Calcineurin–Nuclear Factor of Activated T Cells Pathway–Dependent Cardiac Remodeling in Mice Deficient in Guanylyl Cyclase A, a Receptor for Atrial and Brain Natriuretic Peptides

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Background—Although disruption of guanylyl cyclase (GC) A, a natriuretic peptide receptor, induces cardiac hypertrophy and fibrosis, the molecular mechanism underlying these effects are not well understood. In this study, we examined the role of calcineurin, a calcium-dependent phosphatase, in cardiac remodeling in GCA-knockout (GCA-KO) mice.

Methods and Results—At 14 weeks of age, calcineurin activity, nuclear translocation of nuclear factor of activated T cells c3 (NFATc3), and modulatory calcineurin-interacting protein 1 (MCIP1) gene expressions were increased in the hearts of GCA-KO mice compared with wild-type (WT) mice. Blockade of calcineurin activation by FK506 (6 mg/kg body weight administered subcutaneously once a day from 10 to 14 weeks of age) significantly decreased the heart-to-body weight ratio, cardiomyocyte size, and collagen volume fraction in GCA-KO mice, whereas FK506 did not affect these parameters in WT mice. Overexpression of atrial and brain natriuretic peptides, collagen, and fibronectin mRNAs in GCA-KO mice was also attenuated by FK506. Electrophoretic mobility shift assays demonstrated that GATA4 DNA-binding activity was increased in GCA-KO mice, and this increase was inhibited by calcineurin blockade. In neonatal cultured cardiac myocytes, inhibition of GCA by HS142-1 (100 μg/mL) increased basal and phenylephrine (10^(-6) mol/L)-stimulated calcineurin activity, nuclear translocation of NFATc3, and MCIP1 mRNA expression. In contrast, activation of GCA by atrial natriuretic peptide (10^(-6) mol/L) inhibited phenylephrine (10^(-6) mol/L)-stimulated nuclear translocation of NFATc3.

Conclusions—These results suggest that activation of cardiac GCA by locally secreted natriuretic peptides protects the heart from excessive cardiac remodeling by inhibiting the calcineurin-NFAT pathway. (Circulation. 2005;111:3095-3104.)

Key Words: calcineurin • fibrosis • hypertrophy • natriuretic peptides • remodeling

Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are cardiac hormones that act through guanylyl cyclase A (GCA) to lower blood pressure (BP), induce diuresis/natriuresis, and dilate blood vessels.1,2 Cardiac synthesis and secretion of ANP and BNP are increased during cardiac hypertrophy associated with various cardiovascular diseases.1 We and other groups have demonstrated the existence of natriuretic peptide receptors in cardiac cells.3-5 Therefore, apart from acting as circulating hormones, ANP and BNP may have some functionality as autocrine and/or paracrine factors. Indeed, we have previously reported in an in vitro study that endogenous natriuretic peptides inhibit cardiac myocyte hypertrophy under basal and phenylephrine (PE)-stimulated conditions, probably via a cyclic GMP–dependent process.6 Furthermore, we7 and other groups8 have reported that mice with disrupted GCA exhibit cardiac hypertrophy and interstitial fibrosis as well as hypertension. Cardiac hypertrophy in GCA-knockout (GCA-KO) mice is disproportionate to the increase in BP9 and is resistant to antihypertensive medication.10 We previously reported that overproduction of GCA in the cardiac myocytes of GCA-KO mice reduced cardiac myocyte size without altering BP.11 In addition, mice with cardiomyocyte-restricted GCA disruption exhibited cardiac hypertrophy.12 These findings suggest that GCA plays an in situ role in protecting the heart from abnormal remodeling independent of BP. However, the mo-
molecular mechanism underlying the inhibition of cardiac hypertrophy and fibrosis by GCA is not well understood.

