Chromogranin B Regulates Calcium Signaling, Nuclear Factor κB Activity, and Brain Natriuretic Peptide Production in Cardiomyocytes

Felix M. Heidrich, Kun Zhang,* Manuel Estrada,* Yan Huang, Frank J. Giordano, Barbara E. Ehrlich

Abstract—Altered regulation of signaling pathways can lead to pathologies including cardiac hypertrophy and heart failure. We report that neonatal and adult cardiomyocytes express chromogranin B (CGB), a Ca^{2+} binding protein that modulates Ca^{2+} release by the inositol 1,4,5-trisphosphate receptor ($InsP_3R$). Using fluorescent Ca^{2+} indicator dyes, we found that CGB regulates $InsP_3$-dependent Ca^{2+} release in response to angiotensin II, an octapeptide hormone that promotes cardiac hypertrophy. ELISA experiments and luciferase reporter assays identified angiotensin II as a potent inducer of brain natriuretic peptide (BNP), a hormone that recently emerged as an important biomarker in cardiovascular disease. CGB was found to regulate angiotensin II–stimulated and basal secretion, expression and promoter activity of BNP that depend on the $InsP_3R$. Moreover, we provide evidence that CGB acts via the transcription activity of nuclear factor κB in an $InsP_3/Ca^{2+}$-dependent manner but independent of nuclear factor of activated T cells. In vivo experiments further showed that cardiac hypertrophy induced by angiotensin II, a condition characterized by increased ventricular BNP production, is associated with upregulation of ventricular CGB expression. Overexpression of CGB in cardiomyocytes, in turn, induced the BNP promoter. The evidence presented in this study identifies CGB as a novel regulator of cardiomyocyte $InsP_3/Ca^{2+}$-dependent signaling, nuclear factor κB activity, and BNP production.

Key Words: chromogranin B ■ calcium ■ inositol 1,4,5-trisphosphate receptor ■ nuclear factor NF-κB ■ brain natriuretic peptide

Despite advances in treatment, cardiovascular disease is still the leading cause of morbidity and mortality in the Western world.[1] A prolonged cardiac hypertrophic state leads to heart failure (HF) and is commonly accompanied by complex changes in gene expression.[1,2] This includes the reexpression of fetal cardiac genes,[2] the reciprocal regulation of intracellular Ca^{2+} release channels[3] and an increase in ventricular production of brain natriuretic peptide (BNP), a hallmark of cardiac hypertrophy.[4] Importantly, BNP plasma level elevation in patients with HF correlates with disease severity, as assessed by New York Heart Association functional class, and BNP has recently emerged as important cardiac biomarker.[4]

Myocardial signal-transduction pathways that mediate hypertrophic growth and, eventually, the onset of HF are abundant and complex.[1] One pathway involves Ca^{2+}/calmodulin-activated calcineurin/NFAT (nuclear factor of activated T cells) signaling.[1,5] In this pathway, cytoplasmic NFAT is dephosphorylated by the serine/threonine protein phosphatase calcineurin and subsequently NFAT is translocated to the nucleus to initiate transcription.[1,5] Recently, the importance of nuclear factor (NF)-κB in cardiac hypertrophy was shown, and NF-κB was linked to a variety of cardiovascular pathologies.[1,6] In the resting cell, NF-κB dimers reside in the cytoplasm bound to inhibitor proteins, inhibitor kappa B (IκB).[6] Typically, NF-κB signaling is initiated by stimulus-induced phosphorylation of IκB by IκB kinases (IKKs) that leads to IκB phosphorylation, polyubiquitination, and degradation.[6] This unmasks a nuclear translocation sequence, resulting in translocation of NF-κB into the nucleus to initiate transcription.[6] Even though this NF-κB activation pathway is well described in the immune system,[7] and its existence in the heart is broadly accepted,[6] little is known about NF-κB activation in cardiac cells.

CGB, a Ca^{2+} binding protein that belongs to the granin family of acidic proteins,[8] resides in the endo-/sarcoplasmic reticulum (ER/SR) and functionally interacts with all 3 inositol 1,4,5-trisphosphate receptor ($InsP_3R$) isoforms[9] to shape Ca^{2+} release.[10–12] Local $InsP_3$-dependent Ca^{2+} signaling was only recently linked to cardiac excitation–transcription

From the Departments of Pharmacology (F.M.H., K.Z., B.E.E.) and Medicine/Cardiology (Y.H., F.J.G.), Yale University, New Haven, Conn; Department of Pharmacology and Toxicology (F.M.H.), Dresden University of Technology, Germany; Neuroscience Research Centre (K.Z.), Charité Universitätsmedizin, Berlin, Germany; and Department of Physiology and Biophysics (M.E.), University of Chile, Santiago.

*Both authors contributed equally to this work.

Correspondence to Barbara E. Ehrlich, Department of Pharmacology, Yale University School of Medicine, 333 Cedar St, New Haven, CT 06520-8066. E-mail barbara.ehrlich@yale.edu

coupling (ETC) in adult ventricular myocytes.[13] Because we found that neonatal and adult ventricular cardiomyocytes express CGB, along with all 3 isoforms of the $InsP_3R$, we hypothesized that CGB would be important in the regulation of cardiomyocyte $InsP_3$-dependent Ca^{2+} signaling and would modify ETC. In this study, we show that CGB regulates cardiomyocyte $InsP_3/Ca^{2+}$-dependent signaling, the activity of the transcription factor NF-κB, and the production of BNP.

Materials and Methods

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Cell Culture

All procedures for animal use were in accordance with guidelines approved by the Yale Animal Care and Use Committee. Primary neonatal rat ventricular cardiomyocytes were prepared as described previously with a purity of at least 95%.[14] Cardiac fibroblasts were derived from differential preplating.

Angiotensin II Microosmotic Pump Implantation

Microosmotic pumps were implanted as described in the online data supplement. Mice were constantly infused with angiotensin (Ang) II for 2 weeks at a rate of 1000 ng/kg per minute. Control mice experienced the same surgical procedures without pump implantation and were subsequently treated identically. Cardiac hypertrophy was documented by determination of the left ventricular weight/body weight (LVW/BW) ratios at the time of euthanasia.

Plasmids, Luciferase Reporter Vectors, and Small Interfering RNA

Plasmids, luciferase reporter vectors (hBNPLuc, NF-κB-luc, AdNFAT-luc), and small interfering (si)RNA have been described previously or are commercially available.[5,15–18] Luciferase reporter assays were performed as described.[14] Intracellular chelation of Ca^{2+} was accomplished using BAPTA-AM (200 μmol/L).[19]

Transient Transfection and Adenoviral Infection

Transient transfection and adenoviral infection of cardiomyocytes were previously described.[14] Briefly, transfection was performed at day 2 after culture in OptiMEM using Lipofectamine 2000 for 4 hours, followed by incubation overnight with complete growth medium added. Transfection efficiency in cardiomyocytes using the protocol described is at least 30% to 40% for DNA. Adenoviral infection with NFAT luciferase reporter vector (AdNFAT-luc)[5] was performed for 2 hours.

Brain Natriuretic Peptide ELISA

Secretion and expression of BNP by cardiomyocytes and cardiac fibroblasts were assayed using the AssayMax Rat BNP-45 ELISA Kit (GENTAUR, Brussels, Belgium) according to the protocol of the manufacturer. Experiments were performed 2 days after siRNA transfection (CGB siRNA experiments) or at day 4 after culture, respectively, to match time points. Basal and stimulation studies were performed for 4 hours. Preincubation with inhibitors ($InsP_3R$ inhibitor 2-aminoethoxydiphenylborate [2-APB], 25 μmol/L; AT_1R inhibitor telmisartan, 1 μmol/L) was performed for 1 hour. Samples were collected, and absorbance at 450 nm was measured using a standard microplate reader. Standard points and samples were determined as duplicates or triplicates.

