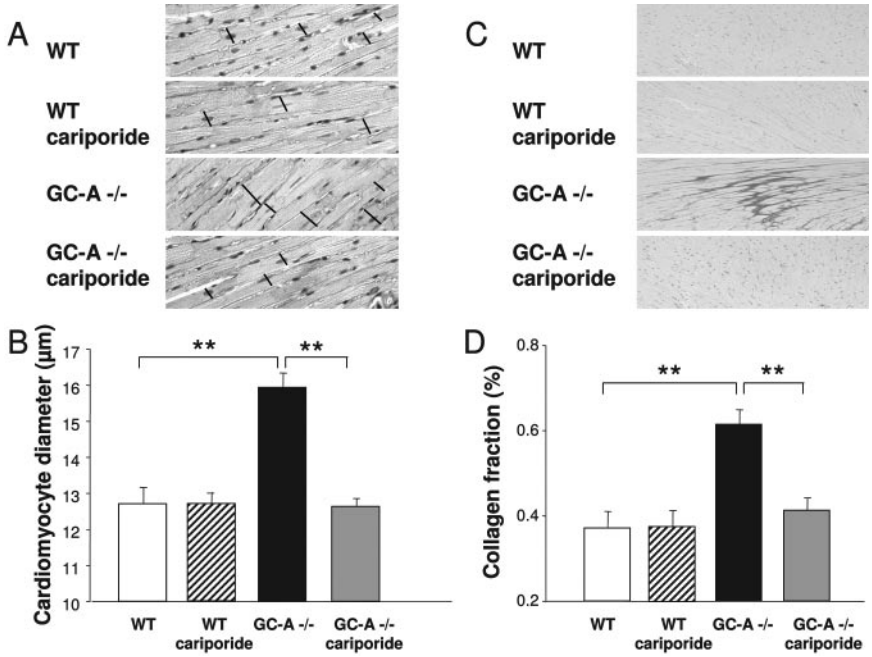

Figure 2. Morphometric analyses of cardiac sections. Cardiomyocyte diameters were estimated by quantitative morphometry of PAS-stained ventricular sections (A). Black lines (A) indicate cardiomyocyte diameters in the region of the cell nucleus, which were significantly increased in hearts of GC-$A^{-/-}$ as compared with WT mice (B). Interstitial collagen fractions, estimated on parallel sirius red–stained sections (C), were also significantly increased in GC-$A^{-/-}$ mice (D). Two-way ANOVA results showed a significant genotype-treatment interaction for both parameters ($P<0.01$). Cardiac hypertrophy and fibrosis in GC-$A^{-/-}$ mice were completely reversed by oral cariporide treatment (A through D) (n=8 per group, $^{**}P<0.01$).

