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REVIEW

Regulation and significance of atrial and brain natriuretic peptides as cardiac hormones

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Abstract. Atrial and brain natriuretic peptides (ANP and BNP, respectively) are cardiac hormones. During cardiac development, their expression is a maker of cardiomyocyte differentiation and is under tight spatiotemporal regulation. After birth, however, their ventricular expression is only up-regulated in response to various cardiovascular diseases. As a result, analysis of ANP and BNP gene expression has led to discoveries of transcriptional regulators and signaling pathways involved in both cardiac differentiation and cardiac disease. Studies using genetically engineered mice have shed light on the molecular mechanisms regulating ANP and BNP gene expression, as well as the physiological and pathophysiological relevance of the cardiac natriuretic peptide system. In this review we will summarize what is currently known about their regulation and the significance of ANP and BNP as hormones derived from the heart.

Key words: Natriuretic peptide, Cardiovascular endocrinology

ATRIAL and brain natriuretic peptides (ANP and BNP, respectively) are polypeptide hormones comprising the cardiac-derived natriuretic peptide system [1, 2]. ANP is usually synthesized in the atria, while BNP is primarily synthesized in the ventricles. These two peptides are markers of cardiac differentiation, and their expression is under tight spatiotemporal regulation during cardiac development. Indeed, analysis of the ANP and BNP promoters and their activity has contributed much to our present understanding of the transcriptional regulation of cardiac development. After birth, ventricular expression of both ANP and BNP is upregulated in several pathological conditions of the heart, and their plasma concentrations are markedly elevated in patients with cardiac hypertrophy or congestive heart failure (CHF) [3]. In fact, measurements of plasma ANP and BNP levels are used clinically to assist in the diagnosis of CHF, assess prognosis, and determine therapeutic strategies [2]. It thus appears that the molecular pathways involved in the reactivation of ANP and BNP gene expression are closely linked to adaptive or maladaptive cardiac signaling pathways induced in response to pathological stress. Because of the importance of these two hormones to the physiology and pathophysiology of the heart, many aspects of the molecular mechanism controlling ANP and BNP gene expression during cardiac development and in disease have been studied.

Upon their release, ANP and BNP act at multiple sites to exert diuretic, natriuretic and vasorelaxant effects [4]. Moreover, recent evidence indicates that ANP and BNP also act as paracrine factors, exerting antihypertrophic and antifibrotic effects in the heart. They exert both their hormonal and paracrine effects through activation of their common receptor, guanylyl cyclase-A (GC-A; also known as natriuretic peptide receptor-A), which is expressed in a variety of tissues, including kidney, blood vessel, adrenal gland and heart, and is coupled to an increase in the intracellular concentration of cGMP [4]. The significance of ANP/BNP-GC-A signaling in various physiological and pathophysiological settings has been examined in a number of studies using mice lacking genes encoding components of this signaling pathway. Here we review what is currently known about the transcrip-

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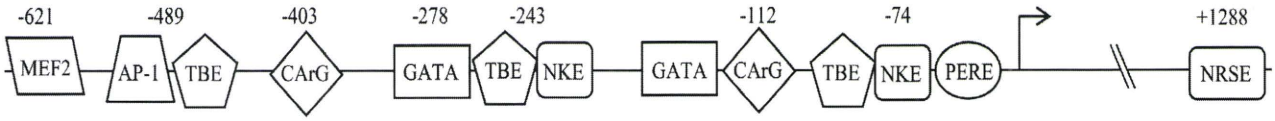

Fig. 1 Schematic representation of rat ANP promoter. Known cis-acting elements are shown.

tional regulation of ANP and BNP gene expression in the myocardium and the physiological and pathophysiological relevance of ANP and BNP as cardiac hormones.

A. Regulation of Natriuretic Peptides Gene Expression

I. Transcriptional regulation of ANP gene expression

Studies using transgenic mice carrying a 500-bp segment of the 5' flanking region (5'-FR) of the human ANP gene fused to a gene encoding SV40 large T antigen, a 2.4-kbp 5'-FR segment of human ANP gene fused to the chloramphenicol acetyltransferase gene or either a 638-bp or 3-kbp 5'-FR segment of the rat ANP gene fused to the luciferase gene have shown that these regions are sufficient to confer cardiac-restricted gene expression, with much higher expression in the atria than in the ventricles [5-7]. The ventricular activities of the 3-kbp, 2.4-kbp and 638-bp 5'-FR segments were down-regulated after birth, while the atrial activity remained high. These observations demonstrate that the proximal 5'-FR of the ANP gene is sufficient to recapitulate the spatial and temporal expression of the endogenous ANP gene and that the region contains sequences important for the regulation of ANP gene expression. Indeed, expression of a reporter gene driven by the proximal 5'-FR of the ANP gene in atrial or ventricular cardiac myocytes at different developmental stages showed that the region confers proper spatial and temporal activity to the ANP promoter [8, 9]. That said, there are some differences in the expression pattern between the proximal 5'-FR of the ANP gene and the intact endogenous ANP gene, which suggests the presence of regulatory elements outside the proximal 5'-FR [10]. It should be noted that in humans and mice, respectively, the ANP gene is located 8 kbp and 12 kbp downstream of the BNP gene on the same chromosome (human, chromosome 1; mouse, chromosome 4) [11, 12]. The proximal 5'-FR of the ANP gene contains two CArG boxes, two NKEs, three TBEs, two GATA sites, an A/T-rich element and a phenylephrine-responsive element (PERE), to which the transcriptional factors SRF, NKX2.5, Tbx5, GATA4/6, MEF2C and Zfp260 have been shown to bind (Fig. 1) [10, 13]. In addition, these elements have been shown to contribute singly or cooperatively to the basal and inducible activation of ANP promoter activity in cardiac cells [13-19]. NRSE, hypoxia-response element (HRE) and glucocorticoid responsive element (GRE), which are located outside the proximal promoter, also reportedly mediate inducible ANP gene transcription [20-23].

