Inhibition of TRPC6 Channel Activity Contributes to the Antihypertrophic Effects of Natriuretic Peptides-Guanylyl Cyclase-A Signaling in the Heart

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Rationale: Atrial and brain natriuretic peptides (ANP and BNP, respectively) exert antihypertrophic effects in the heart via their common receptor, guanylyl cyclase (GC)-A, which catalyzes the synthesis of cGMP, leading to activation of protein kinase (PK)G. Still, much of the network of molecular mediators via which ANP/BNP-GC-A signaling inhibit cardiac hypertrophy remains to be characterized.

Objective: We investigated the effect of ANP-GC-A signaling on transient receptor potential subfamily C (TRPC)6, a receptor-operated Ca\(^{2+}\) channel known to positively regulate prohypertrophic calcineurin–nuclear factor of activated T cells (NFAT) signaling.

Methods and Results: In cardiac myocytes, ANP induced phosphorylation of TRPC6 at threonine 69, the PKG phosphorylation site, and significantly inhibited agonist-evoked NFAT activation and Ca\(^{2+}\) influx, whereas in HEK293 cells, it dramatically inhibited agonist-evoked TRPC6 channel activity. These inhibitory effects of ANP were abolished in the presence of specific PKG inhibitors or by substituting an alanine for threonine 69 in TRPC6. In model mice lacking GC-A, the calcineurin-NFAT pathway is constitutively activated, and BTP2, a selective TRPC channel blocker, significantly attenuated the cardiac hypertrophy otherwise seen. Conversely, overexpression of TRPC6 in mice lacking GC-A exacerbated cardiac hypertrophy. BTP2 also significantly inhibited angiotensin II–induced cardiac hypertrophy in mice.

Conclusions: Collectively, these findings suggest that TRPC6 is a critical target of antihypertrophic effects elicited via the cardiac ANP/BNP-GC-A pathway and suggest TRPC6 blockade could be an effective therapeutic strategy for preventing pathological cardiac remodeling. (Circ Res. 2010;106:00-00.)

Key Words: natriuretic peptides ■ calcium ■ ion channels ■ hypertrophy

In response to pathological stimuli such as prolonged mechanical stress, massive tissue injury, or abnormal neurohumoral activation, hearts show hypertrophic growth and remodeling, which is characterized by an increase in myocyte cell size, assembly of sarcomere proteins, interstitial fibrosis, and reexpression of fetal cardiac genes. Although the hypertrophic response is initially compensatory, it ultimately causes heart failure, which is now a leading cause of morbidity and mortality around the world. Diverse intracellular signaling pathways exerting pro- or antihypertrophic effects have been shown to play important roles in the complex processes of cardiac remodeling, but the details of the molecular mechanisms mediating the crosstalk among these signaling pathways remain uncertain. Unraveling those details should give us a better understanding of the molecular processes underlying the establishment of cardiac hypertro-

Original received August 31, 2009; revision received April 20, 2010; accepted April 22, 2010.

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Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.109.208314
The heart regulates cardiovascular homeostasis in part by secreting 2 peptide mediators, atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP). ANP and BNP bind to their common receptor, guanylyl cyclase (GC)-A (also called NPR-A and NPR1), which then catalyzes the synthesis of cGMP, leading to the activation of protein kinase (PKG). Under pathological conditions in the heart, there is a significant increase in the ventricular expression of both ANP and BNP, which then act as both endocrine and local antihypertrophic factors. Indeed, ANP/BNP have been shown to exert antihypertrophic effects on cardiac myocytes in vitro and in vivo by counteracting multiple prohypertrophic signaling pathways, including the MEK1–ERK1/2 (mitogen-associated protein kinase II pathway), the Akt pathway, and the calcineurin–nuclear factor of activated T cell (NFAT) pathway.

The serine-threonine phosphatase calcineurin functions as a Ca^{2+}-dependent regulator of cardiac hypertrophy and the fetal gene program. Calcineurin dephosphorylates NFAT family transcription factors and induces their translocation to the nucleus, where they bind to the regulatory regions of cardiac genes in conjunction with other cardiac transcription factors and promote hypertrophic growth. Transient receptor potential subfamily C (TRPC)3 and -6 reportedly serve as positive upstream regulators of the calcineurin-NFAT signaling pathway. TRPC3 and 6 form homo- and heteromultimeric cation channels that are activated directly by diacylglycerol and function to couple receptor-phospholipase C activity to Ca^{2+} influx, which in turn activates calcineurin-NFAT signaling pathways and possibly other Ca^{2+}-dependent signaling pathways. NFAT also activates TRPC6 gene transcription, thereby accelerating the calcineurin-NFAT prohypertrophic signaling loop. It was recently shown that TRPC3 and 6 activities are greatly attenuated by PKG-catalyzed phosphorylation of Thr11 and Ser263 in TRPC3 and Thr69 in TRPC6, which are well conserved among mouse, rat and human.

In the present study, we examined the functional crosstalk between the ANP/BNP-GC-A-cGMP-PKG and TRPC6-calcineurin-NFAT pathways during the process of cardiac hypertrophy and characterized its biological significance in cardiac pathophysiology. Our findings demonstrate that TRPC6 is a direct target of ANP/BNP-GC-A-cGMP-PKG antihypertrophic signaling and suggest that inhibition of TRPC6 could represent a novel therapeutic strategy for preventing pathological cardiac hypertrophy and remodeling.

### Methods

#### Plasmid Construction

Regulator of calcineurin (RCAN1)-luciferase (RCAN1-luc), in which RCAN1 intron 3 containing 15 NFAT sites was inserted upstream of the luciferase gene, was kindly provided by B. A. Rothermel (University of Texas, Southwestern Medical Center, Dallas). Expression vectors encoding wild-type (WT) and mutant (T69A) mouse TRPC6 were described previously.

#### Cell Culture

Primary neonatal rat ventricular myocytes were isolated and grown as described previously.

#### Patch Clamp Studies

The details of the patch clamp recording and data analysis were essentially the same as described previously.

#### Animal Experiments

The animal care and all experimental protocols were reviewed and approved by the Animal Research Committee at Kyoto University Graduate School of Medicine. Beginning at 12 weeks of age, GC-A knockout (KO) mice (C57BL/6 background) were left untreated (control) or were treated for 4 weeks with BTP2 (20 mg/kg per day PO) or nitrendipine (40 mg/kg per day PO). BTP2 was dissolved in methylcellulose (Shin-Etsu Chemical) to a concentration of 3.0 mg/mL and was given daily via gastric gavage adjusted to the individual body weight of each mouse. The same amount of 0.5% methylcellulose was given to the other treatment groups in the same manner. Nitrendipine was given as described previously.

#### Echocardiographic Analysis

Echocardiography was carried out as described previously using a Toshiba Power Vision 8000 echocardiography system equipped with a 12-MHz imaging transducer.
Statistical Analysis

Data are presented as means ± SEM. Unpaired t tests were used for comparisons between 2 groups, and ANOVA with post hoc Fisher tests was used for comparisons among groups. Values of P < 0.05 were considered significant.

Results

Signaling via the ANP-cGMP-PKG Pathway Inhibits Endothelin-1–Induced, TRPC6-Mediated Activation of Calcineurin-NFAT Signaling in Cardiac Myocytes

In previous reports, TRPC family ion channels (TRPC3 and TRPC6) were shown to play a central role in activating calcineurin-NFAT signaling in the ventricular myocardium during the process of pathological cardiac hypertrophy. Consistent with those reports, we found that in cultured neonatal rat ventricular myocytes, BTP2, a selective TRPC inhibitor, significantly and dose-dependently inhibited endothelin (ET)-1-induced activation of the NFAT-dependent RCAN1 promoter, with and without overexpression of TRPC6 (Figure 1A). BTP2 also significantly attenuated ET-1–induced hypertrophic responses, including increased expression of ANP, RCAN1 and TRPC6 mRNA and enlargement of cultured cardiac myocytes (Figure 1B and 1C; and Online Figure I, A through C, in the Online Data Supplement, available at http://circres.ahajournals.org).