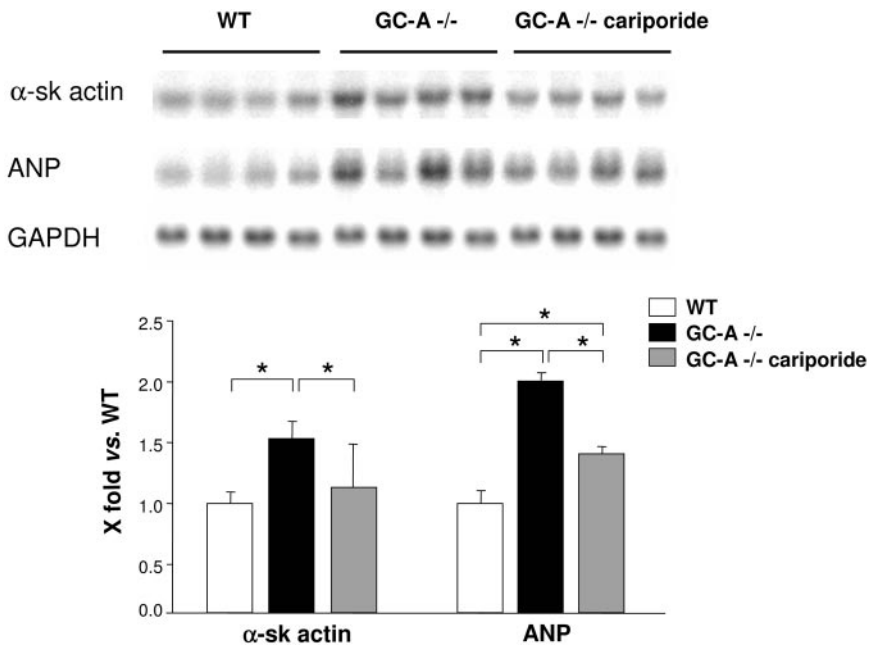

Figure 3. Left ventricular α-sk-actin and ANP mRNA expression in WT and untreated versus cariporide-treated GC-$A^{-/-}$ mice. Top: Representative Northern blots. Bottom: Gene expression levels of ANP and α-sk-actin were normalized to GAPDH and calculated as *X*-fold versus levels in WT mice (n=8; **P*<0.05).

treatment lowered the expression of ctgf and dm15 to 3.2±1.1-fold and 2.5±1.0-fold versus WT, respectively.

Finally, we analyzed the implications of increased cardiac expression and autophosphorylation of CaMKII for calcium handling and single-cell contractility. As depicted in Table 2, the increased baseline Indo-1 Ca^{2+}_i transients in isolated GC-$A^{-/-}$ ventricular myocytes were associated with an increase in contractile function (% single-cell shortening). Inhibition of CaMKII with KN-93 (10 μmol/L, 5 minutes) attenuated Ca^{2+}_i transients and contractility more in GC-$A^{-/-}$ than in WT cardiomyocytes. Chronic oral treatment of GC-$A^{-/-}$ mice with cariporide fully reversed increased baseline Ca^{2+}_i handling and single-cell contractility as well as the enhanced responsiveness to KN-93 (Table 2). The inactive control compound KN-92 did not affect cardiomyocyte calcium handling or contractility in either genotype (not shown).

TABLE 2. Effect of CaMKII Inhibition on Ca^{2+}_i Transients and Contractility of Isolated Ventricular Cardiomyocytes From WT and Untreated Versus Cariporide-Treated GC-$A^{-/-}$ Mice

	WT	GC-$A^{-/-}$	GC-$A^{-/-}$ Cariporide
Ca^{2+}_i peak amplitude			
Baseline	0.11±0.006	0.21±0.029*	0.11±0.012‡
KN-93	0.10±0.006	0.16±0.024*†	0.09±0.010†‡
Cell shortening, %			
Baseline	2.84±0.24	5.34±0.44*	2.52±0.25‡
KN-93	2.27±0.23†	3.62±0.33*†	2.04±0.27†‡

Ca^{2+}_i transients (Indo-1 $ratio_{405/495nm}$, systolic-diastolic) and simultaneously recorded cell length ($L_{max} - L_{min}$, expressed as percent from L_{max}) in field-stimulated cardiomyocytes at baseline and during superfusion with the CaMKII-inhibitory drug KN-93 (10 μmol/L).

**P*<0.05 compared with WT; †compared with baseline (paired *t* test); and ‡compared with untreated GC-$A^{-/-}$ mice (n=6 cells from each of 6 mice per group).

Taken together, these findings support the notion that cardiac CaMKII activity is elevated in $GCA^{-/-}$ mice and that cariporide attenuates CaMKII activation.

Discussion

Principal Findings

The present study demonstrates that increased activity of the cardiac Na^+/H^+ exchanger NHE-1 is involved in the hypertrophic effects of a chronic loss of function of the GC-A receptor for ANP. Particularly, the implications of our study are severalfold. First, NHE-1 activity in GC-A–deficient cardiomyocytes is enhanced, leading to alkaline cardiomyocyte pH_i and increased intracellular free Ca^{2+} levels. Second, the hypertrophic remodeling is accompanied by activation of ERK and p38 MAPKs, Akt, and the Ca^{2+}/calmodulin-dependent signal transducers calcineurin and CaMKII. Third, treatment of GC-$A^{-/-}$ mice with the NHE-1 inhibitor cariporide totally reversed the pH and Ca^{2+} changes and regressed cardiac hypertrophy and fibrosis. Finally, the reversal of these biochemical and histological changes was associated with an inhibition of Akt and CaMKII activity but persistent activation of the MAPK and calcineurin signaling pathways. Notably, the protective cardiac effects of cariporide in GC-$A^{-/-}$ mice were observed in the presence of arterial hypertension. On the basis of these results, we propose that the ANP/GC-A system moderates the cardiac growth response to pressure overload at least in part by preventing excessive activation of NHE-1 and subsequent increases in cardiomyocyte intracellular pH, Ca^{2+}, and CaMKII, as well as Akt activity.