II. Transcriptional regulation of BNP gene expression

The 5'-FR of the BNP gene has also been studied so as to better understand the regulatory mechanisms governing the gene's cardiac-specific and inducible expression. A study using transgenic mice carrying a 5'-FR segment of the human BNP gene extending from -1818 to +100, or from -408 to +100, coupled to a luciferase gene (-1818hBNPluc and -400hBNPluc, respectively) showed that the proximal region of the human BNP promoter is sufficient to mediate ventricle-specific expression [24]. The luciferase activity of -1818hBNPluc was also higher in ventricular myocytes than in atrial myocytes [25]. BNP mRNA has a shorter half-life than ANP mRNA and has an AT-rich region in its 3'-UTR. This makes the gene unstable and implies post-transcriptional control of BNP gene expression [26-29]. In addition, deletion analysis showed that the region extending from -127 to -40 of the human BNP 5'-FR confers cardiac-specific expression [25]. This proximal region of the human BNP promoter contains potential GATA, M-CAT and AP-1/CRE-like elements, which are conserved among humans, rats and mice (Fig. 2) [25, 30, 31]. All of these elements are known to regulate cardiac-specific gene expression [31-38], and have been shown to mediate both basal and inducible BNP gene expression [31, 35, 38-43]. Other sites located in relatively distal regions of the human BNP 5'-FR, including NRSE (-552), SSREs (-652, -641 and 161), TRE (-1000) and NF-AT binding site (-927), have also been shown to par-

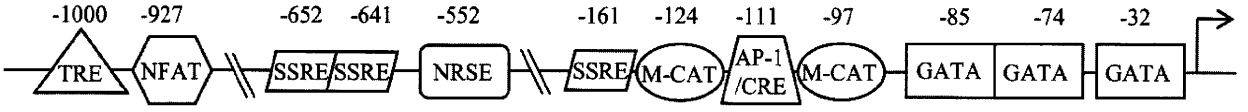

Fig. 2 Schematic representation of human BNP promoter. Known cis-acting elements are shown.

ticipate in the inducible activation of the human BNP promoter (Fig. 2) [44-47].

III. Transcription factor binding sites that regulate ANP and BNP promoter activity

GATA

The proximal human BNP promoter contains three potential sites for GATA binding (-85, -74 and -32) [12]. These sites are generally conserved among species, though the most proximal (-32) is a TATA-box in the mouse BNP promoter [31, 43], which is not seen in the human proximal BNP promoter. It is therefore likely that the -32 GATA site serves as a binding site for different transcription factors, including TATA-binding protein. Mutation of GATA sites in both the human and rat BNP promoter results in a marked reduction in BNP promoter activity in cultured cardiac myocytes [39, 42]. The transcription factor GATA4, which plays an important role in cardiac development [48, 49], can bind to the BNP GATA sites, thereby increasing promoter activity [31, 39, 50]. GATA sites are also present in the ANP proximal promoter, and GATA4 can activate ANP promoter activity [50]. Nonetheless, GATA4-null mice and mutant mice showing a 70% reduction of GATA4 express normal levels of ANP in the heart, suggesting GATA4 is dispensable for natriuretic peptide gene expression during normal cardiac development, though it is plausible that reductions in ANP reflecting the loss of GATA4 are masked by offsetting up-regulation induced by other factors, such as heart failure [48, 49]. Indeed, myocardial ANP gene expression was diminished in hearts with cardiac-restricted deletion of GATA4 accomplished using βMHC-CRE [51].

CArG box/Serum response element (SRE)

Two CArG boxes, also known as SREs, are present in the 5'-FR of the ANP gene, and were shown to mediate hypertrophic signaling [34]. The transcription factor serum response factor (SRF), which is known to play important roles in cardiovascular development and disease, binds to these sequences [52]. At least two signaling pathways are known to modulate SRF activity: one involving the phosphorylation of ternary complex factors in Ets domain family proteins and another controlled by Rho-family small GTPases and actin dynamics [53-56]. It was recently shown that stimulation of Rho- and actin dynamics-dependent signaling results in translocation of a novel SRF co-factor, myocardin-related transcription factor (MRTF)-A (also named as MAL or MKL1), from G-actin in the cytoplasm to the nucleus and in activation of SRF target genes [57, 58]. Although BNP gene expression is also reportedly under the control of SRF, a functional CArG box had not been identified in the proximal 5'-FR of the BNP gene [59]. Recently we have identified a conserved and functional SRF-binding site within the BNP promoter (unpublished observation, 2010).

T-box binding elements (TBE)

Three T-box binding elements (TBE) have been identified in the ANP promoter [60, 61], and the T-box transcription factors Tbx5, Tbx2, Tbx3 and Tbx20 are all thought to be involved in regulating ANP gene transcription [10]. Tbx5, whose mutation causes Holt-Oram syndrome, binds to TBE and interacts with NKX2.5 to synergistically activate ANP gene transcription [60, 61]. Tbx5 haploinsufficient mice showed marked reductions in ANP expression, while Tbx5-null mice expressed no ANP.

Myocyte enhancer factor (MEF) 2

MEF2A-D are MADS box transcription factors that bind to A/T-rich sequences in various muscle-specific genes. Mice lacking MEF2C do not express ANP and show impaired cardiac morphogenesis [62]. MEF2C reportedly binds to the low affinity A/T-rich sequence in the ANP promoter and then stimulates ANP gene transcription [18].

NK-homeobox binding element (NKE)

A homeodomain containing the transcription factor NKx2.5 plays a critical role in cardiac development and cardiac disease [32]. Two such NKEs have been identified in the ANP promoter.

M-CAT

The proximal human BNP promoter contains two M-CAT elements, of which the more proximal is conserved in mouse and rat. This proximal M-CAT site appears to be involved in regulating basal BNP promoter activity in both humans and rats, mediating β-adrenergic-induced human BNP promoter activity and phenylephrine-induced activation of rat BNP promoter activity [40, 42]. Although it is not yet certain which transcription factor binds to the proximal M-CAT site in the BNP promoter, it is likely that one or more TEF family transcription factors are involved, as is the case with the β-MHC and skeletal α-actin promoters [37]. Consistent with that idea, disruption of TEF-1 using a retroviral gene trap leads to the development of cardiac defects [63].