A number of studies have attempted to elucidate the molecular mechanisms of the hypertrophic process in cardiac myocytes. Recently, Molkentin et al\textsuperscript{13} and Vega et al\textsuperscript{14} demonstrated the importance of calcineurin in the development of cardiac hypertrophy. Calcineurin (PP2B) is a calcium/calmodulin-activated serine-threonine phosphatase that is activated by sustained elevations in intracellular calcium.\textsuperscript{15–17} Calcineurin dephosphorylates nuclear factor of activated T cells (NFAT).\textsuperscript{18} The NFAT transcription factor is normally hyperphosphorylated and sequestered in the cytoplasm. However, on stimulation, NFAT is dephosphorylated by calcineurin and rapidly translocates to the nucleus.\textsuperscript{18} In the nucleus, NFAT associates with GATA4, a zinc finger transcription factor, which directly regulates cardiac hypertrophy-related genes.\textsuperscript{13,14} Calcineurin activity is suppressed by association with modulatory calcineurin-interacting protein 1 (MCIP1), which was cloned as the product of the Down syndrome critical region gene on chromosome 21.\textsuperscript{14} MCIP1 is upregulated by calcineurin signaling and has been proposed to function in a negative-feedback loop to modulate calcineurin activity.\textsuperscript{14} Two calcineurin inhibitors, cyclosporine A and FK506, have been shown to prevent cardiac hypertrophy and fibrosis in various models,\textsuperscript{19–23} suggesting that calcineurin may play a pivotal role in cardiac remodeling. However, the physiological and pathophysiological regulation of the calcineurin-NFAT pathway is poorly understood. In this study, we have investigated the molecular mechanism of GCA-mediated inhibition of cardiac remodeling and have focused on the regulation of the calcineurin-NFAT pathway. Our results suggest that the cardiac natriuretic peptides-GCA system is an endogenous mechanism for monitoring cardiac hypertrophy by negatively regulating excessive activation of the calcineurin-NFAT pathway.

**Methods**

**Animals and Treatment**

All experimental procedures conformed to the guidelines for animal experimentation of the National Cardiovascular Center. GCA-KO mice were generated by methods described previously.\textsuperscript{7} The genetic background of the original GCA-KO mice was a mixture of C57BL/6 and 129SVj. Genotypes were determined by using polymerase chain reaction. All comparisons were made among littermates. Male mice were examined at 14 weeks. Pharmacological treatment was begun when the animals reached 10 weeks of age. FK506, a calcineurin inhibitor (a gift from Fujisawa Industries Ltd), was administered subcutaneously at a dose of 6 mg/kg body weight once a day for 4 weeks. Vehicle alone was administered to control mates. Male mice were examined at 14 weeks. Pharmacological treatment was begun when the animals reached 10 weeks of age. FK506, a calcineurin inhibitor (a gift from Fujisawa Industries Ltd), was administered subcutaneously at a dose of 6 mg/kg body weight once a day for 4 weeks. Vehicle alone was administered to control mice.

**Measurement of BP and Heart Rate**

Systolic BP (SBP) and heart rate (HR) were measured just before and after pharmacological treatment in conscious mice by the tail-cuff method (Softron Co Ltd).

**Transthoracic Echocardiographic Study**

Mice were anesthetized with intraperitoneal administration of ketamine (50 mg/kg) and xylazine (10 mg/kg), their chests were shaved, and 2-dimensional guided M-mode tracings of a cross section of the left ventricular minor axis at the tips of the papillary muscles were obtained for calculation with an echocardiographic system equipped with a 15-MHz phased-array transducer (Sonos-5500, Hewlett Packard).

**Assay of Calcineurin Activity**

Calcineurin activity was determined in lysates of whole ventricular tissues and cultured cardiac myocytes as previously described with some modifications.\textsuperscript{24} For the phosphatase assay, RII peptide (Sigma) was phosphorylated by protein kinase A (Calbiochem) in the presence of [γ-\textsuperscript{32}P]ATP overnight at 30°C in the presence of 1 mmol/L Tris-HCl (pH 7.5), 0.1 mmol/L dithiothreitol, and 0.1 mmol/L MgCl\textsubscript{2}. Tissues and cells were homogenized in lysis buffer (50 mmol/L Tris-HCl [pH 7.5], 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L dithiothreitol, 0.2% NP-40, and protease inhibitor cocktail). After the debris was removed by centrifugation, the supernatant was incubated with phosphorylated RII peptide for 30 minutes at 30°C. Okadaic acid (500 mmol/L) was added to the reactions to specifically suppress endogenous protein phosphatase PP1 and PP2A.\textsuperscript{23} The amount of liberated \textsuperscript{32}P was determined by the Chenkov method.