Figure 1. ANP and cGMP inhibit ET-1–induced, TRPC6-mediated NFAT activation in cardiac ventricular myocytes, an effect dependent on phosphorylation of Thr69 of TRPC6. A, Rat neonatal ventricular myocytes cotransfected with an expression plasmid encoding TRPC6 and the RCAN1 promoter-luciferase gene (RCAN1-luc) were maintained in the absence or presence of 10 nmol/L ET-1 and/or 0.03, 0.3, or 3 μmol/L BTP2. Relative luciferase activity in control myocytes transfected with RCAN1-luc alone was assigned a value of 1.0. *P < 0.05 vs control; **P < 0.05 vs myocytes treated with ET-1 alone. B and C, Real-time RT-PCR analysis of the relative levels of ANP (B) and RCAN1 (C) mRNA in cultured ventricular myocytes treated with or without 10 nmol/L ET-1 and/or 3 μmol/L BTP2. The relative mRNA level in control myocytes was assigned a value of 1.0. *P < 0.05 vs control; #P < 0.05 vs myocytes treated with ET-1 alone. D and E, Ventricular myocytes cotransfected with an expression plasmid encoding WT TRPC6 or a TRPC6 T69A mutant and RCAN1-luc were maintained in the absence or presence of 10 nmol/L ET-1 and/or 100 nmol/L ANP (D) or 100 μmol/L 8Br-cGMP (E). Relative luciferase activity in control myocytes transfected with RCAN1-luc alone was assigned a value of 1.0. *P < 0.05; NS; P = NS. In all graphs (A through E), values are shown as means ± SEM. F, Representative Western blots of Thr69-phosphorylated TRPC6 (top) and total TRPC6 (bottom) in cardiac myocytes treated for 1 hour with or without 100 nmol/L ANP and/or 1 μmol/L KT5823. The relative level of Thr69-phosphorylated TRPC6 in control cardiac myocytes was assigned a value of 1.0. Values are shown as means ± SEM (n = 3 each). *P < 0.001 vs control myocytes; **P < 0.05 vs myocytes with ANP alone.
hhibited ET-1–induced RCAN1 promoter activation in cardiac myocytes cotransfected with WT TRPC6 or not, but it failed to inhibit the response in myocytes cotransfected with the TRPC6 T69A mutant (Figure 1E). This suggests that signaling in the ANP-GC-A-cGMP-PKG pathway leads to phosphorylation of TRPC6 on Thr69, which inhibits receptor-mediated TRPC6 channel activation and, in turn, activation of calcineurin-NFAT signaling in cardiac myocytes. Indeed, using a specific antibody against phosphorylated Thr69, we confirmed that ANP stimulates phosphorylation of TRPC6 Thr69 in cardiac myocytes, and that the phosphorylation was blocked in the presence of the selective PKG inhibitor KT5823 (Figure 1F).

ANP-cGMP-PKG Signaling Inhibits TRPC6 Ion Channel Activity

We next examined the effects of ANP-cGMP-PKG signaling on TRPC6 ion channel activity. In HEK293 cells expressing WT TRPC6, the membrane-permeant diacylglycerol analogue oleoyl-2-acetyl-sn-glycerol (OAG) induced a significant increase in Ca^{2+} influx (Figure 2A, top left) that was dramatically inhibited by prior application of ANP (Figure 2A, bottom left). By contrast, in HEK293 cells expressing the TRPC6 T69A mutant, OAG-induced Ca^{2+} influx was unaffected by ANP, indicating that ANP directly inhibits OAG-induced TRPC6 channel activity through the PKG-phosphorylation site.

Figure 2. ANP inhibits agonist-evoked Ca^{2+} influx through phosphorylation of Thr69 in TRPC6. A, Three different representative time courses of OAG-induced Ca^{2+} influx via TRPC6 (left panels) or the TRPC6 T69A mutant (right graphs) in HEK293 cells treated without (top graphs) or with (bottom graphs) 100 nmol/L ANP. B, OAG-evoked Ca^{2+} influx via TRPC6 in HEK293 cells treated with or without ANP in Ca^{2+}-free (white bar) and Ca^{2+}-containing (2 mmol/L) external solution (black bar). *P<0.05. C, Representative time courses of ATP-evoked Ca^{2+} influx in control HEK293 cells treated without (black line) or with (gray line) ANP. Graphs at right show ATP-induced Ca^{2+} influx in control HEK293 cells treated with or without ANP in Ca^{2+}-free (white bar) or Ca^{2+}-containing (2 mmol/L) external solution (black bar). D and E, Representative time courses of ATP-induced Ca^{2+} influx via TRPC6 or the TRPC6 T69A mutant in HEK293 cells treated without (black line) or with (gray line) ANP. Graphs at right show ATP-induced Ca^{2+} influx via TRPC6 or the TRPC6 T69A mutant in HEK293 cells treated with or without ANP in Ca^{2+}-free (white bar) or Ca^{2+}-containing (2 mmol/L) external solution (black bar). *P<0.05. In all graphs, values are shown as means±SEM. F, Inward cation currents (I_{TRPC6}) activated by the muscarinic receptor agonist carbachol (CCh; 100 μmol/L) in the absence (top) and presence (bottom) of 100 nmol/L ANP. Murine TRPC6-expressing HEK293 cells were voltage-clamped at a holding potential of -60 mV. Bath: normal PSS; pipette: Cs-aspartate internal solution. ANP was added to the bath 10 minutes before application of CCh. G, Current density of CCh-induced I_{TRPC6} in HEK293 cells expressing WT or mutant (T69A) TRPC6, with or without ANP; n=12 in TRPC6 without ANP, 7 in TRPC6 with ANP, 7 in T69A without ANP, 5 in T69A with ANP. Values are shown as means±SEM. *P<0.05 vs cells without ANP. NS: P=NS.
tion of Gq upstream of TRPC6, we assessed the endogenous expression of RGS2 and RGS4 in HEK293 cells and confirmed expression of both RGS2 and RGS4 mRNA (Online Figure I, D). Thus our finding that ATP-induced activation of the PKG-resistant TRPC6 T69A mutant in the presence of ANP was similar to seen with WT TRPC6 in the absence of ANP, despite endogenous expression of RGS2 and 4, indicates the existence of an RGS2/4-independent pathway via which ANP inhibits Gq-mediated TRPC6 activation (Figure 2D and 2E).

We also measured cationic currents induced by carbachol in HEK293 cells expressing TRPC6 (Figure 2F) and found that they were significantly inhibited in the presence of ANP (Figure 2F and 2G). Furthermore, the inhibitory effect of ANP was substantially blunted in cells expressing the TRPC6 T69A mutant, again confirming that ANP inhibits Gq-coupled receptor-mediated TRPC6 activation directly through the PKG-phosphorylation site (Figure 2G). Likewise cationic currents carried by TRPC6 channels activated by GTPγS were also inhibited by ANP (Online Figure I, E), and this inhibitory effect was significantly blunted in the presence of DT-3, a selective PKG Iα inhibitor, or by mutation (T69A) of TRPC6 (Online Figure I, F). All of these data indicate that signaling via the ANP-cGMP-PKG pathway inhibits TRPC6 ion channel activity through direct phosphorylation of TRPC6.