Live Cell Calcium Imaging

Calcium-imaging experiments have been described.[14] Briefly, cells were loaded with a cell-permeant indicator dye (Mag-Fluo-4/AM, 5 μmol/L or Fura red/AM, 10 μmol/L) and transferred to a Zeiss LSM 510 NLO laser-scanning confocal microscope. Fluorescence was measured by defining each cell as 1 region of interest and quantified in relation to baseline fluorescence (F/F_0). The change in whole cell F/F_0 was between the nuclear and cytosolic signals and therefore used as representative readout in this study (Figure I in the online data supplement). The low-affinity ($K_{d,Ca2+}$=22 μmol/L) Ca^{2+} indicator Mag-Fluo-4 was used to assess Ca^{2+} release from internal stores. Differential loading of Mag-Fluo-4 into internal stores was verified (supplemental Figure II). In experiments with cells expressing DsRed (cotransfection), the high-affinity Ca^{2+} indicator Fura red was used to assess cytosolic Ca^{2+} changes. This replacement of Mag-Fluo-4 was necessary because of an overlap of Mag-Fluo-4 and DsRed excitation and emission spectra that caused substantial quenching of Mag-Fluo-4 fluorescence by DsRed.

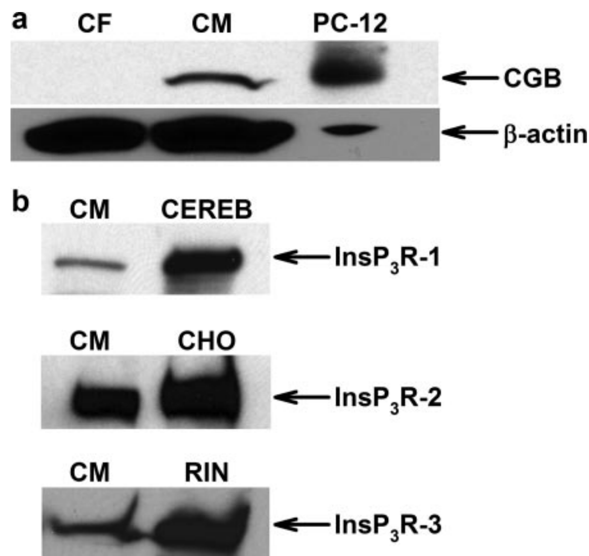

Figure 1. Neonatal cardiomyocytes express CGB and $InsP_3R$ isoforms. a, Representative Western blot shows that cardiomyocytes (CM) but not cardiac fibroblasts (CF) express CGB. PC-12 cells served as positive control and β-actin as loading control. b, Cardiomyocytes express all $InsP_3R$ isoforms; positive controls as indicated: CEREB indicates cerebellar microsomes, CHO, Chinese hamster ovary cells, RIN, rat insulinoma cells.

Immunoblotting

Immunoblotting was performed as described.[20] Primary antibodies used were: CGB (BD Bioscience), $InsP_3R$-1, $InsP_3R$-2, and $InsP_3R$-3[20]; β-actin, GAPDH-HRP (Abcam); and SR/ER-ATPase (SERCA)2a, cardiac ryanodine receptor (RyR) (Affinity Bioreagents). Expressions were quantified by scanning densitometry.

Statistical Analysis

Data are expressed as means±SEM or representative traces (n/N) describes the number of individual experiments (n) in N independent cultures. Statistical analysis of the differences between multiple groups was performed using 1-way ANOVA (Student–Newman–Keuls method) for 2 groups using *t* test (SigmaPlot). Statistical significance is indicated as follows: *$P<0.05$, **$P<0.01$, and ***$P<0.001$.

Results

Cardiomyocytes Express CGB Along With All $InsP_3R$ Isoforms

Neonatal cardiomyocytes but not cardiac fibroblasts express CGB (Figure 1a). CGB also is present in adult mouse ventricular myocardium (Figure 6). Besides CGB, cardiomyocytes express all 3 $InsP_3R$ isoforms ($InsP_3R$-1, $InsP_3R$-2, $InsP_3R$-3) (Figure 1b). CGB resides in the SR and functionally interacts with the $InsP_3R$ to shape Ca^{2+} release.[9–12]

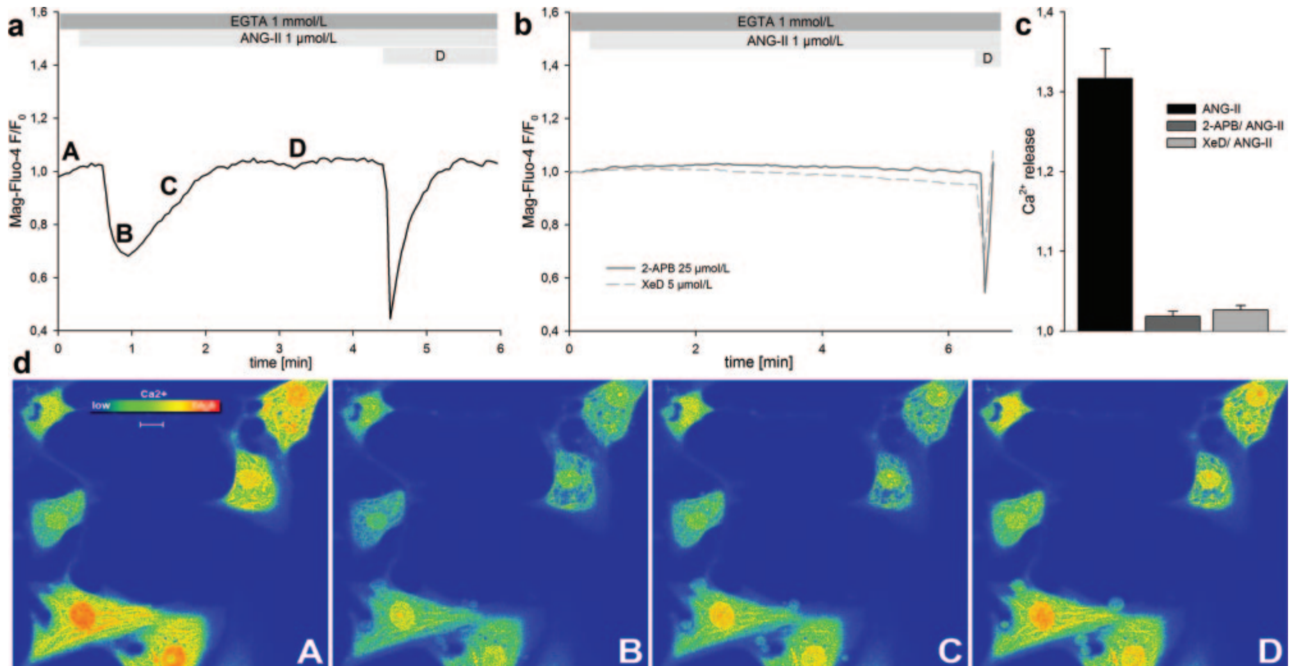

Figure 2. Ang II evokes $InsP_3$-dependent Ca^{2+} release in cardiomyocytes. Ca^{2+} release from internal stores was monitored using the low-affinity Ca^{2+} indicator Mag-Fluo-4/AM. a and b, Representative traces. a, Ang II causes Ca^{2+} release that recovers to baseline. b, No significant Ca^{2+} release was observed in cardiomyocytes pretreated with either of the $InsP_3R$ inhibitors 2-APB or xestospongin D (XeD). c, Quantification of released Ca^{2+}. Ang II causes Ca^{2+} release only in the absence of $InsP_3R$ inhibitors. d, Representative pseudo-colored time course of cardiomyocytes challenged with Ang II. Letters indicate respective time points in a. Scale bar=10 μm.