**Immunoprecipitation/Western Blot Analysis**

Nuclear proteins of ventricles were extracted with NE-PER nuclear and cytoplasmatic extraction reagents (Pierce) according to the manufacturer’s instructions and were immunoprecipitated with anti-NFATc3 polyclonal antibody (Santa Cruz Biotechnology) in low-stringency buffer for 2 hours at 4°C and incubated with protein A beads (Roche) for 1 hour at 4°C. The immunoprecipitates were washed 4 times in phosphate-buffered saline and resuspended with 5% sodium dodecyl sulfate sample buffer. After the immunoprecipitate were heated to 95°C for 5 minutes, the samples were subjected to Western blot analysis. Western blot analyses were performed as described previously.\textsuperscript{5} Samples were electrophoresed through a reducing sodium dodecyl sulfate–polyacrylamide gel and electroblotted onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk and incubated with antibody for NFATc3. The expression levels of the proteins were detected with a Phototope-HRP Western Blot Detection System (Cell Signaling Technology). The band intensity was estimated with the use of NIH Image software.

**Northern Blot Analysis**

Total RNA (15 μg/lane) was extracted from whole ventricles with TRIZol (Invitrogen) reagent, denatured with formaldehyde and for- mamide, and electrophoresed on a 1% agarose gel containing formaldehyde. RNA in the gel was then transferred to a nylon membrane and fixed by UV irradiation. Hybridization and washing of the membrane were performed with cDNA probes for rat MCIP1, murine ANP, rat BNP, rat α1 (type I) collagen, rat α1 (type III) collagen, rat fibronectin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes and an oligonucleotide probe for 18S ribosomal RNA according to methods previously reported.\textsuperscript{6} The band intensity was estimated with a radioimage analyzer (BAS-5000, Fuji Film).

**In Situ Hybridization**

A digoxigenin-labeled cRNA probe specifically hybridized to the murine MCIP1 mRNA was made by reverse transcription–poly- merase chain reaction and in vitro transcription. Deparaffinized sections were permeabilized and digested with protease K (Gibco). After postfixation, acetylated sections were incubated with the cRNA probe at 55°C overnight. After being washed with 0.1× standard saline citrate, the probe was detected with a streptavidin–horseradish peroxidase conjugate and tyramide-based amplification with a diaminobenzidine substrate (Dako).

**Histological Examination**

The ventricles were fixed with 4% paraformaldehyde in phosphate-buffered saline and prepared for routine histological examination. Paraffin sections (2 μm) were stained with hematoxylin and eosin for measurement of myocyte size and diameter and with Sirius red...
F3BA for determination of collagen volume fraction. For measurement of cardiomyocyte cross-sectional area and width, a total of 30 myocytes sectioned transversely at the level of the nucleus were randomly chosen from each section at ×400 magnification and traced. To measure collagen volume fraction, 16 fields of left ventricle wall per section were scanned and digitized with an Optimax 6.5 digital image analyzer (Media Cybernetics) at ×200 magnification. The interstitial collagen volume fraction was measured while omitting fibrosis of the perivascular, epicardial, and endocardial areas from the study. The collagen volume fraction was obtained by calculating the mean ratio of connective tissue to the total tissue area of all measurements of the section.

Electrophoretic Mobility Shift Assay
Electrophoretic mobility shift assays (EMSA) were performed as described previously.26 Nuclear proteins were extracted with NE-PER nuclear and cytoplasmic extraction reagents (Pierce) according to the manufacturer’s instructions, and EMSA was performed with a LightShift chemiluminescent EMSA kit (Pierce). For EMSA, the binding reactions were performed for 20 minutes in 10 mmol/L Tris-HCl (pH 7.5), 50 mmol/L KCl, 5 mmol/L MgCl₂, 1 mmol/L dithiothreitol, 50 ng/µL poly(dI-dC)(dI-dC), 0.05% NP-40, 2.5% glycerol, biotin 3'-end-labeled double-stranded oligonucleotide, and nuclear protein extract. Samples were electrophoresed on a native polyacrylamide gel and then transferred to a nylon membrane. The biotin end-labeled DNA was detected by chemiluminescence. The nucleotide sequence of the sense strand of the double-stranded GATA oligonucleotide was 5’-CAC TTG ATG ACA GAA AGT GAT AAC TCT-3’. Supershift experiment was performed by incubating the nuclear extracts with 2 µg GATA4 polyclonal antibody (Santa Cruz Biotechnology).