AP-1/CRE-like

The human BNP promoter contains multiple AP-1/CRE-like binding sites, of which the proximal -111AP-1/CRE-like binding site has been shown to play an important role in the basal regulation of both human and rat BNP promoter activity [38, 43]. AP-1 site is also identified in the ANP promoter, and a c-fos/c-jun complex was shown to bind to the element [64].

NF-AT

Molkentin *et al.* showed that the NF-AT family transcription factors act with GATA-4 to mediate phenylephrine-induced activation of the human BNP promoter. A functional NF-AT binding site is located at -972 in the human BNP promoter [46]. Mice in which both NF-ATC3 and -C4 were deleted die *in utero* due to abnormal cardiac development [65]. Recent studies have also shown that the TRPC1/3/6-calcineurin pathway is an upstream regulator of NF-AT-dependent transcription [66-69].

Neuron-restrictive silencer element (NRSE)

We have shown that the transcriptional repressor element NRSE, located at -552 in the human BNP promoter, represses basal BNP promoter activity and mediates the hypertrophic signaling evoked with extracellular matrix [70]. This NRSE is conserved in the rat and mouse BNP promoters. A transcriptional repressor, NRSF, binds to the element, thereby repressing promoter activity. Interestingly, NRSE is also located in the 3'UTR of the ANP promoter and is involved in basal and ET-1-inducible activation of the human ANP promoter [21]. That cardiac-restricted inactivation of NRSF through overexpression of a dominant-negative NRSF driven by the cardiac-specific α-MHC promoter leads to up-regulation of ANP and BNP gene expression in the ventricle, cardiomyopathy and sudden death confirms the importance of NRSF in the regulation of cardiac gene expression and cardiac function [22].

Shear stress-responsive element (SSRE)

Multiple SSREs are located in the human BNP promoter at -652, -533 and -162. Mechanical strain reportedly activates the BNP promoter activity via SSRE [71].

Thyroid hormone-responsive element (TRE)

Thyroid hormone (T3) and thyroid hormone receptor activate BNP gene transcription through TRE located at -1000 in the human BNP promoter. T3 and ET-1 act synergistically to stimulate human BNP promoter activity, and mutation of TRE reduces the response to both T3 and ET-1 [47].

IV. Regulation of ANP and BNP in cardiac myocytes

The expression and secretion of ANP and BNP is up-regulated in diseased hearts such as those showing cardiac hypertrophy or cardiomyopathy, and mechanical stress stimulates the synthesis and secretion of ANP and BNP in both atrial and ventricular cells [72-74]. In addition, neurohumoral factors, including ET-1 [75-78], thyroid hormone [79-81], α-adrenergic agonists [29, 82, 83], prostaglandins [84], glucocorticoids [81, 85] and angiotensin II [86], as well as various growth factors [87] and cytokines, including IL-1β [88, 89], LIF, CT-1 [90-92] and TNF-α [93], all stimulate ANP and BNP synthesis in cultured cardiac myocytes (see review in [72]). And multiple signaling pathways comprised of such MAPK family enzymes as ERK1/2, JNK, P38MAPK and ERK5, as well as CaMKII, PKCs, Jak-STATs, Rho-ROCK and calcineurin-NFATs, are reportedly involved in the up-regulation of ANP and/or BNP [43, 46, 78, 94-100]. Thus a broad spectrum of mediators and signaling pathways are thought to contribute to the increased synthesis and secretion of ANP and BNP observed under pathological conditions.

B. Physiological and Pathophysiological Significance of BNP, ANP and Signaling Through GC-A

The physiological functions of ANP and BNP and their receptor, GC-A, have been studied through their genetic ablation or by blocking GC-A. Genetic ablation of ANP leads to salt-sensitive hypertension in homozygous null mice [101]. In heterozygous and homozygous ANP-null mice maintained on low-salt diet, the natriuretic response to acute volume overload is diminished, as compared to wild type mice, suggesting ANP is required for the natriuretic response to volume expansion in animals on a low salt diet [102]. In rats, blockade of GC-A also results in a significant reduction in diuresis and natriuresis in response to acute volume overload [103]. Homozygous GC-A-deficient mice exhibit salt-insensitive hypertension and marked reductions in natriuresis and diuresis in response to acute volume overload [104-107]. All of these findings indicate ANP acts as a circulating hormone regulating systemic blood pressure and cardiorenal homeostasis through GC-A.

GC-A-null mice also show cardiac hypertrophy with extensive interstitial fibrosis [107, 108], and the cardiac hypertrophy observed in GC-A-null mice appears to be at least partially independent of the high blood pressure [109, 110]. Moreover, cardiac hypertrophy is observed in mice in which the CRE/loxP system was used to cardiac-specifically delete GC-A, despite preservation of all endocrine activity, including pressure- and volume-regulating effects [111]. These findings suggest ANP and BNP also act as local antihypertrophic regulators. Consistent with that idea, genetic ablation of angiotensin type IA receptor or blockade of angiotensin type I receptor significantly reduces cardiac hypertrophy and fibrosis in GC-A-null mice, suggesting the cardiac natriuretic peptide-GC-A system antagonizes the prohypertrophic signaling mediated by the angiotensin type I receptor [112]. Interestingly, there is a gender difference in the cardiac hypertrophic and fibrotic responses seen in GC-A-null mice, and androgen contributes to that gender difference in an angiotensin II type I receptor-dependent fashion [113].