Therefore, coexistence of CGB and the $InsP_3R$ in cardiomyocytes suggests a functional role for CGB in shaping cardiomyocyte $InsP_3$-dependent Ca^{2+} signaling.

Ang II Evokes $InsP_3$-Dependent Ca^{2+} Release in Cardiomyocytes

Ang II causes the generation of phosphoinositides in cardiomyocytes through its interaction with the G protein–coupled Ang II type 1 receptor (AT_1R).[21,22] We monitored changes in SR and nuclear envelope Ca^{2+} content in response to Ang II (1 μmol/L) using the low-affinity fluorescent Ca^{2+} indicator Mag-Fluo-4/AM. Experiments were performed in Ca^{2+}-free (0 Ca^{2+}, EGTA 1 mmol/L) extracellular solution. Ang II caused a marked decrease in Mag-Fluo-4 fluorescence intensity that recovered over time to baseline (Figure 2a and 2d), as would be expected for Ca^{2+} release and subsequent reuptake into internal stores. The magnitude of the Ang II–evoked Ca^{2+} release was 1.3±0.04 (30/2) (Figure 2c). Pretreatment of cardiomyocytes with the $InsP_3R$ inhibitors 2-APB (25 μmol/L) or xestospongin D (5 μmol/L) prevents Ca^{2+} release on Ang II stimulation (Figure 2b and 2c). Depolarization served as cell viability control and provided the means to distinguish between excitable myocytes and contaminating fibroblasts ("D"; Figure 2a and 2b). These results show that Ang II evokes $InsP_3$-dependent Ca^{2+} release from internal stores in cardiomyocytes.

CGB Shapes Ang II–Evoked Ca^{2+} Release

CGB is known to modulate Ca^{2+} release by the $InsP_3R$ at the single-channel level and in intact cells.[10–12,17] Because we found that cardiomyocytes express CGB, along with the $InsP_3R$ (Figure 1), we hypothesized that CGB would be important in cardiomyocytes in shaping $InsP_3$-dependent Ca^{2+} signaling. Therefore, CGB expression in cardiomyocytes was silenced by siRNA (Figure 3a). Densitometry revealed a knockdown to 34±7% compared to mock treatment (3/1) ($P<0.001$; Figure 3b). To determine whether this downregulation impacts $InsP_3$-dependent Ca^{2+} release, cells were cotransfected with CGB siRNA and DsRed, and changes in cytosolic Ca^{2+} on Ang II stimulation (1 μmol/L) were monitored using the fluorescent Ca^{2+} indicator Fura red/AM. DsRed-only-transfected cells served as control. The rise in cytosolic Ca^{2+} was blunted after CGB knockdown (Figure 3c). Note that an increase in cytosolic Ca^{2+} causes a decrease in Fura red fluorescence when excited at 488 nm. CGB knockdown diminished peak Ca^{2+} release to 84±2% (17/1) compared to control (100±6% [17/1]) ($P<0.05$; Figure 3d) and decreased the velocity of Ca^{2+} release to 32±13% (12/1) compared to control (100±28.5% [13/1]) ($P<0.05$; Figure 3e). These effects could also be explained by store depletion following CGB knockdown. To address this question, we depleted ER stores by adding 10 μmol/L SERCA inhibitor thapsigargin in Ca^{2+}-free medium and calculated the ER Ca^{2+} content as area under the release curve. Both CGB siRNA-transfected and control cells had similar amounts of Ca^{2+} stored in the ER (Figure 3f). We conclude that CGB shapes $InsP_3$-dependent Ca^{2+} signaling in cardiomyocytes.

Ang II–Stimulated and Basal BNP Secretion Depend on the $InsP_3R$

BNP secretion is regulated at the transcriptional level.[4] Ang II increases cardiomyocyte BNP mRNA levels by its interaction with the G protein–coupled AT_1R that causes the formation of phosphoinositides.[21–25] Therefore, we hypothesized that

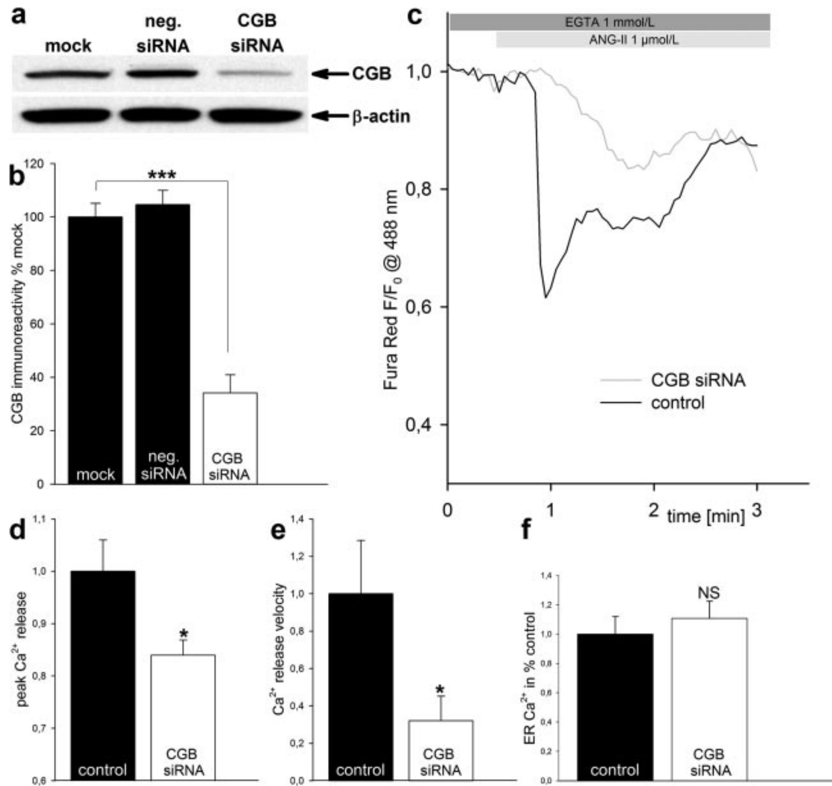

Figure 3. CGB shapes Ang II–evoked Ca^{2+} release. a and b, Documentation of CGB knockdown. a, Representative Western blot. Note the decrease in CGB immunoreactivity in CGB knockdown cells only. b, Quantification of CGB immunoreactivity by densitometry. c, Representative traces. CGB knockdown decreases peak Ca^{2+} release and velocity of Ca^{2+} release on Ang II stimulation. d and e, Quantification of peak Ca^{2+} release (d) and velocity of Ca^{2+} release (e). f, CGB knockdown does not deplete ER-Ca^{2+} stores.

secretion of BNP would be stimulated by Ang II and would depend on the $InsP_3$ signaling pathway. To address this question, we first studied the time course of BNP secretion in cardiomyocytes. Supernatant samples were collected after 1 hour without any treatment (baseline) followed by repetitive collections from cells incubated with Ang II (1 μmol/L) or vehicle for 2, 4, 8, and 12 hours and assayed by ELISA. Cells incubated with vehicle only showed an increase in BNP secretion over time as a result of tonic basal secretion (Figure 4a). However, cells incubated with Ang II showed a markedly different kinetics (Figure 4a). The Ang II–dependent portion of BNP secretion was maximal after 4 hours of incubation (Figure 4b). This time period was used in any further ELISA experiment. Next, we studied the requirement of the AT_1R and Ca^{2+} release by the $InsP_3R$ in Ang II–stimulated BNP secretion. Both the AT_1R inhibitor telmisartan (1 μmol/L) and the $InsP_3R$ inhibitor 2-APB (25 μmol/L) prevented the stimulating effect of Ang II (Figure 4c). Inhibition of the $InsP_3R$ also diminished basal BNP secretion (Figure 4d). These results show that Ang II stimulates BNP secretion in cardiomyocytes and that $InsP_3$-mediated Ca^{2+} release is a component of the pathway for Ang II–stimulated and basal BNP secretion.