ANP Inhibits Agonist-Induced Ca\(^{2+}\) Influx Into Ventricular Myocytes

We next examined the inhibitory effect of ANP on Ca\(^{2+}\) signaling in cardiac myocytes. When we used Fura-2 to measure the frequency of Ca\(^{2+}\) oscillations induced by ET-1 or angiotensin II (Ang II) in cultured neonatal ventricular myocytes, we found that both increased the frequency of Ca\(^{2+}\) oscillation in the cells, that this effect was significantly inhibited by ANP (Figure 3A and 3B), and that the inhibitory effect of ANP was almost completely abolished in the presence of KT5823 (Figure 3C and 3D). ET-1- or Ang II-induced increases in Ca\(^{2+}\) oscillation were also significantly inhibited when TRPC3 and 6 were simultaneously knocked down using small interfering (si)RNAs (Figure 3E and 3F, and Online Figure II, A, D, F, and I). Moreover, knocking down either TRPC3 or 6 had a similar effect (Online Figure II, A, B, C, E, F, G, H, and J). This suggests that TRPC3 and 6 act in concert to mediate ANP-sensitive, ET-1- or Ang II-induced increases in Ca\(^{2+}\) oscillation in cardiac myocytes. ET-1 and Ang II also induced Ca\(^{2+}\) influx into cardiac myocytes that was significantly inhibited by ANP (Figure 4A and 4B). We previously showed that knocking down either TRPC3 or 6 significantly reduced Ang II–induced Ca\(^{2+}\) influx into cardiac myocytes. In the present study, we found that knocking down TRPC3 and 6 significantly inhibited ET-1- and Ang II–induced Ca\(^{2+}\) influx and abolished the inhibitory effect exerted by ANP on this Ca\(^{2+}\) influx, which suggests ANP inhibits TRPC3/6-mediated Ca\(^{2+}\) influx into cardiac myocytes (Figure 4C and 4D; Online Figure III, A through D).

![Graph showing the frequencies of Ca\(^{2+}\) oscillations in cultured cardiac myocytes treated with vehicle, ET-1 or Ang II in the absence (A) or presence of ANP (B) or ANP+KT5823 (C). D, E and F: Effects of double knockdown of TRPC3 and 6 on the frequencies of Ca\(^{2+}\) oscillations in cultured cardiac myocytes treated with ET-1 (E) or Ang II (F). KT5823: a selective PKG inhibitor (200 nmol/L). Values are shown as means±SEM. *P<0.05.](http://circres.ahajournals.org/)
to simultaneously knock down their expression. We initially confirmed that the RGS2 and RGS4 siRNAs efficiently and specifically knocked down RGS2 and RGS4 mRNA levels to 18% and 27%, respectively, of those seen with control siRNA in ventricular myocytes (Figure 5E). In ventricular myocytes cotransfected with RGS2 and RGS4 siRNAs, ANP still significantly inhibited ET-1– and Ang II–induced Ca2+ influx, but this inhibitory effect was significantly attenuated (Figure 5F and 5G; Online Figure IV, A and B). In addition, type1a Ang II receptor densities and the expression levels of mRNAs and proteins related to Gq-coupled receptor-mediated Ca2+ influx were not significantly altered (Online Figure IV, C through E). Collectively then, these results support our notion that ANP inhibits ET-1– and Ang II–induced Ca2+ influx into cardiac myocytes in a manner that is, at least in part, TRPC-dependent.

TRPC Channels Play a Pivotal Role in Cardiac Hypertrophy in GC-A KO Mice

ANP increases intracellular cGMP via its receptor, GC-A, a particulate type of guanylyl cyclase. GC-A KO mice, which lack GC-A, exhibit reduced plasma cGMP levels, salt-resistant hypertension and cardiac hypertrophy.43–45 Activation of calcineurin-NFAT signaling is reportedly involved in the development of the cardiac hypertrophy seen in GC-A KO mice,2,15 and because TRPC6 forms a positive regulatory circuit with the calcineurin-NFAT pathway,23 we examined whether TRPC6 gene expression is induced in the ventricles of GC-A KO mice. Real-time RT-PCR analysis clearly showed a significant increase in the expression of TRPC6, ANP, BNP and TRPC3 mRNA in GC-A KO ventricles (Figure 6A), which is consistent with the notion that GC-A negatively regulates calcineurin-NFAT prohypertrophic signaling. The levels of TRPC3 and 6 protein were also significantly higher in GC-A KO ventricles than WT ventricles (Online Figure V, A and B). To evaluate the contribution made by TRPC6 and 3 to the development of cardiac hypertrophy, we treated GC-A KO mice with BTP2. Although BTP2 did not affect blood pressure or heart rate in GC-A KO mice (Figure 6B), it significantly reduced cardiac hypertrophy assessed based on heart weight (HW), heart weight/body weight (HW/BW) ratios, and myocardial cell diameters measured in histological samples (Figure 6C and 6D). By contrast, nitrendipine did not reduce cardiac hypertrophy in GC-A KO mice, though it modestly reduced blood pressures (WT without nitrendipine, 94.5±0.5 mm Hg; WT with nitrendipine, 89.7±3 mm Hg; GC-A KO without nitrendipine, 119.8±2.1 mm Hg; GC-A KO with nitrendipine, 116.2±1.5 mm Hg; Online Figure V, C through E; Online Table I). BTP2 also reduced cardiac fibrosis in GC-A KO mice (Figure 6E). Consistent with these findings, echocardiographic analysis showed that BTP2 reduced posterior wall thickness and restored left ventricular end-diastolic dimension in GC-A KO ventricles without affecting % fractional shortening (Figure 6F). In addition, the increased ventricular expression of RCAN1 and such hypertrophy marker genes as ANP, BNP, β-myosin heavy chain (βMHC), and skeletal α-actin in GC-A KO ventricles was dramatically attenuated by BTP-2 (Figure 7A), though expression of α-myosin heavy chain (αMHC) and cardiac α-actin was not (Figure 7A). Likewise, the increased

Figure 4. ANP inhibits agonist-evoked Ca2+ influx into cardiac ventricular myocytes. A, Representative time courses of ET-1– or Ang II–evoked Ca2+ influx into cultured ventricular myocytes treated with (gray line) or without (black line) ANP. B, ET-1– and Ang II–evoked Ca2+ influx into cultured ventricular myocytes treated with or without ANP in Ca2+-free (white bar) or Ca2+-containing (black bar) external solution. Values are shown as means±SEM. *P<0.05. C and D, Effects of double knockdown of TRPC3 and 6 on Ca2+ influx in cultured cardiac myocytes treated with ET-1 (C) or Ang II (D) in the presence or absence of ANP. In all of these experiments, 10 nmol/L ET-1, 100 nmol/L Ang II, 100 nmol/L ANP, and 2 mmol/L external Ca2+ were used.
expression of TRPC6 and TRPC3 seen in GC-A KO ventricles was diminished by BTP2 treatment (Figure 7A). Clearly, TRPC-mediated signaling is significantly involved in the development of pathological cardiac hypertrophy induced by a genetic deletion of GC-A.

Exaggerated Cardiac Hypertrophy in Mice With Cardiac Overexpression of TRPC6 Against a GC-A–Null Background

To further confirm the negative functional interaction of the ANP-GC-A-cGMP-PKG and TRPC6-calcineurin-NFAT pathways, we next crossed transgenic mice cardioslectively expressing TRPC6 (TRPC6 Tg; previously referred to as line 16) with GC-A KO mice.23 As previously reported, 12-week-old TRPC6 Tg mice did not show cardiac hypertrophy, as compared to WT mice, whereas GC-A KO mice showed a significant increase in blood pressure, HW and HW/BW ratios (Figure 7B and 7C).23,44 Moreover, TRPC6 Tg mice with a GC-A-null background showed significantly greater HW/BW ratios than GC-A KO mice, without an increase in blood pressure, which is indicative of the hypersensitivity of GC-A KO mice to TRPC6-mediated prohypertrophic signaling (Figure 7B and 7C). Consistent with this finding, echocardiographic analysis showed ventricular wall thicknesses to be greater in GC-A; TRPC6 Tg mice than GC-A KO mice, without a change in systolic function (Figure 7D).