Enhanced NHE-1 Activity Leads to Increased Intracellular Ca^{2+} Levels

The cardiac NHE-1 is one of the key components of the heart to maintain physiological intracellular pH. Many experimental and clinical studies have demonstrated the pathophysio-

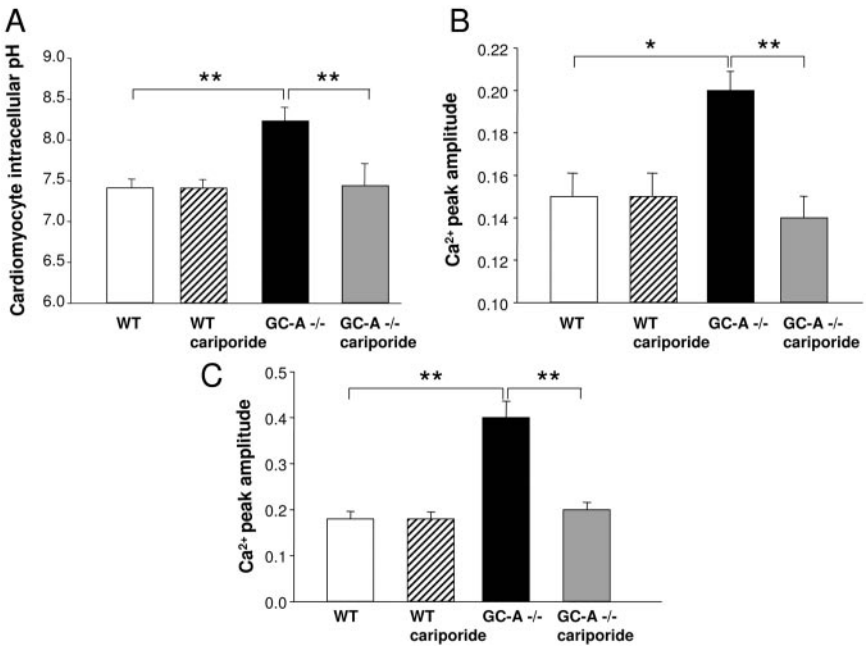

Figure 4. Intracellular pH and Ca^{2+} transients in isolated ventricular cardiomyocytes from untreated and cariporide-treated WT and GC-$A^{-/-}$ mice. Two-way ANOVA results showed a significant genotype-treatment interaction for both pH_i and $Ca^{2+}{}_i$ ($P<0.01$). A, Resting pH_i was markedly alkaline in GC-$A^{-/-}$ as compared with WT cardiomyocytes. Oral cariporide treatment had no effect on cardiomyocyte pH_i of WT and fully reversed altered cardiomyocyte pH_i of GC-$A^{-/-}$ mice. B, Peak amplitudes of $Ca^{2+}{}_i$ transients were markedly increased in GC-$A^{-/-}$ as compared with WT cardiomyocytes. Oral cariporide treatment had no effect on $Ca^{2+}{}_i$ transients in WT mice and fully reversed altered $Ca^{2+}{}_i$ transients of GC-$A^{-/-}$ mice. C, To evaluate SR Ca^{2+} content $Ca^{2+}{}_i$ transients were measured in response to 10 mmol/L caffeine. On average, the peak amplitude of the caffeine-induced Ca^{2+} transient was increased by ≈2-fold in GC-$A^{-/-}$ as compared with WT cardiomyocytes. Oral treatment with cariporide did not affect caffeine-induced Ca^{2+} transients in cardiomyocytes from WT mice and fully reversed increased caffeine responses in GC-$A^{-/-}$ mice (n=10 mice per genotype and condition, $^{**}P<0.01$).