CGB Knockdown Diminishes Basal and Abrogates Ang II–Stimulated BNP Production

Basal and Ang II–stimulated BNP secretion depend on the activation of the $InsP_3R$ (Figure 4d and 4c). We hypothesized that CGB, which shapes $InsP_3$-dependent Ca^{2+} release (Figure 3), would also modify cardiomyocyte BNP production. To address this question, cardiomyocytes were transiently transfected with CGB siRNA, negative siRNA, or mock-treated. Basal BNP secretion was reduced to 65±7% (12/3) after CGB knockdown compared to mock treatment (100±3% [12/3]) ($P<0.001$; Figure 4e). CGB knockdown also prevented the stimulating effect of Ang II (56±8%; 12/3) (Figure 4e). To investigate whether CGB knockdown inhibits BNP secretion by preventing its release or by decreasing its production, we also measured BNP expression. CGB knockdown significantly decreased basal BNP expression to 63±5% (11/3) compared to mock treatment (100±3% [10/3]) ($P<0.01$; Figure 4f). As for secretion, Ang II failed to exert positive effects on BNP expression after CGB knockdown (56±5% [10/2]) (Figure 4f). Negative (nontargeting) siRNA-transfected cells showed slightly increased BNP production (Figure 4e and 4f), which we attribute to cell stress during the transfection. Values measured for BNP expression and secretion of each individual experiment were matched and subjected to bidirectional analysis and linear regression. These data revealed a strong correlation of BNP expression and secretion ($r^2=0.98$) (Figure 4g). This suggests that CGB impacts BNP production at the transcriptional level rather than by preventing the formation of secretory granules or their secretion; either of which would lead to a build up of BNP inside the cell.

CGB Regulates the BNP Promoter

To study the potential role of CGB on BNP production at the transcriptional level, we used a human BNP promoter luciferase reporter (hBNPLuc).[15] To investigate the effect of CGB on basal BNP promoter luciferase activity, cardiomyocytes were transiently transfected with hBNPLuc alone ("mock treatment") or cotransfected with hBNPLuc and negative siRNA or CGB siRNA. Basal luciferase activity after CGB knockdown was significantly diminished to 37±6% (7/2)

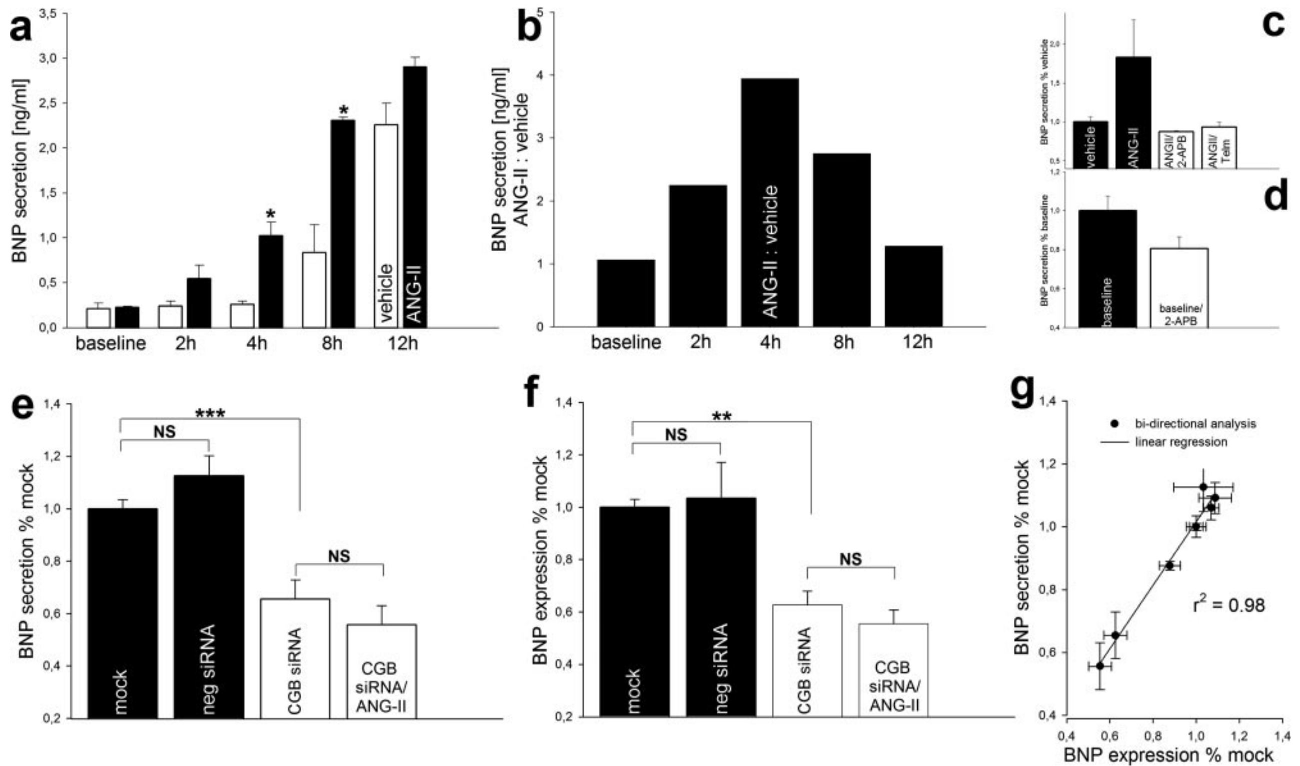

Figure 4. Basal and Ang II–stimulated BNP production depend on the $InsP_3R$ and are regulated by CGB. a, Time course of BNP secretion. Note different kinetics of vehicle and Ang II. b, Ang II–stimulated secretion reaches a relative maximum after 4 hours. c, Ang II–stimulated BNP secretion depends on the AT_1R and the $InsP_3R$. d, Inhibition of the $InsP_3R$ diminishes basal BNP secretion. e and f, CGB knockdown decreases basal BNP secretion and expression and abrogates the stimulating effect of Ang II. g, Linear regression analysis suggests that CGB regulates BNP production at the transcriptional level. Telm indicates telmisartan.

compared to mock treatment (100±5% [6/2]) ($P<0.001$; Figure 5a). Cells transiently transfected with negative siRNA showed a nonsignificant reduction in hBNPLuc activity (87±6% [7/2]) (Figure 5a). Next, we asked whether Ang II would induce BNP and whether this induction would be modified by CGB. Cardiomyocytes were incubated with vehicle (control) or 1 μmol/L Ang II for 4 hours. Ang II increased luciferase activity to 210±9% (3/1) compared to