TRPC Inhibition Prevents Ang II–Induced Cardiac Hypertrophy

The notion that TRPC6 inhibition is a critical component of the antihypertrophic effects exerted via the ANP-GC-A-cGMP-PKG pathway suggests that direct inhibition of TRPC6 could be a novel therapeutic approach to preventing pathological cardiac hypertrophy. Indeed, BTP2 significantly inhibited the cardiac hypertrophy otherwise seen in GC-A KO mice (Figure 6C through 6F and Figure 7A). To further test this hypothesis using models of cardiac hypertrophy in which GC-A-cGMP-PKG signaling is genetically intact, we examined the effects of BTP2 on Ang II–induced cardiac hypertrophy. When we chronically infused Ang II using a subcutaneously implanted osmotic

Figure 5. TRPC channels are involved in ANP-induced inhibition of receptor-mediated Ca2⁺ entry into cardiac ventricular myocytes. A, ET-1- and Ang II–evoked Ca2⁺ influx into cultured ventricular myocytes treated with or without ANP in Ca2⁺-free (white bar) or Ca2⁺-containing external solution (black bar). B and C, Effect of nifedipine (1 μmol/L) (B) or BTP2 (5 μmol/L) (C) on ET-1- and Ang II–evoked Ca2⁺ influx into cultured ventricular myocytes treated with or without ANP in Ca2⁺-free (white bar) or Ca2⁺-containing external solution (black bar). D, Representative traces showing the effect of BTP2-induced (5 μmol/L) on K[Ca²⁺]-induced Ca²⁺ influx into cardiac myocytes. Graphs at right show the effect of BTP2 on K[Ca²⁺]-induced Ca²⁺ influx into cardiac myocytes. E, Effect of RGS2 and RGS4 siRNA on expression of RGS2 (left) and RGS4 (right) mRNA in cultured ventricular myocytes (n=4 each). *P<0.05 vs control siRNA. F, Effect of RGS2 and RGS4 double knockdown on ET-1–evoked (left) and Ang II–evoked (right) Ca²⁺ influx into cultured ventricular myocytes treated with or without ANP in Ca²⁺-free (white bar) or Ca²⁺-containing external solution (black bar). G, Effect of RGS2 and RGS4 double knockdown on the inhibitory effects of ANP in ET-1– or Ang II–evoked Ca²⁺ influx into cultured ventricular myocytes. In all graphs, values are shown as means±SEM *P<0.05. In all these experiments, 10 nmol/L ET-1, 100 nmol/L Ang II, 100 nmol/L ANP, and 2 mmol/L external Ca²⁺ were used.
BTP2 (Figure 8D; Online Figure V, F and G). Affecting in mice treated with or without Ang II and/or cGMP-PKG and TRPC6-calcineurin-NFAT pathways in cardiac remodeling. In the present study, we unraveled the novel therapeutic approaches to preventing pathological cardiac hypertrophy, which could ultimately lead to the discovery of underlying the establishment of cardiac hypertrophy and should lead to a better understanding of molecular processes pathways that promote or antagonize hypertrophic responses.

Characterization of the crosstalk among the cardiac signaling pathways that promote or antagonize hypertrophic responses should lead to a better understanding of molecular processes underlying the establishment of cardiac hypertrophy and heart failure, which could ultimately lead to the discovery of novel therapeutic approaches to preventing pathological cardiac remodeling. In the present study, we unraveled the functionally negative crosstalk between the ANP-GC-A-cGMP-PKG and TRPC6-calcineurin-NFAT pathways in cardiac myocytes using in vitro culture systems and in vivo genetically engineered models. ANP acted via the cGMP-PKG pathway to directly inhibit TRPC6 activity, which in turn suppressed prohypertrophic signaling. Cardiac hypertrophy was significantly attenuated by the selective TRPC inhibitor BTP2 in GC-A KO mice, which were hypersensitive to hypertrophic signaling caused by overexpression of TRPC6. Likewise, BTP-2 significantly inhibited the cardiac hypertrophy induced by chronic Ang II infusion. Our study thus demonstrates that inhibition of TRPC6 activity mediates the antihypertrophic effects of ANP/BNP, and suggests that inhibition of TRPC6 could be an effective therapeutic strategy for preventing pathological cardiac hypertrophy and remodeling.

It was recently reported that RGS4 mediates the antihypertrophic effects of GC-A-catalyzed signaling in the heart.20 RGS2 also reportedly mediates the antihypertrophic effects of inhibiting phosphodiesterase 5, which enhances activity in the NO-cGMP pathway.41 It is thus suggested that RGS proteins mediate the antihypertrophic effects exerted by cGMP. In our study, we confirmed that RGS2 and 4 are significantly involved in the ANP-induced inhibition of agonist-induced Ca2+ influx (Figure 5I). PKG-catalyzed phosphorylation of TRPC6 on Thr69 is significantly involved in ANP-induced inhibition of receptor-mediated TRPC6 activity, despite the expression of RGS2 and RGS4 mRNA in HEK293 cells, however (Figure 2I, 2K and 2N; Online Figure I, D).

**Discussion**

Characterization of the crosstalk among the cardiac signaling pathways that promote or antagonize hypertrophic responses should lead to a better understanding of molecular processes underlying the establishment of cardiac hypertrophy and heart failure, which could ultimately lead to the discovery of novel therapeutic approaches to preventing pathological cardiac remodeling. In the present study, we unraveled the functionally negative crosstalk between the ANP-GC-A-cGMP-PKG and TRPC6-calcineurin-NFAT pathways in cardiac myocytes using in vitro culture systems and in vivo genetically engineered models. ANP acted via the cGMP-PKG pathway to directly inhibit TRPC6 activity, which in turn suppressed prohypertrophic signaling. Cardiac hypertrophy was significantly attenuated by the selective TRPC inhibitor BTP2 in GC-A KO mice, which were hypersensitive to hypertrophic signaling caused by overexpression of TRPC6. Likewise, BTP-2 significantly inhibited the cardiac hypertrophy induced by chronic Ang II infusion. Our study thus demonstrates that inhibition of TRPC6 activity mediates the antihypertrophic effects of ANP/BNP, and suggests that inhibition of TRPC6 could be an effective therapeutic strategy for preventing pathological cardiac hypertrophy and remodeling.

It was recently reported that RGS4 mediates the antihypertrophic effects of GC-A-catalyzed signaling in the heart.20 RGS2 also reportedly mediates the antihypertrophic effects of inhibiting phosphodiesterase 5, which enhances activity in the NO-cGMP pathway.41 It is thus suggested that RGS proteins mediate the antihypertrophic effects exerted by cGMP. In our study, we confirmed that RGS2 and 4 are significantly involved in the ANP-induced inhibition of agonist-induced Ca2+ influx (Figure 5I). PKG-catalyzed phosphorylation of TRPC6 on Thr69 is significantly involved in ANP-induced inhibition of receptor-mediated TRPC6 activity, despite the expression of RGS2 and RGS4 mRNA in HEK293 cells, however (Figure 2I, 2K and 2N; Online Figure I, D).
Agonist-induced activation of the TRPC6 T69A mutant in the presence of ANP was around 80% to 90% of that seen with WT TRPC6 in the absence of ANP. Furthermore, even when we knocked down both RGS2 and RGS4, the significant inhibitory effect of ANP on agonist-induced Ca\(^{2+}\)/H\(^{1}\) influx was preserved in cardiac myocytes (Figure 5G through 5I; Online Figure IV, A and B). Thus, RGS-independent mechanisms also contribute to the antihypertrophic effects of cGMP-dependent signaling. This is not surprising, as multiple mechanisms would be expected to participate in the antihypertrophic effects exerted via the ANP/BNP-GC-A-cGMP-PKG pathway. In that regard, PKG also reportedly inhibits LTCC activity and calcineurin-NFAT signaling.\(^{18}\) Although under our experimental conditions treatment with the LTCC blocker nifedipine did not significantly attenuate the inhibitory effect of ANP on agonist-induced Ca\(^{2+}\) influx into cardiac myocytes (Figure 5G through 5I; Online Figure IV, A and B). Thus, RGS-independent mechanisms also contribute to the antihypertrophic effects of ANP. Indeed, activation of TRPC3/6 has been shown to lead to LTCC activation,\(^{24}\) and ANP may inhibit that activation, thereby inhibiting calcineurin-NFAT signaling. It therefore seems likely that ANP/BNP-GC-A-cGMP-PKG signaling inhibits prohypertrophic signaling pathways at multiple steps. ANP and BNP are already administered clinically to patients with acute heart failure.\(^{46,47}\) But because, currently, these drugs are only administered intravenously, they are not available for use in the treatment of chronic pathological conditions such as cardiac hypertrophy and chronic heart failure.