In mice with smooth muscle cell (SMC)-specific deletion of GC-A accomplished using SM22-CRE, which reduced vascular GC-A gene expression by 80%, the vasodilatory effects of ANP on isolated vessels are abolished. These SMC GC-A knockout mice have normal arterial blood pressure; however, acute volume expansion, which normally causes release of ANP from the heart but does not affect blood pressure in control mice, evokes significant and rapid increases in blood pressure in SMC GC-A knockout mice. Thus SMC GC-A is apparently dispensable for chronic regulation of arterial blood pressure, but is critical for the acute regulation of the response to volume overload [114]. In addition, endothelial cell-specific deletion of GC-A accomplished using Tie2-CRE induces high blood pressure and cardiac hypertrophy, but the direct vasodilatory effect of ANP is preserved, suggesting endothelial GC-A also regulates vascular permeability and is critical for the regulation of the hypovolemic and hypotensive actions of ANP [115].

The role of GC-A in several pathological conditions has also been studied. In a mouse model of ischemia/reperfusion injury of the heart, both genetic loss of GC-A and its pharmacological blockade alleviate ischemia/reperfusion injury, suggesting GC-A plays a role in acute inflammatory responses in the heart [116]. Supporting that notion are the findings that BNP transgenic mice subjected to myocardial infarction show greater accumulation of neutrophils in the heart and a greater susceptibility to cardiac rupture than wild-type mice [117]. On the other hand, GC-A-null mice subjected to myocardial infarction show greater susceptibility to acute heart failure and exacerbation of chronic pathological cardiac remodeling, as compared to wild-type mice. These findings are indicative of the critical role played by GC-A in the regulation of volume during the acute phase after myocardial infarction, as well as the antihypertrophic and antifibrotic effects of GC-A during the chronic phase [118]. GC-A-null mice also show increased susceptibility to heart failure due to volume overload caused by aortocaval fictula, which further highlights the importance of GC-A for volume control under conditions of pathological volume overload [119]. Chronic hypoxia and aortic constriction worsen cardiac hypertrophy in GC-A-null and cardiac-specific GC-A knockout mice, respectively, which also reflects the antihypertrophic effect of GC-A signaling [111, 120]. Finally, BNP knockout mice do not exhibit hypertension or cardiac hypertrophy, but do develop cardiac fibrosis, suggesting BNP acts via GC-A to locally regulate cardiac remodeling [121].

C. Concluding Remarks and Perspective

Studies of ANP and BNP gene expression have led to discoveries of transcriptional regulators and signaling pathways involved in either cardiac differentiation or cardiac diseases. Still, much of molecular mediators via which the expression of ANP and BNP in myocardium is controlled remain to be characterized. It became apparent that the regulation of ANP and BNP gene expression is more complex than initially thought. More efforts to elucidate molecular mechanisms regulating ANP and BNP expression, in particular mechanisms regulating the perinatal suppression and the reactivation in diseased hearts of the expression may be necessary. Studies using genetically engineered mice have shed light on the physiological and pathophysiological relevance of the cardiac natriuretic peptide system and its downstream signaling. Continued efforts to identify molecular targets of natriuretic peptide system will further enhance our understanding of the role of natriuretic peptides in cardiovascular system and also of the molecular basis for the development and progression of cardiovascular diseases.

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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;106:1849-1860.)

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,[1,2] 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-

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Non-standard Abbreviations and Acronyms	
Ang II	angiotensin II
ANP	atrial natriuretic peptide
BNP	brain natriuretic peptide
GC-A	guanylyl cyclase-A
HW/BW	heart weight/body weight
KO	knockout
LTCC	L-type voltage-dependent Ca^{2+} channel
NFAT	nuclear factor of activated T cells
PKG	protein kinase G
RCAN	regulator of calcineurin
RGS	regulator of G-protein signaling
siRNA	small interfering RNA
TRPC	transient receptor potential subfamily C
WT	wild type

phy and heart failure, which could ultimately lead to the discovery of novel therapeutic targets for prevention of pathological cardiac remodeling.

The heart regulates cardiovascular homeostasis in part by secreting 2 peptide mediators, atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP).[3,4] 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 (PK)G.[5,6] Under pathological conditions in the heart, there is a significant increase in the ventricular expression of both ANP and BNP,[4,7,8] which then act as both endocrine and local antihypertrophic factors.[9] Indeed, ANP/BNP have been shown to exert antihypertrophic effects on cardiac myocytes in vitro and in vivo[10–14] by counteracting multiple prohypertrophic signaling pathways, including the MEK1–ERK1/2 (mitogen-associated/extracellular regulated kinase 1–extracellular regulated kinase 1/2) pathway, the Ca^{2+}/calmodulin-dependent kinase II pathway, the Akt pathway, and the calcineurin-nuclear factor of activated T cell (NFAT) pathway.[1,2,15–17] ANP/BNP reportedly antagonize prohypertrophic signaling by inhibiting receptor-mediated Ca^{2+} influx into cells through activation of regulator of G-protein signaling (RGS) proteins and inhibition of L-type voltage-dependent Ca^{2+} channels (LTCCs) and the Na^+/H^+ exchanger.[10,17–20] Still, much of the network of molecular mediators via which ANP/BNP inhibit cardiac hypertrophy remains to be characterized.

The serine-threonine phosphatase calcineurin functions as a Ca^{2+}-dependent regulator of cardiac hypertrophy and the fetal gene program.[21] 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.[21] Transient receptor potential subfamily C (TRPC)3 and -6 reportedly serve as positive upstream regulators of the calcineurin-NFAT signaling pathway.[22–24] TRPC3 and 6 form homo- and heteromultimeric cation channels that are activated directly by diacylglycerol[25,26] 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.[26] NFAT also activates TRPC6 gene transcription, thereby accelerating the calcineurin-NFAT prohypertrophic signaling loop.[23] 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.[27,28]

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 (RCAN)1-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).[29] Expression vectors encoding wild-type (WT) and mutant (T69A) mouse TRPC6 were described previously.[28]

Cell Culture

Primary neonatal rat ventricular myocytes were isolated and grown as described previously.[30]

Patch Clamp Studies

The details of the patch clamp recording and data analysis were essentially the same as described previously.[31]

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.[32]

Echocardiographic Analysis

Echocardiography was carried out as described previously[33,34] using a Toshiba Power Vision 8000 echocardiography system equipped with a 12-MHz imaging transducer.