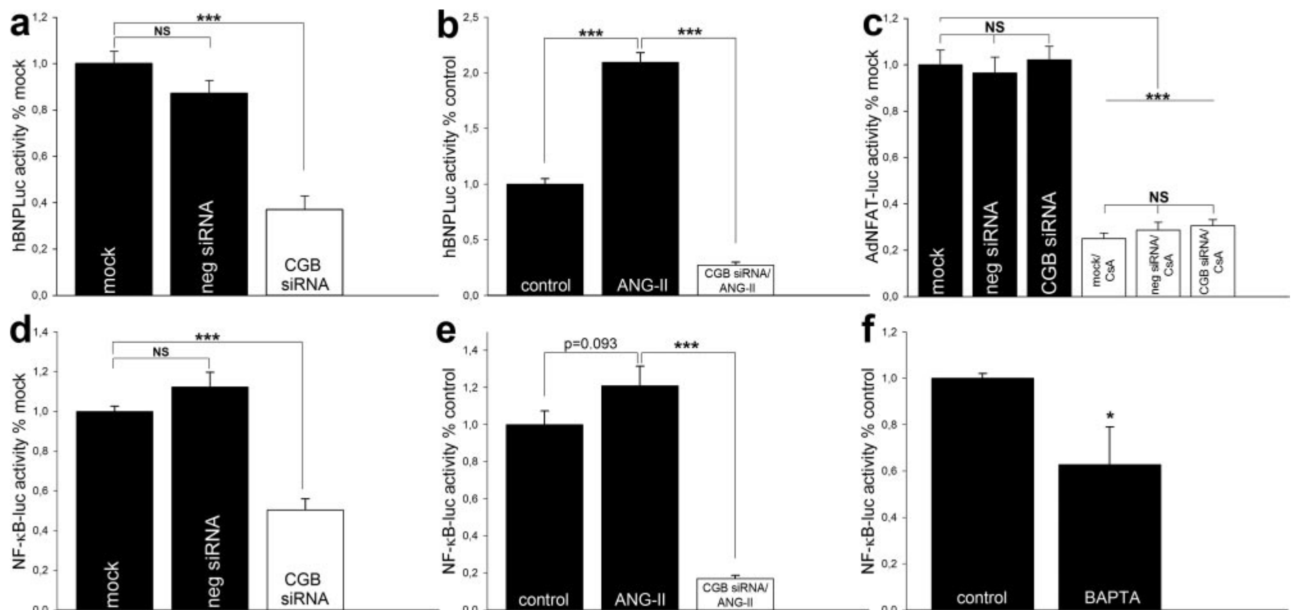

Figure 5. CGB regulates the BNP promoter and NF-κB. a and b, BNP promoter luciferase activities. CGB knockdown decreases basal promoter activity and prevents its induction by Ang II. c, Basal NFAT activity is not affected by CGB knockdown. CsA served as control. d and e, NF-κB luciferase activities. CGB knockdown decreases basal NF-κB activity and prevents its activation by Ang II. f, Basal NF-κB activity depends on Ca^{2+}.

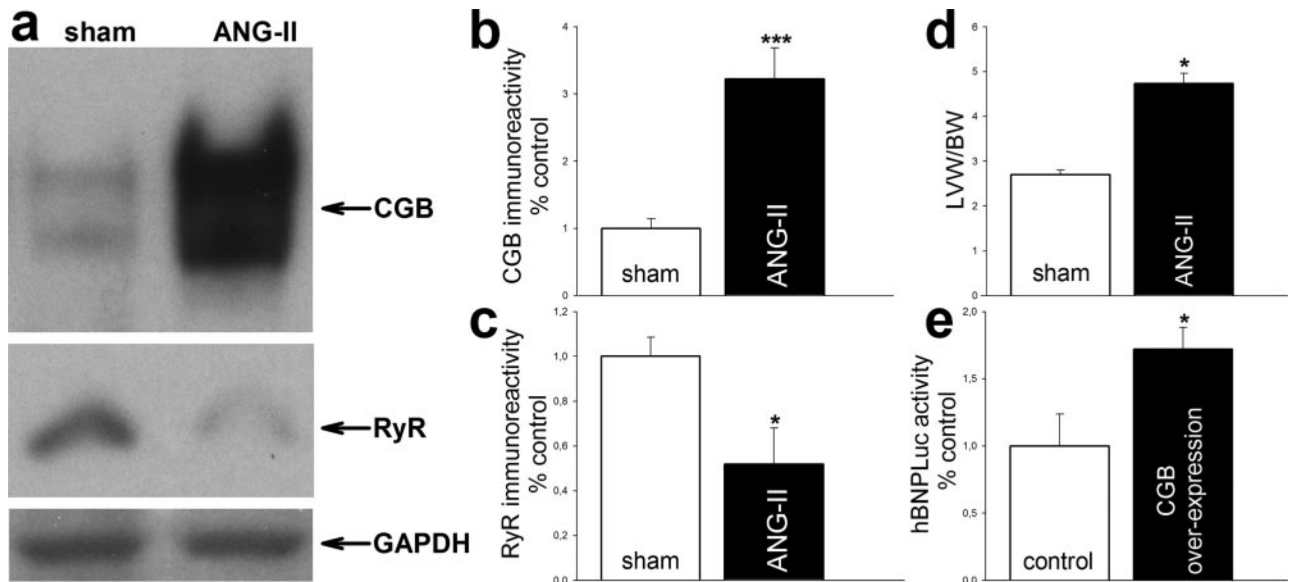

Figure 6. CGB is upregulated in Ang II–induced cardiac hypertrophy in vivo. a, Representative Western blot shows ventricular expression of CGB and cardiac RyR. GAPDH served as loading control. b and c, Quantifications by densitometry. b, Ang II induces ventricular CGB expression ≈3-fold. c, In contrast, cardiac RyR expression is decreased to ≈50%. d, LVW/BW ratio documents hypertrophy. e, CGB overexpression in cardiomyocytes induces the BNP promoter.

control (100±5% [3/1]) ($P<0.001$; Figure 5b). In contrast, Ang II failed to exert stimulating effects in CGB knockdown cells (Figure 5b). Remarkably, CGB knockdown kept luciferase activity below basal levels even with Ang II present (27±3% of control [3/1]) ($P<0.001$; Figure 5b). These results show that CGB regulates the BNP promoter under both basal and Ang II–stimulated conditions.

CGB Regulates NF-κB Activity but Does Not Affect NFAT

We aimed to identify the transcription factor that mediates the regulatory role of CGB on the BNP promoter. We first focused on NFAT, a Ca^{2+}-dependent transcription factor that plays a central role in cardiac hypertrophy.[1,5] Cardiomyocytes were infected with adenoviral NFAT-luciferase reporter (AdNFAT-luc)[5] alone (mock) and after transient transfection with CGB siRNA or negative siRNA. We did not observe any significant change in basal NFAT activity after CGB knockdown (mock 100±6% [7/2]; negative siRNA 96±7% [7/2]; CGB siRNA 102±6% [15/2]) (Figure 5c). However, in control experiments using the calcineurin inhibitor cyclosporine A (CsA) (1 μmol/L), basal NFAT activity was significantly decreased, with no significant differences among the 3 groups (mock/CsA 25±2% [7/2]; negative siRNA/CsA 29±3% [7/2]; CGB siRNA/CsA 30±3% [7/2]) ($P<0.001$; Figure 5c). These results suggest an NFAT-independent mechanism.