logical implications of increased NHE-1 activity during ischemia and in hypertrophy and fibrosis of nonischemic origin.[8,18–25] Increased NHE-1 activity exerts detrimental effects on the myocardium probably by increasing intracellular Na^+ load, which finally results in elevated intracellular Ca^{2+} through the Na^+/Ca^{2+} exchanger NCX-1.[14,18,22] In some studies, increased cardiac protein expression levels of NHE-1 appear to be involved in the subsequent pathological changes.[8] In other studies, and in particular, in human heart failure, enhanced NHE-1 activity is not correlated with an increase in expression,[21,22] suggesting a role for posttranslational mechanisms. In accordance with the latter, in GC-$A^{-/-}$ mice the cardiac NHE-1 expression levels were not altered. The following observations indicate that NHE-1 activity was markedly enhanced in GC-$A^{-/-}$ cardiomyocytes, finally resulting in elevated intracellular Ca^{2+} handling: (1) Cardiomyocytes from GC-$A^{-/-}$ mice exhibit alkaline pH and increased Ca^{2+} levels, and both changes are reversed by chronic oral

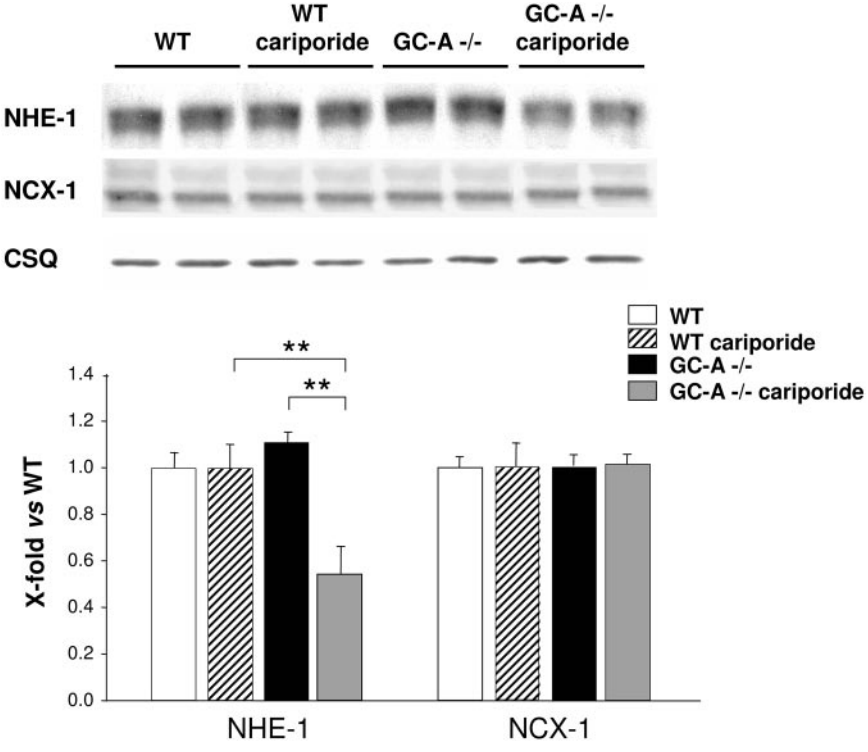

Figure 5. Left ventricular expression levels of NHE-1 and NCX-1 in untreated versus cariporide-treated WT and GC-$A^{-/-}$ mice. Top: Representative Western blots. Bottom: Protein levels were normalized to the cardiomyocyte-specific protein calsequestrin (CSQ) and calculated as *X*-fold respective WT. NHE-1 expression levels were not different between WT and GC-$A^{-/-}$ hearts and were significantly diminished by cariporide only in the later (n=8; $^{**}P<0.01$). Two-way ANOVA results showed a significant genotype-treatment interaction for NHE-1 expression ($P<0.01$).