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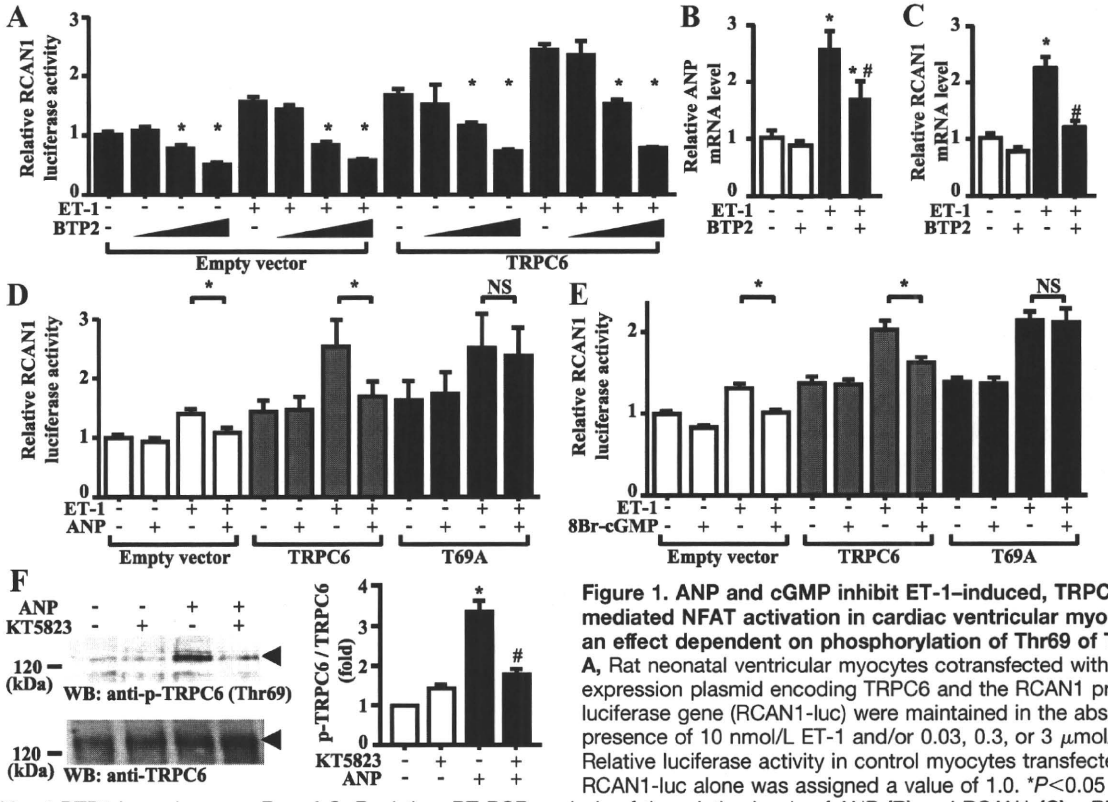

Figure 1. ANP and cGMP inhibit ET-1–induced, TRPC6-mediated NFAT activation in cardiac ventricular myocytes, an effect dependent on phosphorylation of Thr69 of TRPC6. **A,** Rat neonatal ventricular myocytes cotransfected with an expression plasmid encoding TRPC6 and the RCAN1 promoter-luciferase gene (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 cells treated without BTP2 in each group. **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 are shown at **left**. Graphs at the **right** show the relative levels of Thr69-phosphorylated TRPC6 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.

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.[22–24,35] Consistent with those reports, we found that in cultured neonatal rat ventricular myocytes, BTP2, a selective TRPC inhibitor,[36–40] 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). To determine whether ANP inhibits calcineurin-NFAT signaling through TRPC6 inhibition, we examined the effect of ANP on ET-1–induced, TRPC6-mediated activation of calcineurin-NFAT signaling in cultured ventricular myocytes transfected with a *RCAN1* promoter-reporter gene and/or an expression vector for TRPC6. Even without overexpression of TRPC6, ET-1 induced *RCAN1* promoter activity in ventricular myocytes, and this activity was significantly inhibited by ANP (Figure 1D). With overexpression of TRPC6, basal *RCAN1* promoter activity was higher than without it, and ET-1 increased the promoter's activity still further (Figure 1D). ANP then significantly inhibited the ET-1–induced, TRPC6-enhanced *RCAN1* promoter activity. Moreover, when cells were transfected with a TRPC6 T69A mutant, in which the PKG-phosphorylation site, Thr69, was substituted with an Ala, the basal and ET-1–induced activities of the *RCAN1* promoter were comparable to those obtained with overexpression of WT TRPC6, but the inhibitory effects of ANP were almost completely abolished (Figure 1D). 8Br-cGMP, a membrane-permeant cGMP analogue, also significantly in-

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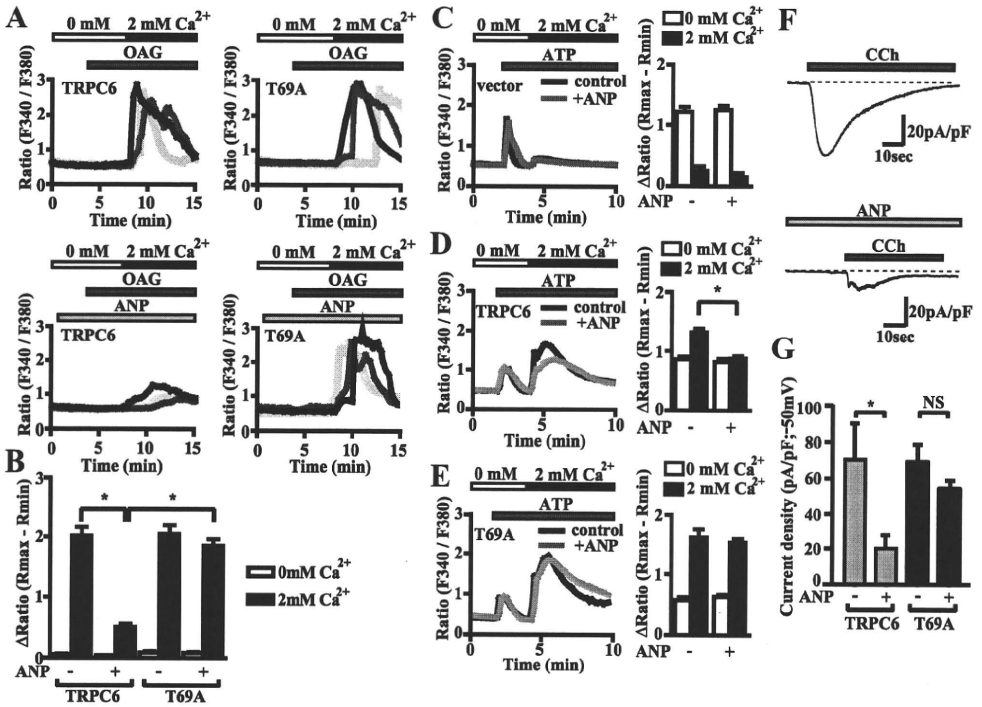