Cell Culture
Primary cultures of neonatal ventricular myocytes were prepared as described previously.6 In brief, apical halves of cardiac ventricles from 1- to 2-day-old Wistar rats were separated, minced, and dispersed with 0.1% collagenase type II (Worthington). To segregate myocytes from nonmyocytes, a discontinuous gradient of Percoll (Sigma) was prepared. After centrifugation, the upper layer consisted of a mixed population of nonmyocyte cell types, and the lower layer consisted almost exclusively of cardiac myocytes. After the myocytes were incubated twice on uncoated 10-cm culture dishes for 30 minutes to remove any remaining nonmyocytes, the nonattached viable cells were plated on gelatin-coated glass slides or culture dishes and then cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco BRL) supplemented with 10% fetal calf serum (DMEM, Gibco BRL) supplemented with 10% fetal calf serum (DMEM, Gibco BRL). After a 24-hour incubation in DMEM with fetal calf serum, the culture medium was changed to serum-free DMEM, and all experiments were performed 24 hours later. This purification procedure has well been established,26,27 and in fact, immunostaining with anti-rat sarcomeric antibody revealed that >99% of the cells obtained by this method were cardiomyocytes.27

Immunocytochemistry
Cardiac myocytes were grown on glass slides. The cells were fixed with 4% paraformaldehyde in phosphate-buffered saline for 15 minutes at room temperature. Immunocytochemical staining for NFATc3 and GATA4 was performed with the indirect immunofluorescence method. Cells were incubated with anti-NFATc3 polyclonal antibody (Santa Cruz Biotechnology) and anti-GATA4 polyclonal antibody (Santa Cruz Biotechnology) at dilutions of 1:50 and 1:200, respectively. The NFATc3 and GATA4 signals were detected with anti-rabbit fluorescein isothiocyanate–conjugated secondary antibody and an anti-goat tetramethylrhodamine B isothiocyanate–conjugated secondary antibody, respectively. A fluorescence microscope was used to visualize the cells at ×1000 magnification.

Statistical Analysis
All values are shown as mean±SEM. Statistical significance between the 2 groups was determined with an unpaired t test. For multiple comparisons, the data were subjected to 1-way ANOVA followed by the Bonferroni/Dunn test. Probability values of <0.05 were considered statistically significant.

Results
Calcineurin-NFAT Pathway Is Upregulated in GCA-KO Mice
At 14 weeks of age, there was no significant difference in HR between the genotypes (WT, 633.3±18.0 versus GCA-KO, 608.0±15.7 bpm; n=4 per group); however, moderate increases in SBP were observed in the GCA-KO mice (WT, 105.5±3.2 versus GCA-KO, 130.6±2.1 mm Hg [P<0.05], n=4 per group). As shown in Figure 1A, disruption of the GCA gene induced a significant increase in the heart-to-body weight ratio (WT, 3.92±0.05 versus GCA-KO, 5.99±0.21 [P<0.05]).

To determine whether deletion of GCA changed calcineurin activity, we performed calcineurin enzymatic assays. As shown in Figure 1B, a significant increase in calcineurin activity was observed in GCA-KO mice (WT, 168.8±12.0 versus GCA-KO, 252.6±5.6 cm³/µg protein [P<0.05]).

It has been shown that the NFATc3 transcription factor is a direct downstream effector of calcineurin in the heart and that NFATc3 translocates to the nucleus after calcineurin-mediated dephosphorylation.28 Therefore, we examined whether NFATc3 translocation to the nucleus was increased in the hearts of GCA-KO mice. As shown in Figure 1C, the amount of NFATc3 protein in nuclear extracts was significantly higher in GCA-KO mice compared with their WT littermates (1.5-fold, P<0.05).