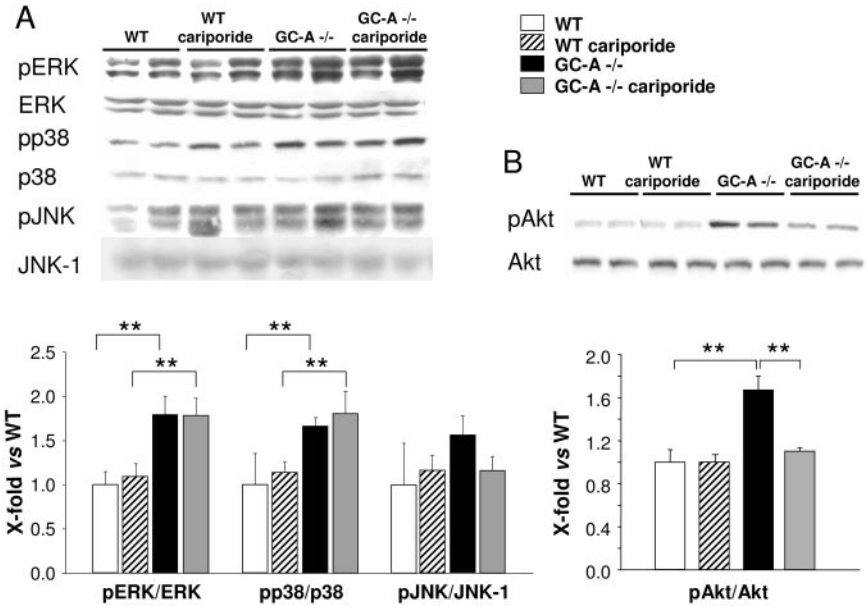

Figure 6. Increased phosphorylation of the MAPKs ERK and p38 (A) and of Akt (B) in hearts from GC-A$^{-/-}$ mice. Top: Representative Western blots. Bottom: Protein levels were normalized to the cardiomyocyte-specific protein calsequestrin (CSQ) and calculated as *X*-fold versus WT. Data demonstrate that cariporide treatment inhibited the increased phosphorylation of Akt but not the increased phosphorylation of ERK and p38 in GC-A$^{-/-}$ hearts (n=8; $^{**}P<0.01$). Two-way ANOVA results showed a significant genotype-treatment interaction for pAkt/Akt ($P<0.01$).

cariporide treatment; (2) acute superfusion of isolated cardiomyocytes with cariporide did not affect Ca^{2+} levels of WT cardiomyocytes but normalized the increased Ca^{2+} levels of GC-A$^{-/-}$ cardiomyocytes; and (3) this acute, Ca^{2+}-lowering effect of cariporide was abolished in cardiomyocytes obtained from GC-A$^{-/-}$ mice chronically treated with cariporide. Notably, similar to other studies,[8] oral cariporide treatment led to a marked reduction in the cardiac expression levels of NHE-1. The exact mechanism is unknown, but it is possible that diminished NHE-1 expression and activity both contributed to normalize cardiomyocyte pH_i and Ca^{2+} handling in cariporide-treated GC-A$^{-/-}$ mice.

Mechanisms for Increased NHE-1 Activity in GC-A–Deficient Cardiomyocytes

The mechanism through which chronic GC-A ablation results in increased NHE-1 activity is not yet apparent. In isolated cardiomyocytes, ANP was shown to inhibit NHE-1 in a GC-A/cGMP-dependent way.[13] Thus, disruption of cardiomyocyte ANP/GC-A signaling might directly enhance NHE activity. This notion is supported by our observation that cardiomyocytes from mice with conditional, cardiomyocyte-restricted (instead of global) GC-A disruption exhibit similar changes in pH_i and intracellular calcium handling, as do cardiomyocytes from mice with global GC-A deletion. On the other hand, sarcolemmal NHE activity is stimulated by various neurohumoral stimuli such as α_1-adrenergic agonists, endothelin, and angiotensin II,[26,27] which appear to increase NHE activity by increasing the pH_i sensitivity of the exchanger. ANP, through GC-A, is known to antagonize both the endocrine and local cardiac actions of catecholamines, endothelin, and angiotensin II at the level of their release and/or function.[1,28] GC-A disruption, causing an imbalance