Figure 2. ANP inhibits agonist-evoked Ca^{2+} influx through phosphorylation of Thr69 in TRPC6. A, Three different representative time courses of OAG-induced Ca^{2+} influx via TRPC6 **(left graphs)** or the TRPC6 T69A mutant **(right graphs)** in HEK293 cells treated without **(top graphs)** or with **(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 HEK 293 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.

hibited 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 2A, right graphs, and 2B).

To evaluate the effect of ANP on receptor-mediated activation of TRPC6, we next stimulated HEK293 cells using ATP and examined the effect of ANP on ATP-induced TRPC6 activation. In control cells, which did not express TRPC6, ATP did not induce sustained Ca^{2+} influx, and ANP had no effect (Figure 2C). In cells expressing WT TRPC6, by contrast, ATP induced sustained Ca^{2+} influx, which was significantly inhibited by ANP (Figure 2D). Moreover, mutation of the PKG-phosphorylation site (T69A) in TRPC6 abolished the ANP-induced inhibition of ATP-evoked Ca^{2+} influx (Figure 2E). This means that ANP acts in a PKG-dependent manner to inhibit TRPC6 activation via Gq-coupled receptors. RGS2 and RGS4 are reportedly involved in the cGMP-mediated inhibition of Gq-coupled receptor signaling pathways, including the calcineurin-NFAT pathway.[20,41,42] Because RGS2 and 4 are known to block activa-

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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).[24] 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.[24] 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).

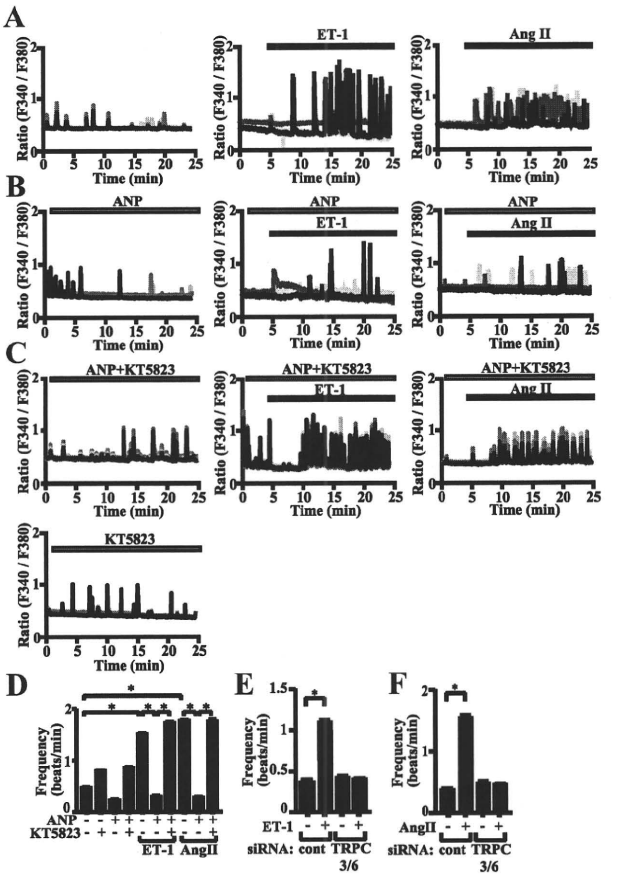

Figure 3. ANP inhibits agonist-induced increases in Ca^{2+} oscillation in cardiac ventricular myocytes. A through C, Three representative traces showing Ca^{2+} oscillations in cultured cardiac myocytes treated with vehicle, ET-1 or Ang II in the absence **(A)** or presence of ANP **(B)** or ANP+KT5823 **(C)**. **D,** Graph shows the frequencies of the Ca^{2+} oscillations under the above conditions. Values are shown as means±SEM. *$P<0.05$. **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. *$P<0.05$.

To further determine whether TRPC channels or LTCCs were responsible for the ANP-induced inhibition of agonist-induced Ca^{2+} influx in cultured ventricular myocytes, we tested the effects of nitrendipine, a selective LTCC inhibitor, and BTP2 on the Ca^{2+} influx. Although nitrendipine reduced the overall Ca^{2+} influx induced by ET-1 or Ang II, the inhibitory effect of ANP was preserved (Figure 5A and 5B). By contrast, ANP-induced inhibition of agonist-evoked Ca^{2+} influx was almost completely blocked in the presence of BTP2 (Figure 5C). The dose of BTP2 we used in this study did not affect KCl-induced, nitrendipine-sensitive Ca^{2+} influx (Figure 5D). These findings further confirm that inhibition of TRPCs, not LTCCs, is a critical component of the inhibitory effect of ANP on ET-1 and Ang II–induced Ca^{2+} influx into cultured ventricular myocytes.