Development of nonpeptide GC-A agonists that can be administered orally may lead to the development of new therapeutic agents for preventing pathological cardiac hypertrophy and remodeling. It has been shown in separate studies that GC-A is desensitized in failing human hearts\(^{48}\) and that Ang II and ET-1 act to desensitize GC-A.\(^{49}\) This makes it unlikely that under pathological conditions, endogenous cardiac ANP/BNP-GC-A-cGMP signaling would be sufficient to block the pathological signaling activity. Such disruption of the balance between anti- and prohypertrophic signaling would lead to further activation of TRPC3/6-dependent prohypertrophic signaling, thereby promoting the pathological cardiac remodeling. Thus inhibition of TRPC3/6 channel activity could be an effective therapeutic strategy for preventing cardiac remodeling under these pathological conditions. Indeed, our finding that BTP2 attenuated cardiac hypertrophy in GC-A KO and Ang II–infused mice may support this notion. In that regard, Pyr3 is a pyrazole compound recently identified as a specific inhibitor for TRPC3 that blocks Ca\(^{2+}\)/H\(^{1}\) influx carried by TRPC3/6 heteromeric complexes and by TRPC3 homomeric complexes. Moreover, Pyr3 also inhibited Ang II–induced hypertrophic responses in cultured cardiac myocytes more
potently than BTP2. In our study, knocking down either TRPC3 or -6 in cardiac myocytes significantly inhibited ET-1- and Ang II–induced increases in Ca^{2+} oscillation to levels comparable to those seen when both TRPC3 and -6 were knocked down simultaneously (Online Figure II, A through J). This raises the possibility that a TRPC3/6 heteromeric complex plays a key role in mediating agonist-induced prohypertrophic signaling in cardiac myocytes. Development of highly specific TRPC6 inhibitors could lead to the development of more potent and safer agents with which to prevent pathological cardiac remodeling and heart failure.

Acknowledgments
We thank Yukari Kubo for excellent secretarial work. We also thank E. N. Olson for providing us TRPC6 Tg mice.

Sources of Funding
This research was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (to K.K., H. Kinoshita and K.N.); a grant from the Japanese Ministry of Health, Labour and Welfare (to K.N.); grants from the Mitsubishi Foundation, the Mochida Memorial Foundation for Medical and Pharmaceutical Research, the Uehara Memorial Foundation, the Japan Heart Foundation/Novartis Grant for Research Award on Molecular and Cellular Cardiology, the Japan Foundation for Applied Enzymology, the Mitsubishi Pharma Research Foundation, the Astellas Foundation for Research on Metabolic Disorders, the Kaneue Foundation for the Promotion of Medical Science, the Ichiro Kanehara Foundation, the Suzuken Memorial Foundation, the Vehicle Racing Commemorative Foundation, the Japan Research Promotion Society for Cardiovascular diseases, the Takeda Medical Research Foundation, the Hoh-ansha Foundation (to K.K.), and the Kimura Memorial Heart Foundation (to H. Kinoshita).

Disclosures
None.

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**Novelty and Significance**

**What Is Known?**

- Under pathological conditions, the ventricular expression of 2 peptide mediators, atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), is increased in the heart. These peptides act as both endocrine and local antihypertrophic factors. However, the molecular mechanisms by which ANP/BNP inhibit cardiac hypertrophy remain unclear.

- Transient receptor potential subfamily C (TRPC)3 and -6 form homo- and heteromultimeric cation channels that are activated directly by diacylglycerol following receptor activation and reportedly serve as positive upstream regulators of the pathological calcineurin-NFAT signaling pathway in cardiac myocytes.

**What New Information Does This Article Contribute?**

- We demonstrate that ANP/BNP acts via the guanylyl cyclase (GC)-A-cGMP-protein kinase (PKG) pathway to inhibit TRPC6 channel activity, which in turn suppresses the subsequent activation of the prohypertrophic calcineurin-nuclear factor of activated T cells (NFAT) signaling pathway.

- The present study suggests that inhibition of TRPC6 could be an effective therapeutic strategy for preventing pathological cardiac hypertrophy and remodeling.

Characterization of the crosstalk among the cardiac signaling pathways that promote or antagonize cardiac hypertrophy should lead to a better understanding of molecular processes underlying the development of heart failure and ultimately to the discovery of novel therapeutic approaches for preventing pathological cardiac remodeling and heart failure. The cardiac hormones ANP and BNP reportedly exert antihypertrophic effects on the heart via their common receptor, GC-A, which catalyzes the synthesis of cGMP, leading to activation of PKG. Details of molecular mechanisms via which ANP/BNP-GC-A signaling inhibit cardiac hypertrophy are not well understood. The present study demonstrates that ANP/BNP-GC-A-cGMP-PKG signaling pathway inhibits TRPC6 activity by phosphorylation of threonine 69, which in turn suppresses prohypertrophic calcineurin-NFAT signaling. In mice lacking GC-A, BTP2, a selective TRPC channel blocker, significantly attenuated the cardiac hypertrophy otherwise seen. Conversely, overexpression of TRPC6 in mice lacking GC-A exacerbated cardiac hypertrophy. BTP2 also significantly inhibited angiotensin II-induced cardiac hypertrophy in mice. The present study reports the novel finding that inhibition of TRPC6 contributes to the antihypertrophic effects exerted by ANP/BNP-GC-A-cGMP-PKG signaling. These results suggest that inhibition of TRPC6 could be an effective therapeutic strategy for preventing pathological cardiac hypertrophy.
Inhibition of TRPC6 Channel Activity Contributes to the Antihypertrophic Effects of Natriuretic Peptides-Guanylyl Cyclase-A Signaling in the Heart

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Circ Res. published online May 6, 2010;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2010/05/06/CIRCRESAHA.109.208314.citation

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Supplemental Detailed Methods

Plasmid construction
RCAN1-luciferase (RCAN1-luc), in which RCAN1 intron 3 containing 15 NFAT sites was inserted upstream of the luciferase gene, was kindly provided from B.A. Rothermel (University of Texas, Southwestern Medical Center)\(^1\). Expression vectors encoding wild-type and mutant (T69A) mouse TRPC6 were described previously\(^2\).

Cell Culture
Primary neonatal rat ventricular myocytes were isolated and grown as described previously\(^3\). Twenty-four hours after plating, the myocytes were transfected for 12 h with 200 ng of reporter plasmid and 200 ng of expression vectors using Fugene 6 (Roche). Renilla luciferase driven by the TK promoter was included in all transfections as an internal control. After transfection, the serum was removed from the growth medium, and after 24 h of serum deprivation, endothelin-1 (ET-1) (10 nmol/L) (Peptide Institute, Inc.), angiotensin II (AngII) (100 nmol/L) (Peptide Institute, Inc.), ANP (100 nmol/L) (Peptide Institute, Inc.), 8Br-cGMP (100 μmol/L) (Calbiochem), BTP2 (3 μmol/L) (Calbiochem) or vehicle was added, and the cells were maintained for an additional 48 h.

Human embryonic kidney 293 (HEK293) cells were maintained and transfected with expression vectors as described previously\(^2\).

For knockdown of rat RGS2 and 4 proteins, cells were transfected with Stealth siRNA oligo (Invitrogen) (100 nmol/L each) for RGS2 (sequence; AGA AAU AGC UCA AAC GGG UCU UCC A) and RGS4 (sequence; UUU GAA AGC UGC CAG UCC ACA UUC A) or control scrambled siRNA using lipofectamine 2000 for 72 h. For knockdown of rat TRPC3 and TRPC6, cells were transfected with Stealth siRNA oligo (Invitrogen) (100 nmol/L each) for TRPC3 and TRPC6 or control scrambled siRNA using lipofectamine 2000 for 72 h as previously described\(^4\).