Recently, the NF-κB signaling pathway has been recognized as an important mediator of cardiac hypertrophic signaling.[1,6] Little is known about Ca^{2+}-dependent activation of NF-κB beyond its well-characterized Ca^{2+}-dependent function in the immune system and recent reports showing Ca^{2+}-dependent activation of NF-κB in hippocampal neurons and skeletal muscle.[7,19,26] To study the effect of CGB on NF-κB activity in cardiomyocytes, we used a NF-κB luciferase reporter (NF-κB-luc).[16] Cardiomyocytes were transiently transfected with NF-κB-luc alone (mock) or cotransfected with NF-κB-luc and CGB siRNA or negative siRNA. Following CGB knockdown, basal NF-κB luciferase activity was significantly decreased to 50±6% (8/2) compared to mock (100±3% [8/2]) ($P<0.001$; Figure 5d). The nonsignificant increase in NF-κB activity following negative siRNA transfection is most likely attributable to cell stress during the transfection and consistent with our observations on BNP production (Figure 4e and 4f). Next, we investigated whether NF-κB would be induced by Ang II and whether this induction was regulated by CGB as we found for the BNP promoter (Figure 5b). In fact, Ang II (1 μmol/L) increased NF-κB luciferase activity to 121±10% (3/1) compared to control (100±7% [3/1]) ($P=0.093$; Figure 5e), and CGB knockdown kept NF-κB luciferase activity below basal levels even with Ang II present (17±2% of control [3/1]) ($P<0.001$; Figure 5e). The Ca^{2+} dependence of basal NF-κB activity was tested by chelation of intracellular Ca^{2+} with BAPTA. Basal luciferase activity following Ca^{2+} chelation was reduced to 63±16% (5/1) compared to control (100±2% [6/1]) ($P<0.05$; Figure 5f). These results provide evidence for a novel role of CGB in the regulation of basal and Ang II–stimulated BNP promoter and NF-κB activities in a Ca^{2+}-dependent manner that is independent of NFAT signaling.

Cardiac Hypertrophy Induces Ventricular CGB In Vivo

Cardiac hypertrophy is commonly associated with increased ventricular BNP production and elevated BNP plasma levels in patients.[4] Because we found that CGB is an important modulator of BNP transcription, expression, and secretion in cardiomyocytes (Figures 4 and 5), we studied ventricular CGB expression in vivo during cardiac hypertrophy. Cardiac hypertrophy was induced in adult mice by chronic Ang II treatment using microosmotic pumps. Hypertrophy was doc-

umented by determination of the LVW/BW ratios (Figure 6d). Chronic Ang II treatment increased LVW/BW ratio by 1.7-fold (4.7±0.3 in Ang II–treated mice compared to 2.7±0.1 in sham-treated matched littermates) ($P<0.05$; Figure 6d). Hypertrophy was associated with a marked upregulation of ventricular CGB expression (Figure 6a). Densitometry revealed an increase in CGB expression to 322±46% (5/1) in hypertrophied ventricles compared to control (100±14% [9/1]) ($P<0.001$; Figure 6b). Moreover, consistent with previous reports on decreased cardiac RyR mRNA levels in failing human hearts,[3] we found the expression of cardiac RyR in the same samples significantly decreased to 52±16% (5/1) compared to control (100±9% [9/1]) ($P<0.05$; Figure 6a and 6c). No significant change in the expression of any of the $InsP_3R$ isoforms or SERCA 2a was observed (data not shown).

CGB Overexpression Increases BNP Promoter Activity in Cardiomyocytes

Because we found CGB induced in vivo in adult mice ventricular myocardium following the induction of cardiac hypertrophy (Figure 6a and 6b), we next asked whether elevated CGB expression would, in turn, increase BNP promoter activity. To address this question, we overexpressed CGB. Cardiomyocytes were cotransfected with the hBNPLuc reporter plasmid[15] and full-length CGB[18] or empty vector (control). In fact, CGB overexpression increased BNP promoter luciferase activity to 172±16% (4/1) compared to control (100±24% [3/1]) ($P<0.05$; Figure 6e).

Discussion

In the present study, we provide evidence for a novel role of the Ca^{2+} binding protein CGB in the regulation of cardiomyocyte signaling. We show that CGB shapes $InsP_3$-dependent Ca^{2+} release in cardiomyocytes and that CGB also regulates NF-κB activity and BNP production. Our in vitro results are supported by in vivo experiments that show the involvement of CGB in Ang II–mediated cardiac hypertrophy.

Local $InsP_3$-dependent Ca^{2+} signaling was only recently linked to cardiac ETC in adult ventricular myocytes, and it was also shown that these local Ca^{2+} signals act in a manner that is segregated from the global Ca^{2+} transients that mediate excitation–contraction coupling.[13] $InsP_3Rs$ are ubiquitous intracellular Ca^{2+} release channels that are present in the heart, although at lower levels than the related RyRs, which mediate excitation–contraction coupling.[27,28] The $InsP_3R$-2 is the most $InsP_3$-sensitive and the predominant isoform in cardiomyocytes.[27,29,30] In atrial cardiomyocytes, the $InsP_3R$-2 is believed to colocalize with "junctional" RyRs at the subsarcolemmal space, possibly modifying excitation–contraction coupling and thereby triggering arrhythmias,[27] whereas in ventricular cardiomyocytes, the $InsP_3R$-2 was found throughout the cell but elevated within or in close proximity to the nuclear envelope,[31] and an important role in cardiac ETC was reported.[13]

CGB, which is a member of the granin family of acidic proteins,[8] interacts with all 3 $InsP_3R$ isoforms[9] and modulates $InsP_3$-dependent Ca^{2+} release.[10–12] Here, we show that CGB is expressed in neonatal cardiomyocytes (Figure 1), as well as adult mouse ventricular myocardium (Figure 6), and impacts $InsP_3$-dependent Ca^{2+} release on Ang II stimulation that could not be explained by store depletion following CGB knockdown (Figures 2 and 3). Signaling specificity of the multifunctional second-messenger Ca^{2+} is achieved by variations in and combinations of magnitude, frequency, and duration of Ca^{2+} signals.[32] Our results that CGB simultaneously modifies 2 of these modalities (ie, magnitude and velocity of Ca^{2+} release; Figure 3) suggests a crucial role for CGB in fine-tuning of the local $InsP_3$-dependent Ca^{2+} signals that target gene transcription in cardiomyocyte ETC. In fact, the decrease in basal and the prevention of Ang II–stimulated BNP secretion and expression after CGB knockdown (Figure 4) is also seen at the transcriptional level in similarly impaired basal and stimulated BNP promoter activities (Figure 5). Notably, both basal and Ang II–stimulated BNP production depend on Ca^{2+} release by the $InsP_3R$ (Figure 4) that is modified by CGB (Figure 3). Although CGB impacts $InsP_3$-dependent Ca^{2+} release (Figure 3), and the calcineurin/NFAT signaling pathway is considered among the central players in integrating cardiac $InsP_3/Ca^{2+}$-mediated hypertrophic inputs,[1,5] we did not observe any significant impairment in basal NFAT activity after CGB knockdown (Figure 5) that would explain the consistent decrease in basal BNP production (Figure 4) and promoter activity (Figure 5). However, basal (Ca^{2+}-dependent [Figure 5f]) and Ang II–stimulated activity of another transcription factor, NF-κB, were markedly decreased following CGB gene-silencing (Figure 5), similar to the observed impairments in basal and Ang II–stimulated BNP production and promoter activity (Figures 4 and 5). Therefore, linkage of NF-κB signaling and BNP production in cardiomyocytes seems very likely. Specific NF-κB binding sites identified on the human BNP promoter further suggest that both are linked directly.[33] CGB knockdown consistently kept BNP secretion, expression, and promoter activity and NF-κB activity below basal/control levels even with the inducer Ang II present (Figures 4 and 5). This suggests that Ang II recruits the same CGB-regulated pathway that is responsible for basal BNP production to exert its positive effects, most likely the $InsP_3R$, $InsP_3$, and Ca^{2+}.