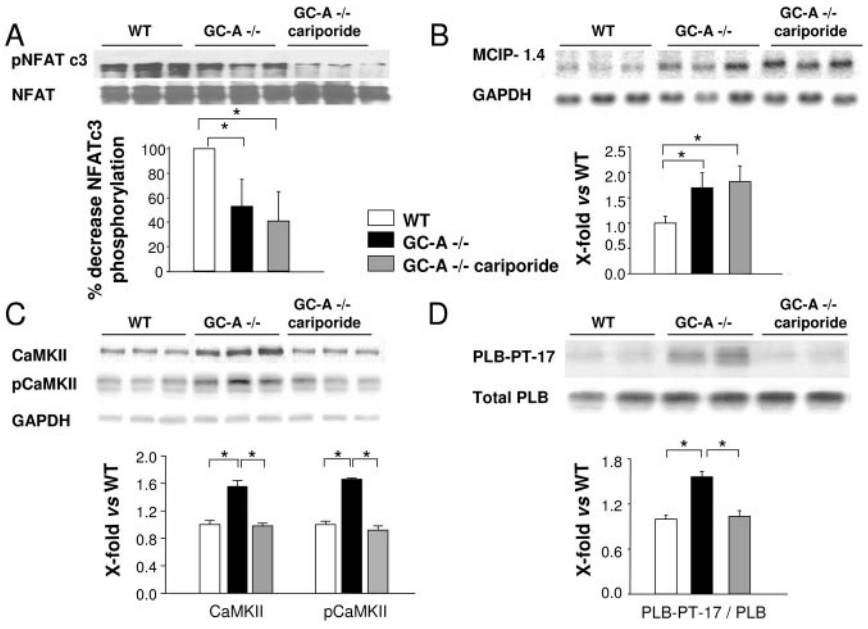

Figure 7. Activation of the calcineurin-NFAT and CaMKII signaling pathways in hearts from GC-A$^{-/-}$ mice. A, Representative Western blots for the cardiac level of hyperphosphorylated NFATc3. Data demonstrate decreased left ventricular phospho-NFAT levels in untreated and cariporide-treated GC-A$^{-/-}$ as compared with WT mice. As a control for loading, levels of total NFATc3 are also shown. B, Representative Northern blot demonstrating significantly increased ventricular expression of MCIP1.4 in untreated and cariporide-treated GC-A$^{-/-}$ compared with WT. GAPDH was used as loading control. C and D, Representative Western blots for the cardiac levels of CaMKII and autophosphorylated CaMKII (C, both normalized to GAPDH) and of Thr_{17}-phosphorylated phospholamban (D, PLB-PT-17 normalized to the levels of total PLB). Data demonstrate increased left ventricular CaMKII, pCaMKII, and PLB-PT-17 levels in untreated GC-A$^{-/-}$ mice and attenuation by cariporide treatment. (n=8 for all conditions; $^{*}P<0.05$).