Luciferase assay
Cells were harvested, and luciferase activities were measured according to the manufacture’s protocol (Promega). All assays were performed at least twice in triplicate.

Measurement of intracellular Ca\(^{2+}\) concentrations
The intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in cardiomyocytes or HEK293 cells was determined as described previously\(^4,5\). Briefly, HEK293 cells (5 x 10\(^5\) cells) were transfected for 48 hours with 2.5 μg of vector (pCI-neo), wild-type TRPC6 (WT) or TRPC6 mutant (T69A), and 0.5 μg of GFP (pEGFP-F) using Fugene 6 (6 μl). Cardiomyocytes (1 x 10\(^6\) cells) plated on gelatin-coated glass-bottom 35-mm dishes were loaded with 1 μM fura-2/AM for 30 min at 37°C. During the incubation with fura-2, the cells were also pretreated with KT5823 (200 nM), after which they were
washed twice with HEPES-buffered saline, with or without KT5823 (200 nM). Bars indicate the times during which agents were applied. The transient increase in Ca seen in the Ca-free condition at the time agents was applied shows Ca release from the intracellular Ca store. The cells were imaged by alternate excitation at 340 nm and 380 nm every 5 s, and the intensity of the fluorescence emission (510 nm) was measured using a video image analysis system (Aquacosmos, Hamamatsu Photonics).

**Patch clamp studies**

The details of the patch clamp recording and data analysis were essentially the same as described previously. In brief, patch electrodes with a resistance of 2.5-4 MΩ (when filled with internal solution) were made from 1.5-mm borosilicate glass capillaries using an automated electrode puller (Sutter Instruments) and heat polished. Voltage generation and current signal acquisition were accomplished using a high-impedance low-noise patch clamp amplifier (EPC9; HEKA Electronics, Germany) in conjunction with an A/D, D/A converter (Digidata 1200; Axon Instruments). Sampled data were low-pass filtered at 1 kHz, digitized at 5 kHz and analyzed using Clampfit v. 9.2 (Axon Instruments). Longer time-frame recordings (e.g., the whole-cell current traces in Figure 3) were made using PowerLab/400 (AD Instruments, Australia; sampling rate, 100 Hz), after which the data were evaluated using the accessory software Chart v3.6. The magnitudes of noisy currents were defined as their 5-10 s averages over the time period of interest.

**Animal experiments**

The animal care and all experimental protocols were reviewed and approved by the Animal Research Committee at Kyoto University Graduate School of Medicine. Beginning at 12 weeks of age, GC-A KO mice (C57BL/6 background) were left untreated (control) or were treated for 4 weeks with BTP2 (20 mg/kg/day P.O.) or nitrendipine (40 mg/kg/day P.O.). BTP2 was dissolved in methylcellulose (Shin-Etsu Chemical) to a concentration of 3.0 mg/ml, and was given daily via gastric gavage adjusted to the individual body weight of each mouse. The same amount of 0.5% methylcellulose was given to the other treatment groups in the same manner. Nitrendipine was given as described previously. Transgenic mice expressing rat TRPC6 driven by the cardiac-specific α-MHC promoter (C57BL/6 background) were kindly provided by E. N. Olson (University of Texas, Southwestern Medical Center). Mice lacking GC-A (GC-A KO) were kindly provided by D. L. Garbers (University of Texas, Southwestern Medical Center).

Wild-type (WT), GC-A−/−, TRPC6 Tg and TRPC6 Tg; GC-A−/− mice were generated by crossing GC-A+/− mice and TRPC6 Tg; GC-A−/− mice and compared among the littermates.

For chronic AngII infusion, AngII (Peptide Institute, Inc.) was dissolved in 0.01mol/L acetic acid and subcutaneously infused at a rate of 0.6 mg/kg/day for 2 weeks using an osmotic minipump (Alzet model 2002; Alza Corp.) implanted in each mouse (C57BL/6). As a control, 0.01mol/L acetic acid alone was similarly infused for 2 weeks. One week after implantation, blood pressure was measured in conscious mice.
Noninvasive Blood Pressure Measurements
Systolic blood pressure (SBP) were measured in conscious mice using the tail-cuff method (Muromachi Kikai Co., Ltd) as described previously.\textsuperscript{10}

Echocardiographic analysis
Echocardiography was carried out as described previously\textsuperscript{11,12} using a Toshiba Power Vision 8000 echocardiography system equipped with a 12-MHz imaging transducer.

Histological Examination
Hearts were fixed in 10% formalin and prepared for histological analysis as described previously.\textsuperscript{10}

Quantitative RT-PCR analysis
Using 50-ng samples of total RNA prepared from ventricles, relative levels of mouse ANP, BNP, RCAN1, \(\beta\)MHC, \(\alpha\)MHC, skeletal \(\alpha\)-actin, cardiac \(\alpha\)-actin, TRPC3, TRPC6 and GAPDH mRNA were determined by quantitative real-time PCR according to the manufacture’s protocol (Applied Biosystems, Zaventam, Belgium), as previously described.\textsuperscript{12} The primers and a probe sets for ANP, BNP, RCAN, \(\beta\)MHC, \(\alpha\)MHC, skeletal \(\alpha\)-actin, cardiac \(\alpha\)-actin, TRPC3, TRPC6 and GAPDH were purchased from Applied Biosystems. In particular, to assess levels of TRPC3 and TRPC6 mRNA in GC-A KO ventricular myocardium, we used three different sets of primers and a probe, and confirmed that all three sets yielded similar results.

Using 20-ng samples of total RNA prepared from rat neonatal ventricular myocytes, relative levels of rat type1a angiotensin II receptor (AT1R), guanine nucleotide binding protein alpha q subunit (Gaq), phospholipase C-\(\beta\)3 (PLC\(\beta\)), TRPC3, TRPC6 and 18S mRNA were determined by quantitative real-time PCR carried out according to the manufacture’s protocol (Applied Biosystems, Zaventam, Belgium). The primers and a probe sets for AT1R, Gaq, PLC\(\beta\), TRPC3, TRPC6 and 18S were purchased from Applied Biosystems.

Western blot analysis
Whole cell lysates were prepared, after which Western blotting for Thr69-phosphorylated TRPC6 and total TRPC6 was carried out using anti- phospho-Thr69 TRPC6 antibody and anti-TRPC6 antibody (Alomone labs), as described previously.\textsuperscript{13} Anti-phospho-Thr69 TRPC6 antibody was generated using the peptide CHRRQ(P)TILREK corresponding to TRPC6\textsuperscript{65-74}. We confirmed the specificity of the anti-phospho-Thr69 TRPC6 antibody using whole cell lysates prepared from HEK293 cells expressing wild-type or mutant (T69A) TRPC6 incubated with or without 8br-cGMP (data not shown).
Whole cell lysates prepared from rat neonatal ventricular myocytes or ventricular myocardium were immunoblotted for RGS4 (Santa Cruz), Gaq/11 (Calbiochem), Gβ (Santa Cruz), PLCβ3 (Santa Cruz), TRPC3 and TRPC6 (Alamone Labs) as described previously.  

**RT-PCR analysis for RGS2 and 4 mRNA**

Single-stranded cDNA was synthesized using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems) with 2 µg of total RNA prepared from HEK293 cells or human left ventricles (purchased from Applied Biosystems) and the following primers: RGS2 sense, 5'-GGG TGT TCA GGA AAC ATC AC-3’; RGS2 antisense, 5’-GTA GCT GCC TTC AAG ACA GA-3’; RGS4 sence, 5’-GAT GGG AAA GAC CCT AGG TG-3’; RGS4 antisense, 5’-GTA ACT TAC GGC AGG TGT TG-3’. The PCR products were analyzed on a 1.5% agarose gel.