MCIP1 is upregulated by calcineurin signaling and has been proposed to function in a negative-feedback loop to modulate calcineurin activity.14 MCIP1 gene expression was significantly increased in the hearts of GCA-KO mice compared with WT hearts (3.5-fold, P<0.05; Figure 1D). Furthermore, we used in situ hybridization to investigate the cell types that expressed the MCIP1 gene. As shown in Figure 1E, MCIP1 gene expression was observed primarily in cardiac myocytes. Taken together, these results suggest that calcineurin is activated in cardiac myocytes of GCA-KO mice.

FK506 Suppresses Activation of Calcineurin in GCA-KO Mice Without Affecting Body Weight, SBP, or HR
Because the calcineurin-NFAT pathway was activated in GCA-KO mice, we next examined whether inhibition of calcineurin activity by FK506 could prevent cardiac hypertrophy and fibrosis in GCA-KO mice. Elevation of calcineurin activity in GCA-KO mice was suppressed completely by FK506 administration (6 mg/kg body weight administered subcutaneously once a day from 10 to 14 weeks of age, Figure 2A). Calcineurin activity in the WT mice was mildly but significantly suppressed. FK506 treatment had no effect on physiological increases in body weight, SBP, and HR in both WT and GCA-KO mice (Table 1). No remarkable side effects of FK506 treatment were seen in mice with either genotype, and all mice survived throughout the treatment protocol.
FK506 Attenuates Cardiac Hypertrophy in GCA-KO Mice

As shown in Figure 2B, treatment with FK506 partially, but significantly attenuated the increase in the heart-to-body weight ratio in GCA-KO mice (GCA-KO/vehicle, 6.07±0.18 versus GCA-KO/FK506, 4.59±0.17 [P<0.05]). In contrast, FK506 treatment did not influence the heart-to-body weight ratio in WT mice (WT/vehicle, 3.80±0.11 versus WT/FK506, 3.62±0.13 [NS]).

As shown in Table 2, echocardiographic analysis demonstrated an increase in the thickness of the interventricular septum (IVSth) and left ventricle posterior wall (LVPWth) and an increase in the left ventricle diastolic dimension (LVDd) in GCA-KO mice. Fractional shortening did not differ significantly between WT and GCA-KO mice. Treatment with FK506 partially but significantly attenuated the increases in IVSth, LVPWth, and LVDd in GCA-KO mice but did not affect fractional shortening. In WT mice, FK506 did not change cardiac function or morphology. Representative images of M-mode echocardiograms are shown in Figure 2C.

Histological Analysis of FK506-Treated Mice

We next examined myocyte size and interstitial fibrosis in the left ventricle. In hematoxylin and eosin–stained sections, we observed an increase in cross-sectional myocyte area and width in the GCA-KO mice compared with WT mice; this increase was partially but significantly prevented by treatment with FK506 (Figure 3A-C). In contrast, FK506 did not affect myocyte area and width in WT mice.

To determine the degree of fibrosis, we performed Sirius red staining. Left ventricular interstitial fibrosis was 7-fold higher in GCA-KO mice than in WT mice. Excessive interstitial fibrosis in GCA-KO mice was also prevented by FK506 treatment (Figure 4A, 4B), whereas FK506 did not affect interstitial fibrosis in WT mice.

Calcineurin Activation Is Involved in Cardiac Gene Expression in GCA-KO Mice

ANP gene expression is increased by a variety of hypertrophic stimuli, and both the expression and secretion of BNP are elevated in patients with cardiac hypertrophy. As shown in Figure 5, ANP and BNP mRNA levels in the ventricle were...
significantly elevated in GCA-KO mice compared with WT. FK506 treatment strongly suppressed the expression of ANP and BNP in GCA-KO mice but had no effect in WT mice. Ventricular expression of collagen I, collagen III, and fibronectin was also significantly elevated in GCA-KO mice compared with WT. FK506 treatment strongly suppressed the expression of these genes in GCA-KO mice but had no effect in WT mice.