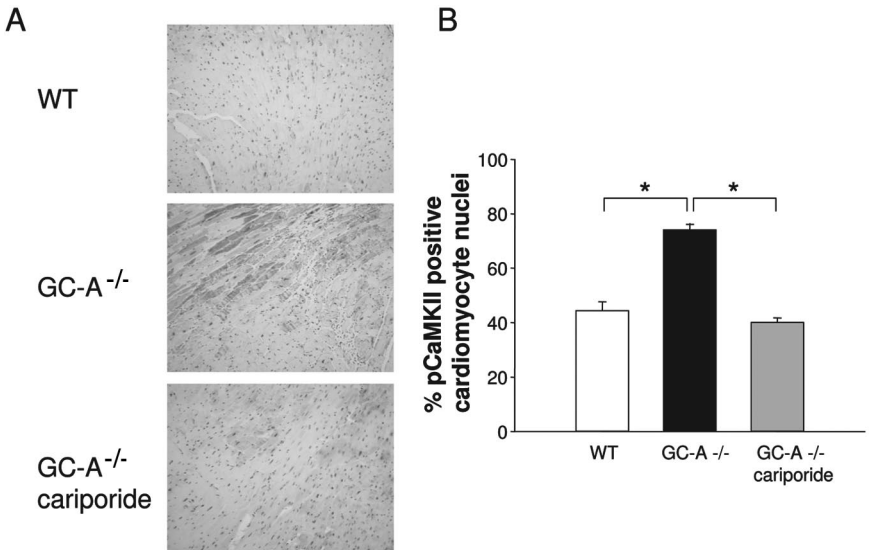

Figure 8. Intracellular distribution of autophosphorylated CaMKII in the hearts of WT and untreated versus cariporide-treated GC-A$^{-/-}$ mice. Left: Representative images showing increased cytosolic and nuclear pCAMKII staining in GC-A$^{-/-}$ hearts and attenuation by cariporide treatment. Right: Increased nuclear accumulation of pCAMKII in cardiomyocytes from untreated GC-A$^{-/-}$ mice and reversal by cariporide treatment (number of positive cardiomyocyte nuclei calculated as percent from total myocyte nuclei; n =8 for all conditions; *P<0.05 vs WT).

between ANP and these neurohumoral growth factors, may facilitate stimulation of cardiac NHE activity by the latter. Because most experiments in the present study were conducted in mice with global GC-A deletion, in our future studies we will take advantage of mice with cardiomyocyte-specific GC-A-deletion to further characterize the interactions between ANP/GC-A and cardiac NHE-1.

Downstream Signaling Pathways Leading to Increased Cardiomyocyte Growth

Increased NHE-1 activity might be involved in hypertrophic remodeling through cellular alkalinization, which together with increased Na^+ and increased Ca^{2+} can directly accelerate protein synthesis.[14,29,30] In addition, increases in intracellular Ca^{2+} can activate critical hypertrophic pathways leading to alterations in gene expression, such as MAPKs, calcineurin,[15] and CaMKII.[17] Furthermore, a recent study demonstrated that activated NHE-1 functions as a scaffold for recruitment of a signalplex that includes ezrin/radixin/moesin (ERM), phosphoinositide 3-kinase (PI3K), and Akt.[31] In cardiomyocytes, activated Akt has both an antiapoptotic[31] and a hypertrophic effect.[32] Indeed, our study showed that all 4 pathways (MAPKs, calcineurin, CaMKII, and Akt) were activated in hearts of GC-A$^{-/-}$ mice. However, only the activation of CaMKII and Akt correlated with both the enhanced cardiac NHE-1 activity and the hypertrophic phenotype, being stimulated during induction of these parameters and repressed during pharmacological inhibition of NHE-1 and concomitant regression of hypertrophy. These observations suggest that enhanced CaMKII and Akt signaling are both involved in cardiac hypertrophy of GC-A$^{-/-}$ mice. Both, and especially CaMKII, are multifunctional protein kinases that can phosphorylate a wide range of substrates and regulate numerous cellular functions.[33] The downstream signaling pathways ultimately stimulating myocyte growth are not revealed by our present experiments and remain an important question for future studies.

In contrast to CaMKII and Akt signaling, the MAPK and calcineurin/NFAT signaling pathways remained activated during regression of GC-A$^{-/-}$ hypertrophy by cariporide. In the context of recent studies indicating cooperative interactions between ERK1/2 and calcineurin-NFAT in many forms of cardiac hypertrophy,[15] our observations are in line with the notion that the activation of these pathways per se (in the absence of other hypertrophic signals) is not sufficient to fully maintain cardiac hypertrophy in GC-A$^{-/-}$ mice. In fact, increased calcineurin activity in the absence of myocyte hypertrophy has been observed previously.[34] The reason for this dissociation remains unclear, but it should be noted that in latter model and in our own study calcineurin activity was only moderately increased by ≈1.5- to 2-fold, which is considerably less than in other models of hypertrophy such as aortic banding.[35]