**AT1R binding assay**

After various treatments for 24 h, cardiac myocytes were rinsed with 10 ml of ice-cold phosphate-buffered saline and mechanically detached in 1 ml of ice-cold lysis buffer containing 10 mM Tris (pH 7.4), 5 mM EDTA, 5 mM EGTA, 1 µg/ml benzamidine, 10 µg/ml soybean trypsin inhibitor (type II-S) and 5 µg/ml leupeptin. The cell lysate was centrifuged at 45,000 g for 10 min at 4°C. The pellet containing the crude membrane fraction was resuspended in 1 ml of ice-cold lysis buffer using a Potter type homogenizer, after which the buffer was frozen and stored at -80°C until use. After measuring the concentration of membrane protein in the buffer, aliquots containing 20 µg of protein were used in binding assays. The membrane samples were incubated with 0.1 nM ¹²⁵I-Ang II (Amersham) in 75 mM Tris (pH 7.4), 12.5 mM MgCl₂, 2 mM EDTA and increasing concentrations of unlabeled Ang II (0 to 14 nM) for 1 h at 25°C. Nonspecific binding was determined in the presence of 1 µM unlabeled Ang II. The reaction mixture was filtered over Whatman GF/C filters, which were then washed with ice-cold buffer containing 25 mM Tris (pH 7.4) and 1 mM MgCl₂. The ¹²⁵I-Ang II bound to the filters was measured in a gamma counter, after which the values of Kd and Bmax were calculated using Prism software (GraphPad Software, San Diego, CA).

**Statistical Analysis**

Data are presented as means ± standard errors of the means (SEM). Unpaired t tests were used for comparisons between two groups, and ANOVA with post hoc Fisher tests was used for comparisons among groups. Values of P<0.05 were considered significant.
Supplemental Figure IV

A

0 mM Ca$^{2+}$ 2 mM Ca$^{2+}$

ET-1

ANP

colorbar: control siRNA

Ratio (F340 / F380)

0 5 10 15

Time (min)

(-) ANP (+) ANP

B

0 mM Ca$^{2+}$ 2 mM Ca$^{2+}$

Ang II

ANP

colorbar: control siRNA

Ratio (F340 / F380)

0 5 10 15

Time (min)

(-) ANP (+) ANP

C

Relative AT1R mRNA level

siRNA: cont RGS4 2/4

Relative Goq mRNA level

siRNA: cont RGS4 2/4

Relative PLCβ mRNA level

siRNA: cont RGS4 2/4

Relative TRPC3 mRNA level

siRNA: cont RGS4 2/4

Relative TRPC6 mRNA level

siRNA: cont RGS4 2/4

D

diagram: cont RGS4 2/4

RGS4

Gαq/11

Gβ

PLCβ3

TRPC3

TRPC6

E

AT1R binding

(fmol/mg protein)

siRNA: cont RGS4 2/4
Supplemental Figure I.  Effects of BTP2 on ET-1-induced hypertrophic responses in cultured ventricular myocytes.  (A) Real-time RT-PCR analysis of the relative expression of TRPC6 mRNA in cultured ventricular myocytes treated with or without 10 nmol/L ET-1 and/or 10 μmol/L BTP2. Relative mRNA levels in control myocytes (without ET-1 or BTP2) were assigned a value of 1.0. *p<0.05 vs. control.  #p<0.05 vs. myocytes treated with ET-1 alone.  (B) Representative fluorescence micrographs of cultured ventricular myocytes maintained in the absence or presence of 10 nmol/L ET-1 and/or 3 μmol/L BTP2 (400x).  Green: anti-α-sarcomeric actinin.  Bars show 50 μm.  (C) Cell surface area of cultured ventricular myocytes treated with or without 10 nmol/L ET-1 and/or 10 μmol/L BTP2.  Values are means±SEM.  *p<0.05 vs. control.  #p<0.05 vs. myocytes treated with ET-1 alone.  n=100 each.  (D) Representative levels of RGS2 (top panel) and RGS4 (bottom panel) mRNA in HEK293 cells and human left ventricular myocardium (LV), analyzed by RT-PCR.  RT(-): RT minus control.  (E) Representative I TRPC6 elicited by GTPγS at a holding potential of -60 mV.  GTPγS (100 μmol/L) was added into the patch pipette.  100nmol/L ANP was added.  At the end of the trace, external Na+ was quickly substituted with a large impermeant cation, N-methyl, D-glucamine (NMDG), to determine the baseline current.  (F) Summary for the inhibition of I TRPC6 by ANP (100 nmol/L) under the indicated conditions.  The rightmost bar shows the inhibitory effects of ANP on the TRPC6 T69A mutant.  Each column represents the relative I TRPC6 amplitude 10 min after initiation of ANP treatment.  Note that there is a progressive decline in I TRPC6, even under control conditions.  n=6-9.  DT3: a selective PKGIα inhibitor (1 μmol/L).  Values are shown as means±SEM.  *P<0.01.  #p<0.05.

Supplemental Figure II.  Effect of TRPC3/6 knockdown on agonist-evoked Ca2+ influx into cultured ventricular myocytes.  (A, B, C, D, F, G, H and I) Representative traces showing ET-1- (A, B, C and D) and AngII-induced (F, G, H and I) increases in Ca2+ oscillation in cultured ventricular myocytes transfected with control siRNA (A and F), TRPC3 siRNA (B and G), TRPC6 siRNA (C and H) or a combination of TRPC3 and TRPC6 siRNAs (D and I).  (E and J) Effects of knocking down TRPC3, TRP6 or both TRPC3 and 6 on the frequency of Ca2+ oscillation in cultured cardiac myocytes treated with ET-1 (E) or AngII (J).  *p<0.05 vs. control siRNA.

Supplemental Figure III.  Effect of TRPC3/6 knockdown on agonist-evoked Ca2+ influx into cultured ventricular myocytes.  (A-D) Representative traces showing ET-1- (A and B) and AngII-evoked (C and D) Ca2+ influx into cultured ventricular myocytes transfected with control siRNA (A and C) or TRPC3 and TRPC6 siRNAs (B and D).  In all these experiments, 10 nmol/L ET-1, 100 nmol/L AngII, 100 nmol/L ANP and 2 mmol/L external Ca2+ were used, unless indicated otherwise.
Supplemental Figure IV. Effect of RGS2/4 knockdown on agonist-evoked Ca²⁺ influx into cultured ventricular myocytes.  (A-B) Representative traces showing ET-1- (A) and AngII-evoked (B) Ca²⁺ influx into cultured ventricular myocytes transfected with control siRNA (left panel) or RGS2 and RGS4 siRNAs (right panel).  In all these experiments, 10 nmol/L ET-1, 100 nmol/L AngII and 100 nmol/L ANP were used.  (C) Graphs show the relative levels of AT1R, Gαq, PLCβ, TRPC3 and TRPC6 mRNAs in cultured ventricular myocytes transfected with control siRNA or with RGS2 and RGS4 siRNAs.  In each case, no significant difference was observed between the two groups.  n=4 each.  (D) Western blotting of RGS4, Gαq/11, Gβ, PLCβ3, TRPC3 and TRPC6 in cultured ventricular myocytes transfected with control siRNA or a combination of RGS2 and RGS4 siRNAs.  With the exception of RGS4, whose protein levels showed a 50% reduction in myocytes transfected with a combination of RGS2 and 4 siRNAs, there was no statistical difference in the expression levels of any of the tested proteins between myocytes transfected with control siRNA and those transfected with RGS2/4 siRNAs (n=3 each).  (E) AT1R binding assay showing no significant difference between myocytes transfected with control siRNA and those transfected with RGS2/4 siRNAs.