Ca^{2+}-dependent NF-κB activation was recently shown in neurons and skeletal muscle.[19,26] Here, we provide evidence for the existence of a similar Ca^{2+}-dependent NF-κB activation in cardiomyocytes that is independent of the Ca^{2+}/calcineurin-activated NFAT pathway, even though both transcription factors use the same second messenger: Ca^{2+}. Therefore, our results provide another example for how precisely Ca^{2+} signals can activate different signaling pathways in cardiomyocytes, possibly similar to the differential activation of transcription factors by Ca^{2+} described in lymphocytes.[32]

In neurons, Ca^{2+}-dependent activation of NF-κB was shown to require Ca^{2+}/calmodulin-dependent kinase (CaMK)II.[19] However, when using the general CaMK inhibitor KN-62 (10 μmol/L), we did not observe any significant suppression in basal NF-κB activity that could explain the suppressive effect of CGB knockdown (data not shown). This suggests that CGB impacts NF-κB signaling independent of CaMKs in

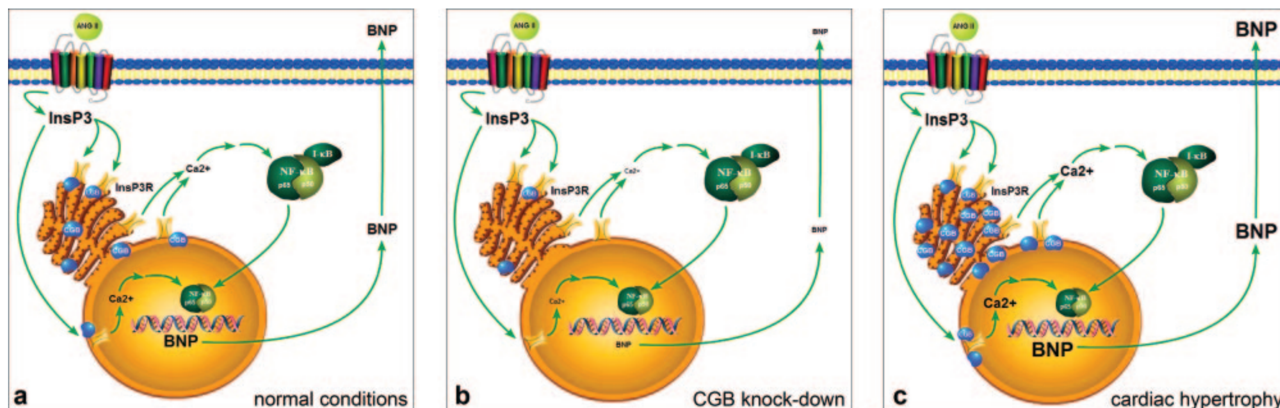

Figure 7. Proposed model for the role of CGB in cardiac hypertrophic signaling. a, Physiologically, CGB resides inside the SR and shapes Ca^{2+} release from internal stores through its interaction with the $InsP_3R$. Released Ca^{2+} activates transcription factors including NF-κB that initiate BNP transcription. Nuclear Ca^{2+} release by the $InsP_3R$ might exert direct effects. b, Decreased CGB expression impairs CGB–$InsP_3R$ coupling and leads to decreased $InsP_3$-dependent Ca^{2+} release, NF-κB activity, and BNP production. c, Upregulation of CGB, as observed in Ang II–induced ventricular hypertrophy, improves CGB–$InsP_3R$ coupling and leads to increased $InsP_3$-dependent Ca^{2+} release, NF-κB activity, and BNP production.

cardiomyocytes and that NF-κB activation is regulated differently in various cell types.

In vivo experiments showed that Ang II–induced cardiac hypertrophy, a condition characterized by increased ventricular BNP production, is associated with an impressive upregulation of CGB in ventricular myocardium (Figure 6). Because we also show in this study that CGB is an important regulator of basal and Ang II–stimulated BNP transcription, expression and secretion in cardiomyocytes in vitro (Figures 4 and 5), this result suggests that CGB may be of similar importance in the regulation of BNP production in vivo, possibly contributing to the induction of ventricular BNP in cardiac hypertrophy. This hypothesis becomes even more likely because CGB overexpression in cardiomyocytes significantly induces the BNP promoter (Figure 6e).

Based on the evidence presented in this study, we propose that CGB is of crucial importance in the $InsP_3/Ca^{2+}$-dependent regulation of hypertrophic signaling in cardiomyocytes (Figure 7a through 7c). Physiologically, CGB resides in the SR and shapes Ca^{2+} release from internal stores by modulating the activity of the $InsP_3R$. The generation of $InsP_3$ is triggered by various hypertrophic agonists, including Ang II. Ca^{2+} released from the SR by the $InsP_3R$ triggers the activation of Ca^{2+}-dependent transcription factors, including NF-κB, that initiate BNP transcription (ETC) (Figure 7a). Decreased CGB expression levels in cardiomyocytes lead to impaired CGB-$InsP_3R$ coupling that results in decreased $InsP_3$-dependent Ca^{2+} release, NF-κB activity, and BNP production (Figure 7b). Because we found that CGB expression is markedly upregulated in Ang II–induced cardiac hypertrophy in vivo and that CGB overexpression in cardiomyocytes leads to enhanced activity of the human BNP promoter, we hypothesize that CGB contributes to the increase in ventricular BNP production commonly associated with cardiac hypertrophy (Figure 7c). In this scenario, the upregulation of CGB expression in hypertrophic ventricles improves the CGB-$InsP_3R$ coupling, leading to increased $InsP_3$-dependent Ca^{2+} release, NF-κB activity, and BNP production.

In the present study, we provide insights into how CGB regulates $InsP_3$-dependent Ca^{2+} release, NF-κB activity, and BNP production at the cellular level in neonatal cardiomyocytes. Our results also show that upregulation of CGB occurs in vivo in adult hypertrophic cardiomyocytes, suggesting that CGB plays a role in the complex cellular processes involved in cardiac hypertrophy.

Acknowledgments

We are grateful to Wolfgang Boehmerle, Craig J. Gibson, Andjelka Celic, and Brenda DeGray for invaluable advice regarding the design of the experiments and thoughtful discussions and comments on the manuscript. We thank Peter Burch and Dr Anton Bennett for help with the adenoviral infection and Dr Richard Wojcikiewicz for generously providing the $InsP_3R$-2 antibody. We thank Dr Margot C. LaPointe (hBNPLuc), Dr Gabriel Nùñez (NF-κB-luc), and Dr Jeffery D. Molkentin (AdNFAT-luc) for kindly providing reporter constructs, as well as Dr Jean-Pierre Julien and Dr Makoto Urushitani for providing the full-length CGB plasmid.

Sources of Funding

Supported by grants from the NIH (to B.E.E. and F.J.G.), scholarships from the German National Merit Foundation (to F.M.H. and K.Z.), the Roland Ernst Foundation (to F.M.H.), and Fondo Nacional de Desarrollo Científico y Tecnológico grant 1060077 (to M.E.).

Disclosures

None.

References

1. Heineke J, Molkentin JD. Regulation of cardiac hypertrophy by intracellular signalling pathways. *Nat Rev Mol Cell Biol*. 2006;7:589–600.
2. Swynghedauw B. Phenotypic plasticity of adult myocardium: molecular mechanisms. *J Exp Biol*. 2006;209:2320–2327.
3. Go LO, Moschella MC, Watras J, Handa KK, Fyfe BS, Marks AR. Differential regulation of two types of intracellular calcium release channels during end-stage heart failure. *J Clin Invest*. 1995;95:888–894.
4. de Lemos JA, McGuire DK, Drazner MH. B-type natriuretic peptide in cardiovascular disease. *Lancet*. 2003;362:316–322.
5. Wilkins BJ, Dai YS, Bueno OF, Parsons SA, Xu J, Plank DM, Jones F, Kimball TR, Molkentin JD. Calcineurin/NFAT coupling participates in pathological, but not physiological, cardiac hypertrophy. *Circ Res*. 2004;94:110–118.
6. Hall G, Hasday JD, Rogers TB. Regulating the regulator: NF-kappaB signaling in heart. *J Mol Cell Cardiol*. 2006;41:580–591.