Of note, cariporide-treated GC-A$^{-/-}$ mice exhibited increased cardiac calcineurin/NFAT signaling despite normalization of cardiomyocyte-free cytosolic Ca^{2+} transients. It has been suggested that calcineurin is preferentially activated by a sustained, low Ca^{2+} plateau[36] or by specific subcellular Ca^{2+} pools.[37] If these types of subtle changes occur in GC-A–deficient cardiomyocytes in vivo, they may have been unappreciated by our fluorometric measurements in isolated cardiomyocytes in vitro. In addition, calcineurin-regulating mechanisms beyond calcium/calmodulin, such as redox events, have been described,[38] but their functional relevance in cardiomyocytes remains speculative.

GC-A–Deficient Cardiomyocytes Exhibit Enhanced SR Ca^{2+} Storage and Release

In contrast to many other models of cardiac hypertrophy/cardiac failure, only the systolic Ca^{2+} levels were markedly enhanced in isolated GC-A$^{-/-}$ cardiomyocytes, whereas diastolic free Ca^{2+} was normal. Concomitantly, single-myocyte contractility was enhanced. This is surprising because increased NHE-1 activity and subsequent increases in intracellular Na^+ through NCX in general are associated with increased end-diastolic Ca^{2+} and prolongated $Ca^{2+}{}_i$ transients, ultimately abrogating in cardiac failure.[18] Our in vitro experiments with caffeine indicate that contrasting with other models of cardiac hypertrophy, SR Ca^{2+} handling in GC-A$^{-/-}$ cardiomyocytes is not disturbed but even improved. The

CaMKII inhibitory drug KN-93 depressed Ca^{2+}_i transients and contractility more in isolated cardiomyocytes obtained from GC-$A^{-/-}$ mice as compared with WT or cariporide-treated GC-$A^{-/-}$ mice. Thus, a putative mechanism of the increased SR Ca^{2+} handling involves CaMKII-dependent phosphorylation of phospholamban (PLB) at Thr_{17} and subsequent activation of the SR Ca^{2+} pump (SERCA2a). However, KN-93 only partially reversed increased Ca^{2+}_i transients and contractility in isolated ventricular GC-$A^{-/-}$ myocytes, indicating that additional mechanisms are involved. Because cardiac-specific overexpression of active Akt in mice led to enhanced Ca^{2+}_i transients and contraction of isolated myocytes,[39] it is conceivable that enhanced cardiac Akt activity also participates in the changes in Ca^{2+}_i handling and contractility of GC-$A^{-/-}$ cardiomyocytes.

Preservation of Cardiac Function In Vivo Despite Pronounced Hypertrophy

Because cariporide treatment regressed cardiac hypertrophy of GC-$A^{-/-}$ mice despite persistent arterial hypertension and reversed increased Ca^{2+}_i transients and contraction of isolated GC-$A^{-/-}$ myocytes, it was particularly important to assess whether or not this would ultimately be beneficial or detrimental for cardiac function in vivo. As shown, the echocardiographic data of untreated GC-$A^{-/-}$ mice at 6 months of age indicate that cardiac hypertrophy in this model is associated with preserved and even slightly enhanced cardiac contractility. Reversal of cardiac hypertrophy by cariporide treatment did not affect the contractile parameters in vivo. The occurrence of marked cardiac hypertrophy with preserved or even enhanced cardiac function even at very late stages is in accordance with previous studies by our own and other groups[3,9,10] and constitutes one of the most intriguing aspects of this mouse model. The aforementioned improvement in intracellular SR calcium handling might be critically involved.

Implications

Patients with cardiac hypertrophy and/or congestive heart failure have elevated plasma levels of ANP and BNP. However, the cardiovascular and cGMP responses to these hormones are markedly attenuated, indicating impaired receptor or postreceptor responsiveness of GC-A.[1,5] Our present findings in GC-A–deficient mice indicate that an inhibition of cardiac ANP/GC-A signaling might contribute to the increased activity of myocardial NHE-1 that has been observed in these patients[21] and thereby to the progression of cardiac remodeling and dysfunction.

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Disclosure

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