Supplemental Figure V. Nitrendipine, a selective LTCC blocker, did not reduce cardiac hypertrophy in GC-A KO mice.  (A) Representative Western blots showing TRPC6 and 3 expression in ventricular myocardium from 8-week-old wild-type (WT) and GC-A KO (KO) mice.  (B) Graphs show the relative levels of TRPC6 and 3 protein in ventricular myocardium from 8-week-old wild-type and GC-A KO mice.  *p<0.05 vs. wild-type (n=4 each).  Relative protein levels in control wild-type mice were assigned a value of 1.0.  (C-E) Blood pressures (C), heart weight to body weight ratio (HW/BW; D), and posterior wall thickness (PWTh; E) assessed by echocardiography in 16 weeks old WT and GCA-KO mice treated for 4 weeks with or without nitrendipine (Nit).  N=3 for WT with or without nitrendipine, n=5 for GCA-KO with or without nitrendipine.  Two-way ANOVA revealed that in GCA-KO mice blood pressure, HW/BW and PWTh were all higher than in WT mice, and that nitrendipine modestly reduced blood pressure, but had no effect on HW/BW or PWTh.  No mouse status/medication status interaction was observed (in C, P<0.0001 between WT and GCA-KO, p=0.04 between without and with nitrendipine, interaction p=718; in D, p<0.0001 between WT and GCA-KO, p=0.789 between without and with nitrendipine, interaction p=0.822; in E, p<0.001 between WT and GCA-KO, p=0.821 between without and with nitrendipine, interaction p=0.434).  (F and G) Real-time RT-PCR analysis of relative expression of TRPC6 (F) and TRPC3 (G) mRNA in ventricular myocardium from 10-week-old mice treated for 14 days with or without AngII (0.6 mg/kg/day) and/or BTP2 (20 mg/g/day).  Relative mRNA levels in control mice without AngII and BTP2 were assigned a value of 1.0.  In all graphs, values are shown as means±SEM.
**Supplemental Table I. Hemodynamic parameters in WT and GCA-KO mice treated with or without nitrendipine.**

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>GCA-KO</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+ Nit</td>
<td>-</td>
<td>+ Nit</td>
</tr>
<tr>
<td>Blood Pressure (mmHg)</td>
<td>94.5±0.5</td>
<td>89.7±3.0</td>
<td>119.8±2.1</td>
<td>116.2±1.5</td>
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<tr>
<td>Heart Rate (/min)</td>
<td>583.5±21.5</td>
<td>643.0±18.6</td>
<td>541.0±13.3</td>
<td>628.0±40.5</td>
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<tr>
<td>Echocardiographic data</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>LVDd (mm)</td>
<td>3.27±0.07</td>
<td>3.13±0.07</td>
<td>3.08±0.10</td>
<td>2.98±0.17</td>
</tr>
<tr>
<td>LVDs (mm)</td>
<td>1.83±0.26</td>
<td>1.80±0.12</td>
<td>1.80±0.08</td>
<td>1.54±0.12</td>
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<td>PWTh (mm)</td>
<td>1.03±0.02</td>
<td>1.02±0.04</td>
<td>1.31±0.02</td>
<td>1.34±0.02</td>
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<tr>
<td>FS (%)</td>
<td>44.0±6.93</td>
<td>43.3±2.85</td>
<td>40.6±2.36</td>
<td>48.0±2.81</td>
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<tr>
<td>EF (%)</td>
<td>80.7±6.74</td>
<td>82.0±2.52</td>
<td>78.2±2.73</td>
<td>86.0±2.10</td>
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<td>Body Weight (g)</td>
<td>32.0±0.4</td>
<td>32.3±1.4</td>
<td>33.5±1.1</td>
<td>33.6±1.7</td>
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<tr>
<td>Heart Weight (g)</td>
<td>0.149±0.008</td>
<td>0.145±0.008</td>
<td>0.251±0.011</td>
<td>0.249±0.007</td>
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<td>Lung Weight (g)</td>
<td>0.148±0.008</td>
<td>0.158±0.008</td>
<td>0.207±0.007</td>
<td>0.197±0.010</td>
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<tr>
<td>HW/BW (mg/g)</td>
<td>4.66±0.24</td>
<td>4.50±0.09</td>
<td>7.49±0.30</td>
<td>7.47±0.36</td>
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<tr>
<td>LW/BW (mg/g)</td>
<td>4.63±0.18</td>
<td>4.90±0.04</td>
<td>6.19±0.26</td>
<td>5.91±0.39</td>
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</table>

Values are means ± SEM. Numbers of mice tested are 3 for WT, with or without nitrendipine, and 5 for GCA-KO, with or without nitrendipine. Nit, nitrendipine; LVDd, left ventricular diastolic dimension; LVDs, left ventricular systolic dimension; PWTh, posterior wall thickness; FS, fractional shortening; EF, ejection fraction; HW/BW, heart weight-to-body weight ratio; LW/BW, lung weight-to-body weight ratio.
### Supplemental Table II. Hemodynamic parameters in mice treated with or without angiotensin II and/or BTP2

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<tr>
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<th>Angiotensin II -</th>
<th>+ BTP2</th>
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<tr>
<td>Blood Pressure (mmHg)</td>
<td>106.4±0.7</td>
<td>115.9±4.9</td>
<td>119.6±4.2</td>
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<td>Heart Rate (/min)</td>
<td>720.8±6.6</td>
<td>712.4±10.5</td>
<td>643.4±35.0</td>
<td>714.1±7.7</td>
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**Echocardiographic data**

<table>
<thead>
<tr>
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<th>Angiotensin II -</th>
<th>+ BTP2</th>
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</thead>
<tbody>
<tr>
<td>LVDd (mm)</td>
<td>2.52±0.06</td>
<td>2.58±0.14</td>
<td>2.26±0.07</td>
<td>2.44±0.08</td>
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<tr>
<td>LVDs (mm)</td>
<td>1.20±0.08</td>
<td>1.14±0.11</td>
<td>1.10±0.09</td>
<td>1.09±0.07</td>
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<tr>
<td>PWTh (mm)</td>
<td>0.94±0.04</td>
<td>0.94±0.02</td>
<td>1.27±0.02</td>
<td>1.03±0.03</td>
</tr>
<tr>
<td>FS (%)</td>
<td>50.6±2.96</td>
<td>55.2±2.87</td>
<td>51.3±2.92</td>
<td>56.0±2.94</td>
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<td>EF (%)</td>
<td>87.6±2.11</td>
<td>88.6±0.81</td>
<td>87.4±2.09</td>
<td>90.7±1.54</td>
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<table>
<thead>
<tr>
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<th>Angiotensin II -</th>
<th>+ BTP2</th>
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</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td>28.8±0.7</td>
<td>24.8±0.5</td>
<td>27.9±1.0</td>
<td>25.1±0.7</td>
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<tr>
<td>Heart Weight (g)</td>
<td>0.151±0.006</td>
<td>0.123±0.009</td>
<td>0.168±0.010</td>
<td>0.130±0.005</td>
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<tr>
<td>Lung Weight (g)</td>
<td>0.148±0.005</td>
<td>0.127±0.006</td>
<td>0.155±0.008</td>
<td>0.137±0.012</td>
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<tr>
<td>HW/BW (mg/g)</td>
<td>5.25±0.21</td>
<td>4.97±0.33</td>
<td>6.02±0.22</td>
<td>5.16±0.12</td>
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<tr>
<td>LW/BW (mg/g)</td>
<td>5.14±0.14</td>
<td>5.14±0.18</td>
<td>5.56±0.16</td>
<td>5.44±0.10</td>
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</table>

Values are means ± SEM. Numbers of mice tested are 5 for mice without AngII and 7 for mice with AngII. LVDd, left ventricular diastolic dimension; LVDs, left ventricular systolic dimension; PWTh, posterior wall thickness; FS, fractional shortening; EF, ejection fraction; HW/BW, heart weight-to-body weight ratio; LW/BW, lung weight-to-body weight ratio. Heart rates and echocardiographic parameters are from 12-week-old WT and dnNRSF-Tg (Tg) mice treated with or without efionidipine (Efo) or nitrendipine (Nit).
Supplemental References