7. Baeuerle PA, Henkel T. Function and activation of NF-kappa B in the immune system. *Annu Rev Immunol*. 1994;12:141–179.
8. Helle KB. The granin family of uniquely acidic proteins of the diffuse neuroendocrine system: comparative and functional aspects. *Biol Rev Camb Philos Soc*. 2004;79:769–794.
9. Yoo SH, Oh YS, Kang MK, Huh YH, So SH, Park HS, Park HY. Localization of three types of the inositol 1,4,5-trisphosphate receptor/Ca(2+) channel in the secretory granules and coupling with the Ca(2+) storage proteins chromogranins A and B. *J Biol Chem*. 2001;276: 45806–45812.
10. Thrower EC, Choe CU, So SH, Jeon SH, Ehrlich BE, Yoo SH. A functional interaction between chromogranin B and the inositol 1,4,5-trisphosphate receptor/Ca2+ channel. *J Biol Chem.* 2003;278:49699–49706.
11. Choe CU, Ehrlich BE. The inositol 1,4,5-trisphosphate receptor (IP3R) and its regulators: sometimes good and sometimes bad teamwork. *Sci STKE.* 2006;2006:re15.
12. Jacob SN, Choe CU, Uhlen P, DeGray B, Yeckel MF, Ehrlich BE. Signaling microdomains regulate inositol 1,4,5-trisphosphate-mediated intracellular calcium transients in cultured neurons. *J Neurosci*. 2005;25: 2853–2864.
13. Wu X, Zhang T, Bossuyt J, Li X, McKinsey TA, Dedman JR, Olson EN, Chen J, Brown JH, Bers DM. Local InsP3-dependent perinuclear Ca2+ signaling in cardiac myocyte excitation-transcription coupling. *J Clin Invest*. 2006;116:675–682.
14. Uhlen P, Burch PM, Zito CI, Estrada M, Ehrlich BE, Bennett AM. Gain-of-function/Noonan syndrome SHP-2/Ptpn11 mutants enhance calcium oscillations and impair NFAT signaling. *Proc Natl Acad Sci U S A.* 2006;103:2160–2165.
15. LaPointe MC, Wu G, Garami M, Yang XP, Gardner DG. Tissue-specific expression of the human brain natriuretic peptide gene in cardiac myocytes. *Hypertension*. 1996;27:715–722.
16. Inohara N, Koseki T, Lin J, del Peso L, Lucas PC, Chen FF, Ogura Y, Nunez G. An induced proximity model for NF-kappa B activation in the Nod1/RICK and RIP signaling pathways. *J Biol Chem*. 2000;275: 27823–27831.
17. Huh YH, Jeon SH, Yoo JA, Park SY, Yoo SH. Effects of chromogranin expression on inositol 1,4,5-trisphosphate-induced intracellular Ca2+ mobilization. *Biochemistry*. 2005;44:6122–6132.
18. Urushitani M, Sik A, Sakurai T, Nukina N, Takahashi R, Julien JP. Chromogranin-mediated secretion of mutant superoxide dismutase proteins linked to amyotrophic lateral sclerosis. *Nat Neurosci.* 2006;9: 108–118.
19. Meffert MK, Chang JM, Wiltgen BJ, Fanselow MS, Baltimore D. NF-kappa B functions in synaptic signaling and behavior. *Nat Neurosci*. 2003;6:1072–1078.
20. Johenning FW, Zochowski M, Conway SJ, Holmes AB, Koulen P, Ehrlich BE. Distinct intracellular calcium transients in neurites and somata integrate neuronal signals. *J Neurosci*. 2002;22:5344–5353.
21. Dostal DE, Hunt RA, Kule CE, Bhat GJ, Karoor V, McWhinney CD, Baker KM. Molecular mechanisms of angiotensin II in modulating cardiac function: intracardiac effects and signal transduction pathways. *J Mol Cell Cardiol*. 1997;29:2893–2902.
22. Sadoshima J, Izumo S. Signal transduction pathways of angiotensin II-induced c-fos gene expression in cardiac myocytes in vitro. Roles of phospholipid-derived second messengers. *Circ Res*. 1993;73:424–438.
23. Wiese S, Breyer T, Dragu A, Wakili R, Burkard T, Schmidt-Schweda S, Fuchtbauer EM, Dohrmann U, Beyersdorf F, Radicke D, Holubarsch CJ. Gene expression of brain natriuretic peptide in isolated atrial and ventricular human myocardium: influence of angiotensin II and diastolic fiber length. *Circulation*. 2000;102:3074–3079.
24. Nakagawa O, Ogawa Y, Itoh H, Suga S, Komatsu Y, Kishimoto I, Nishino K, Yoshimasa T, Nakao K. Rapid transcriptional activation and early mRNA turnover of brain natriuretic peptide in cardiocyte hypertrophy. Evidence for brain natriuretic peptide as an "emergency" cardiac hormone against ventricular overload. *J Clin Invest*. 1995;96:1280–1287.
25. Lokuta AJ, Cooper C, Gaa ST, Wang HE, Rogers TB. Angiotensin II stimulates the release of phospholipid-derived second messengers through multiple receptor subtypes in heart cells. *J Biol Chem*. 1994;269: 4832–4838.
26. Valdes JA, Hidalgo J, Galaz JL, Puentes N, Silva M, Jaimovich E, Carrasco MA. NF-kappaB activation by depolarization of skeletal muscle cells depends on ryanodine and IP3 receptor-mediated calcium signals. *Am J Physiol Cell Physiol.* 2007;292:C1960–C1970.
27. Lipp P, Laine M, Tovey SC, Burrell KM, Berridge MJ, Li W, Bootman MD. Functional InsP3 receptors that may modulate excitation-contraction coupling in the heart. *Curr Biol*. 2000;10:939–942.
28. Mackenzie L, Roderick HL, Proven A, Conway SJ, Bootman MD. Inositol 1,4,5-trisphosphate receptors in the heart. *Biol Res*. 2004;37: 553–557.
29. Thrower EC, Hagar RE, Ehrlich BE. Regulation of Ins(1,4,5)P3 receptor isoforms by endogenous modulators. *Trends Pharmacol Sci*. 2001;22: 580–586.
30. Perez PJ, Ramos-Franco J, Fill M, Mignery GA. Identification and functional reconstitution of the type 2 inositol 1,4,5-trisphosphate receptor from ventricular cardiac myocytes. *J Biol Chem*. 1997;272:23961–23969.
31. Bare DJ, Kettlun CS, Liang M, Bers DM, Mignery GA. Cardiac type 2 inositol 1,4,5-trisphosphate receptor: interaction and modulation by calcium/calmodulin-dependent protein kinase II. *J Biol Chem.* 2005;280: 15912–15920.
32. Dolmetsch RE, Lewis RS, Goodnow CC, Healy JI. Differential activation of transcription factors induced by Ca2+ response amplitude and duration. *Nature*. 1997;386:855–858.
33. Liang F, Gardner DG. Mechanical strain activates BNP gene transcription through a p38/NF-kappaB-dependent mechanism. *J Clin Invest*. 1999; 104:1603–1612.