**FK506 Attenuates GATA4 DNA-Binding Activity in GCA-KO Mice**

Previous studies have demonstrated that GATA4 is involved in reactivation of the fetal gene program in response to a variety of hypertrophic stimuli and that NFAT may cooperate with GATA4 in the activation of hypertrophic gene markers. We therefore assessed whether GATA4 DNA-binding activity was enhanced in GCA-KO mice. The identity of the GATA4-specific band was confirmed by competition and supershift analyses with unlabeled oligonucleotide and anti-GATA4 antibody (Figure 6A). A representative EMSA with a GATA consensus oligonucleotide and quantitative analysis are shown in Figure 6B and 6C. GATA4 DNA-binding activity was significantly enhanced in GCA-KO mice compared with WT. FK506 treatment strongly suppressed GATA4 DNA-binding activity in GCA-KO mice and mildly suppressed this activity in WT mice.

**Inhibitory Regulation of the Calcineurin-NFAT Pathway by Locally Secreted Natriuretic Peptides in the Heart**

We previously reported that endogenous natriuretic peptides inhibit cardiac myocyte hypertrophy in vitro with use of the natriuretic receptor antagonist HS-142-1. We therefore investigated whether locally secreted natriuretic peptides were able to inhibit the calcineurin-NFAT pathway in cardiac myocytes in an autocrine manner. As shown in Figure 7A, HS-142-1 significantly elevated calcineurin activity under basal (121% versus control) and PE-stimulated (125% versus PE) conditions. In addition, HS-142-1 significantly elevated MCIP1 gene expression under basal (151% versus control) and PE-stimulated (130% versus PE) conditions (Figure 7B). Furthermore, we investigated whether HS-142-1 treatment was able to induce the translocation of cytoplasmic NFATc3 to the nucleus and whether exogenous ANP had an effect on NFATc3 translocation. As shown in Figure 7C, NFATc3 was detected in the cytoplasm of nearly all control cardiac

**Table 1. Body Weight, SBP, and HR in Each Experimental Group**

<table>
<thead>
<tr>
<th></th>
<th>WT + Vehicle</th>
<th>WT + FK506</th>
<th>KO + Vehicle</th>
<th>KO + FK506</th>
</tr>
</thead>
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<tr>
<td>BW, g</td>
<td>28.9±0.8</td>
<td>31.7±0.9</td>
<td>28.7±0.6</td>
<td>30.8±0.7</td>
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<tr>
<td></td>
<td>10 wk</td>
<td>14 wk</td>
<td>10 wk</td>
<td>14 wk</td>
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<tr>
<td>SBP, mmHg</td>
<td>100.6±1.7</td>
<td>104.4±1.7</td>
<td>99.7±1.4</td>
<td>101.5±1.8</td>
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<tr>
<td></td>
<td>10 wk</td>
<td>14 wk</td>
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<td>HR, bpm</td>
<td>616±6</td>
<td>620±5</td>
<td>618±7</td>
<td>609±10</td>
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</table>

BW indicates body weight. Data are expressed as mean±SEM. n=15 or 16.

*P<0.05 vs vehicle-treated WT at 10 weeks.
†P<0.05 vs vehicle-treated WT at 14 weeks.
‡P<0.05 vs FK506-treated WT at 10 weeks.
§P<0.05 vs FK506-treated WT at 14 weeks.
myocytes. However, treatment of cardiac myocytes with HS-142 (100 μg/mL) induced the nuclear translocation of NFATc3 (Figure 7D). PE (10−6 mol/L) stimulation also caused the nuclear translocation of NFATc3 (Figure 7E). However, pretreatment of cardiac myocytes with ANP (10−6 mol/L) prevented PE-induced NFATc3 translocation (Figure 7F). In addition, pretreatment of cardiac myocytes with the cyclic GMP analog 8-bromo-cyclic GMP (10−3 mol/L) also inhibited PE-induced NFATc3 translocation (Figure 7G). GATA4 was stably detected in the nucleus under all experimental conditions.

### Discussion

This study has demonstrated for the first time that disruption of the GCA gene results in activation of the cardiac calcineurin-NFAT pathway. Moreover, pharmacological blockade of calcineurin activity markedly inhibited cardiac hypertrophy and fibrosis in GCA-KO mice. We have also demonstrated that blockade of endogenous GCA induces activation of the calcineurin-NFAT pathway in cultured cardiac myocytes.