We also assessed the contribution made by RGS2 and RGS4 to ANP-induced inhibition of Gq-coupled receptor-mediated Ca^{2+} influx into cardiac myocytes by using siRNAs

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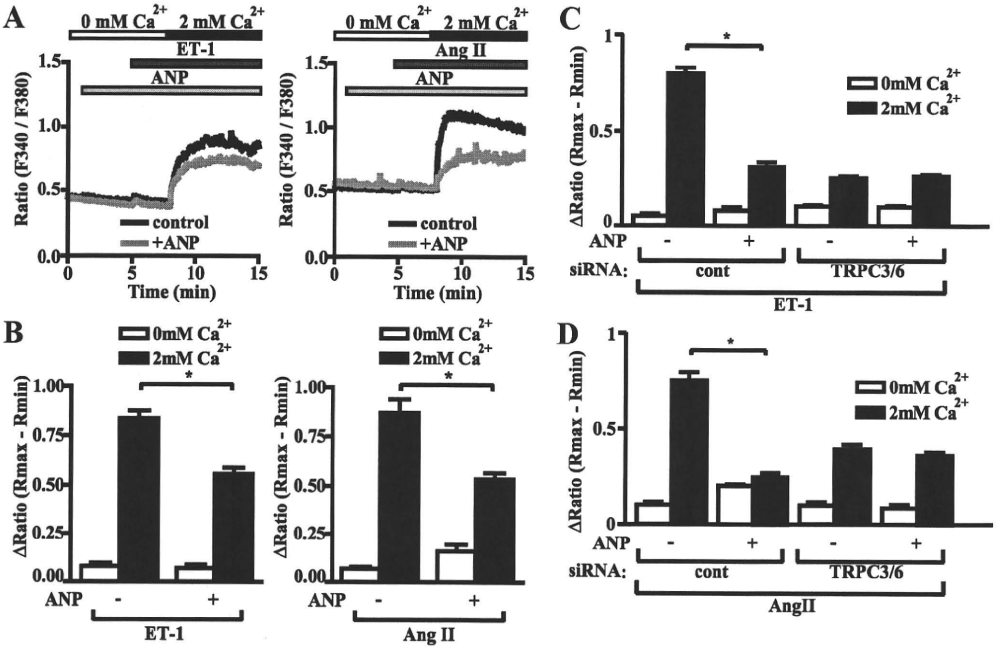

Figure 4. ANP inhibits agonist-evoked Ca^{2+} influx into cardiac ventricular myocytes. A, Representative time courses of ET-1– or Ang II–evoked Ca^{2+} influx into cultured ventricular myocytes treated with **(gray line)** or without **(black line)** ANP. **B,** ET-1– and Ang II–evoked Ca^{2+} influx into cultured ventricular myocytes cells treated with or without ANP in Ca^{2+}-free **(white bar)** or Ca^{2+}-containing external solution **(black bar).** Values are shown as means±SEM. *$P<0.05$. **C and D,** Effects of double knockdown of TRPC3 and 6 on Ca^{2+} 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 Ca^{2+} were used.

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 Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} 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

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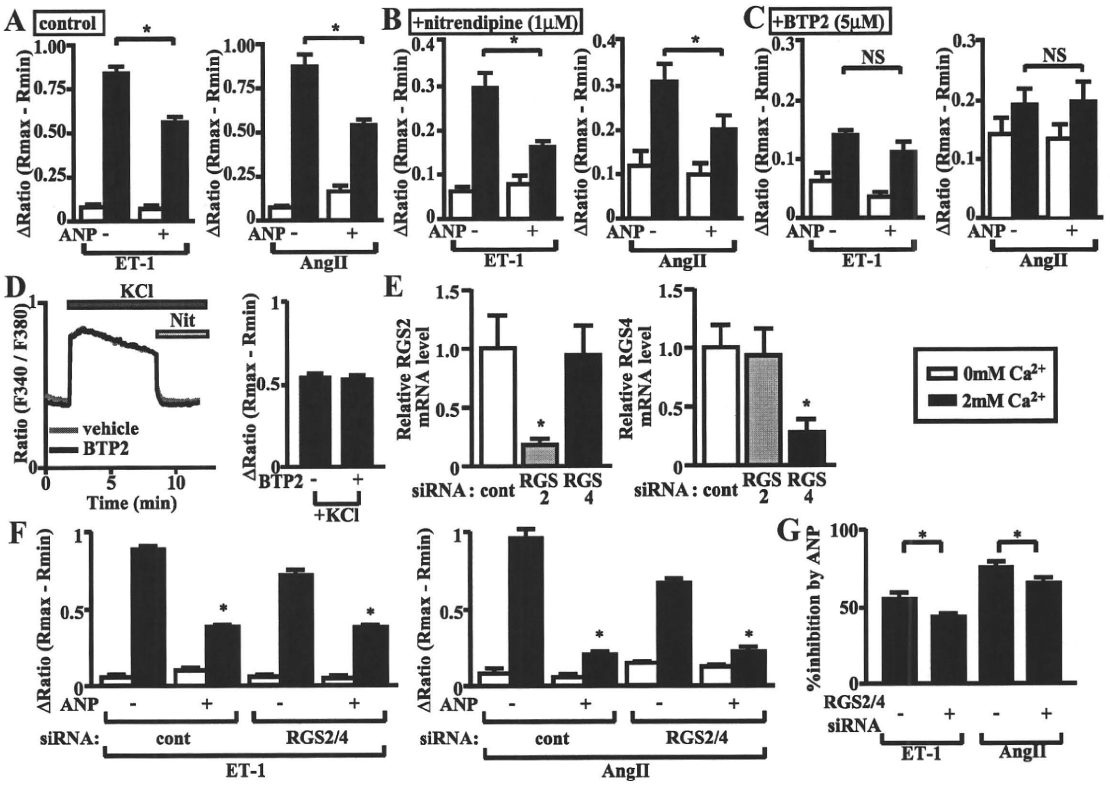