As has been reported previously, GCA plays a primary role in moderating cardiac hypertrophy in vivo, independent of its effects on BP regulation. However, which intracellular signaling pathway contributes to GCA-mediated inhibition of cardiac hypertrophy has not been elucidated. Recent studies have recognized the importance of the calcineurin-NFAT signaling pathway in cardiac growth. Molkentin et al13 and Vega et al14 showed that cardiac hypertrophy was induced by the calcium-dependent phosphatase calcineurin, which dephosphorylates the transcription factor NFAT, enabling it to translocate to the nucleus. NFAT then interacts with the zinc finger transcription factor GATA4 and synergistically activates cardiac embryonic gene reprogramming. Therefore, it is tempting to speculate that GCA exerts its antihypertrophic action by antagonizing the calcineurin-NFAT pathway. However, the role of endogenous GCA in the regulation of the calcineurin-NFAT pathway has not been elucidated.

### Table 2. Echocardiographic Characteristics in Each Experimental Group

<table>
<thead>
<tr>
<th>Group</th>
<th>IVSth, mm</th>
<th>LVPWth, mm</th>
<th>LVd, mm</th>
<th>LVds, mm</th>
<th>FS, %</th>
<th>HR, bpm</th>
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<tr>
<td>WT + Vehicle</td>
<td>0.55±0.02</td>
<td>0.56±0.03</td>
<td>3.88±0.05</td>
<td>2.46±0.07</td>
<td>37.2±1.3</td>
<td>268±12</td>
</tr>
<tr>
<td>WT + FK506</td>
<td>0.54±0.02</td>
<td>0.56±0.03</td>
<td>3.76±0.07</td>
<td>2.42±0.06</td>
<td>35.7±1.3</td>
<td>272±13</td>
</tr>
<tr>
<td>KO + Vehicle</td>
<td>0.87±0.05*</td>
<td>0.92±0.05*</td>
<td>4.31±0.08*</td>
<td>2.60±0.07</td>
<td>39.7±1.4</td>
<td>262±9</td>
</tr>
<tr>
<td>KO + FK506</td>
<td>0.66±0.02†</td>
<td>0.68±0.02†</td>
<td>3.94±0.08†</td>
<td>2.44±0.07</td>
<td>38.1±1.6</td>
<td>279±11</td>
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</table>

LVDs indicates left ventricular end-systolic diameter; FS, fractional shortening. n=15 or 16.

*P<0.05 vs vehicle-treated WT.
†P<0.05 vs vehicle-treated KO.
Therefore, in this study, we investigated the significance of the calcineurin-NFAT pathway in cardiac remodeling in mice deficient for GCA. We found that calcineurin activity, NFATc3 translocation to the nucleus, and MCIP1 gene expression were upregulated in the absence of GCA. In addition, chronic pharmacological inhibition of calcineurin by FK506 administration suppressed cardiac hypertrophy and fibrosis. FK506 attenuated not only cardiac hypertrophy but also cardiac dilation without affecting cardiac contractility. Moreover, the cross-sectional areas and widths of cardiac myocytes and interstitial fibrosis, which were significantly increased in GCA-KO mice, were all attenuated by FK506 treatment. Marked increases in ventricular expression of the genes for ANP, BNP, collagen I, collagen III, and fibronectin were observed in GCA-KO mice; these effects were significantly attenuated by FK506 treatment. GATA4 is a transcriptional regulator of the hypertrophic response and cooperates with NFAT to activate the BNP promoter in cardiac myocytes.13,31 In this study, we have demonstrated that GATA4 DNA-binding activity was augmented in GCA-KO mice and that FK506 treatment diminished this activity. Taken together, these results suggest that activation of the calcineurin-NFAT pathway and the subsequent activation of GATA4 play an important role in cardiac hypertrophy and fibrosis in GCA-KO mice.