Figure 5. TRPC channels are involved in ANP-induced inhibition of receptor-mediated Ca^{2+} entry into cardiac ventricular myocytes. A, ET-1– and Ang II–evoked Ca^{2+} influx into cultured ventricular myocytes treated with or without ANP in Ca^{2+}-free **(white bar)** or Ca^{2+}-containing external solution **(black bar). B and C,** Effect of nitrendipine (1 μmol/L) **(B)** or BTP2 (5 μmol/L) **(C)** on ET-1– and Ang II–evoked Ca^{2+} influx into cultured ventricular myocytes treated with or without ANP in Ca^{2+}-free **(white bar)** or Ca^{2+}-containing external solution **(black bar). D,** Representative traces showing the effect of BTP2 (5 μmol/L) on KCl-induced (5 mmol/L), nitrendipine-sensitive (1 μmol/L) Ca^{2+} influx into cardiac myocytes. Graphs at **right** show the effect of BTP2 on KCl-induced Ca^{2+} 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)** Ca^{2+} influx into cultured ventricular myocytes treated with or without ANP in Ca^{2+}-free **(white bar)** or Ca^{2+}-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 Ca^{2+} 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 Ca^{2+} 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 cardioselectively 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

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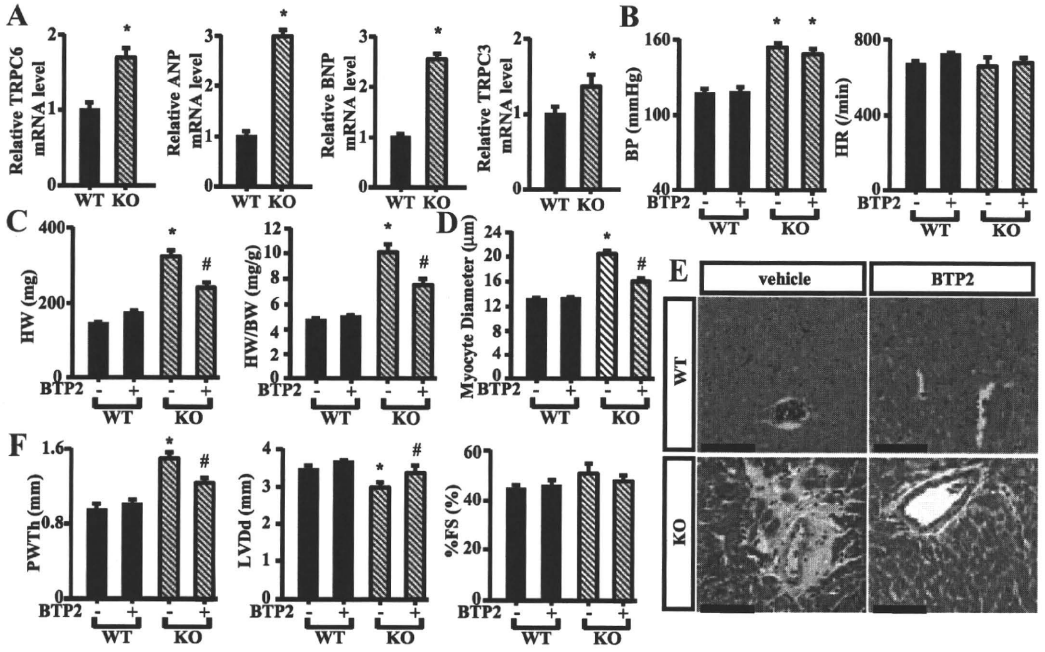

Figure 6. TRPC6 plays an important role in cardiac hypertrophy in GC-A KO mice. A, Real-time RT-PCR analysis of the relative expression of TRPC6, ANP, BNP, and TRPC3 mRNA in ventricular myocardium from WT and GC-A KO (KO) mice at 12 weeks of age. Relative mRNA levels in WT were assigned a value of 1.0. *$P<0.05$ vs WT. Values are shown as means±SEM. *$P<0.05$. **B,** Blood pressure (BP) and heart rate (HR) in WT and KO mice treated for 28 days with or without BTP2 (20 mg/g per day). **C,** Heart weight (HW) and HW/BW (mg/g) ratios in WT and KO mice treated for 28 days with or without BTP2 (20 mg/g per day). Values are shown as means±SEM. *$P<0.05$ vs WT without BTP2; #$P<0.05$ vs KO without BTP2. **D,** Myocardial cell diameters in left ventricles were measured for 200 cells in each group. Values are shown as means±SEM. *$P<0.05$ vs WT without BTP2; #$P<0.05$ vs KO without BTP2. **E,** Histology of 16-week-old WT and GC-A KO ventricles treated for 28 days with or without BTP2. **Scale bars:** 100 mm. **F,** Echocardiographic parameters in WT and KO mice treated for 28 days with or without BTP2 (20 mg/g per day). Graphs show posterior wall thickness (PWTh) (mm), left ventricular end diastolic dimension (LVDd) (mm), and percentage fractional shortening (%FS). Values are shown as means±SEM. *$P<0.05$ vs WT without BTP2; #$P<0.05$ vs KO without BTP2.

minipump, we observed a significant increase in blood pressure and HW/BW ratios (Figure 8A and 8B). Administration of BTP2 significantly inhibited Ang II–induced cardiac hypertrophy assessed based on HW/BW ratios, without affecting blood pressure (Figure 8A and 8B). Echocardiographic analysis confirmed that BTP2 inhibited the Ang II–induced hypertrophic response in the heart (Figure 8C and Online Table II), without affecting systolic function (Online Table II). In addition, the increase in the cardiac expression of the hypertrophy marker genes *RCAN1*, *BNP*, *βMHC*, and skeletal α-actin, which was observed in Ang II–treated mice, was significantly inhibited by BTP2 treatment (Figure 8D), whereas expression of cardiac α-actin, *TRPC6*, and *TRPC3* was not significantly affected in mice treated with or without Ang II and/or BTP2 (Figure 8D; Online Figure V, F and G).

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 Ca^{2+} influx (Figure 5G). 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 2D, 2E, and 2G; Online Figure I, D).

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