Although the calcineurin-NFAT pathway was activated in GCA-KO mice, the upstream factor(s) that activates the calcineurin-NFAT pathway has not been identified. We suggest that the renin-angiotensin (Ang) II system may contribute to calcineurin activation in GCA-KO mice. We previously reported that targeted deletion or pharmacological blockade of the Ang II type 1A receptor ameliorated cardiac hypertrophy and interstitial fibrosis in GCA-KO mice.32 Furthermore, it has been reported that a nonantihypertensive dose of the selective Ang II type 1A receptor blocker candesartan attenuated cardiac calcineurin activity and the development of cardiac hypertrophy and fibrosis in hyperten-
This suggests that GCA inhibits calcineurin activity at least in part by interacting with Ang II signaling. However, other factors are probably involved in calcineurin activation in GCA-KO mice, because targeted deletion or pharmacological blockade of the Ang II type 1A receptor significantly reduced, but did not completely inhibit, cardiac remodeling in GCA-KO mice.  

Because we cannot exclude the involvement of high BP in calcineurin activation in GCA-KO mice, we next examined whether locally secreted natriuretic peptides/GCA signaling was able to directly inhibit the calcineurin-NFAT pathway in an autocrine manner by using HS-142-1, a natriuretic receptor antagonist, in cultured cardiac myocytes. Treatment with HS-142-1 increased calcineurin activity and MCIP1 gene expression under both basal and PE-stimulated conditions. Furthermore, HS-142-1 induced the nuclear translocation of NFATc3. These in vitro results confirm that inhibition of GCA leads to activation of the calcineurin pathway. Importantly, a cyclic GMP analog and exogenous ANP both inhibited PE-induced nuclear translocation of NFATc3. Because ANP- or BNP-induced activation of GCA leads to an increase in intracellular cyclic GMP levels, GCA-mediated inhibition of the calcineurin-NFAT pathway might occur in part through activation of cyclic GMP and its effectors. In fact, Fiedler et al recently demonstrated in vitro that cyclic GMP–dependent protein kinase inhibits calcineurin-NFAT signaling upstream of calcineurin and may also inhibit the pathway downstream of calcineurin. In addition, we previously reported that blockade of endogenous natriuretic peptides induced hypertrophy in cultured cardiac myocytes, probably via a cyclic GMP–dependent mechanism. Taken together, these results suggest that ANP or BNP may play a role as an autocrine factor in the regulation of cardiac myocyte hypertrophy, in part via cyclic GMP–dependent protein kinase–mediated inhibition of the calcineurin-NFAT pathway. A schematic diagram depicting the signaling supported by the present study is shown in Figure 8.

Although inhibition of calcineurin attenuated cardiac fibrosis and markers of fibrosis in this study, the mechanism underlying these observations is not yet well understood. Transforming growth factor (TGF)-β1 is a potent stimulator of extracellular matrix protein synthesis (eg, collagen and fibronectin). We previously reported that TGF-β1 gene expression is significantly increased in the hearts of GCA-KO mice. Recently, the involvement of calcineurin in TGF-β–mediated regulation of extracellular matrix accumulation in other cell types has been reported. Therefore, inhibition of TGF-β1 signaling in cardiac fibroblasts might be one mechanism whereby FK506 treatment induces attenuation of cardiac fibrosis.

In the present study, the inhibitory effect of FK506 treatment on cardiac hypertrophy was more potent than on cardiac fibrosis. Although the exact mechanisms are unknown, the calcineurin pathway might be differently activated depending on cell type. In fact, MCIP expression, which is regulated by calcineurin activation, was observed primarily in cardiac myocytes.

It has recently been suggested that various signaling molecules coordinate with the calcineurin-NFAT pathway. The involvement of mitogen-activated protein kinase (MAPK) pathways, including c-Jun-NH2-terminal kinase (JNK), extracellular regulated kinase (ERK), and P38 MAPK, have been well characterized in cardiac hypertrophy. However, it had been reported that the activities of JNK, ERK, and P38 MAPK were not activated in GCA-KO mice. Further investigations are necessary to identify effectors that play a role in the enhanced cardiac hypertrophy observed in GCA-KO mice.

In the present study, the HR of mice during echocardiography was lower than that observed in conscious mice. In fact,
Insight into endogenous mechanisms for protection of the heart by natriuretic peptides/GCA signaling.

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