Online Data Supplement

Expanded Materials and Methods

Culture of neonatal rat ventricular cardiomyocytes

Cardiomyocytes were isolated from neonatal rat hearts as described previously.[1] Briefly, after anesthesia (dry ice/CO_2) neonatal rats were decapitated, the hearts removed, ventricles dissected from atrias and the tissue was pooled and rinsed. Dissociation of ventricular cardiomyocytes was achieved by sequential digestion of the minced ventricles with collagenase type II and pancreatin. Following enzymatic dissociation, the population of cardiomyocytes was selectively enriched by differential pre-plating for 2 hours at 37°C. After pre-plating which left fibroblasts on the initial plate, myocytes were seeded on gelatin coated vessels and coverslips. The protocol produces cultures of cardiomyocytes that are at least 95% pure.[1] To prevent fibroblast growth, growth medium was supplemented with 25 µmol/L 5-Bromo-2′-deoxyuridine (BrdU). Cardiomyocytes were cultured in growth medium containing D-MEM and Medium 199 in a 4:1 ratio supplemented with 10% FBS and 1% Penicillin-Streptomycin.

ANG-II micro-osmotic pump implantation

Age and sex-matched C57BL6 littermate mice were anesthetized with ketamine (100mg/kg) and xylazine (5mg/kg). An incision was made in the skin between the scapulae, and a small pocket was created by spreading the subcutaneous connective tissues apart using a hemostat. In each mouse a miniature osmotic pump (model 1002, Alza Co, Palo Alto, CA, USA), filled with ANG-II was inserted into the pocket with the flow moderator pointing away from the incision. The infusion rate was calculated as 1000ng/kg/min for 14 days. Control mice underwent the same surgical intervention without implantation of the osmotic pump. Cardiac hypertrophy was documented by heart left ventricular weight/body weight (LVW/BW) ratios measured at the time of sacrifice. Mice were anesthetized, hearts removed, and flash

frozen in liquid nitrogen and stored at -80℃ until ventricles were homogenized for use in assays.

Plasmids, luciferase reporter vectors, siRNA

The chimeric full-length (-1818 to +100) human BNP promoter luciferase reporter plasmid (hBNPLuc) was generously provided by Dr. Margot LaPointe (Henry Ford Hospital, Detroit, MI) and has been described in detail.[2] The NF-κB luciferase reporter plasmid carrying 6 tandem κB-sites, pBVIx-luc (NF-κB-luc), was generously provided by Dr. Gabriel Nùñez (The University of Michigan Medical School, Ann Arbor, MI).[3] The adenoviral NFAT–luciferase reporter construct (AdNFAT-luc) was kindly provided by Dr. Jeffery D. Molkentin (University of Cincinnati, Cincinnati, OH).[4] The pcDNA3 expression vector harbouring the full length murine CGB was kindly provided by Dr. Jean-Pierre Julien and Dr. Makoto Urushitani (Laval University, Quebec, Canada).[5] Empty pcDNA3 vector was purchased from Invitrogen (Carlsbad, CA). The pIRES2-DsRed2 Express Vector (DsRed) was purchased from Clontech (Mountain View, CA). CGB siRNA and negative control (i.e. non-targeting) siRNA were purchased from Ambion (Austin, TX). The CGB specific siRNA duplex sense and antisense sequences are described.[6]

Transient transfection and adenoviral infection

Transient transfection of DNA and siRNA was performed with Lipofectamine 2000 in OptiMEM using the manufacturer's protocol for DNA and siRNA transfections and DNA-siRNA co-transfections, respectively. Cardiomyocytes were transfected at day two after culture. Transfection was performed for 4 hours in OptiMEM followed by incubation overnight with complete growth-medium added. Adenoviral infection of cardiomyocytes with AdNFAT-luc was previously described.[1] Briefly, infection was performed for two hours 24 hours after siRNA transfection. Thereafter, medium was replaced with fresh growth medium and cultures were further incubated for 24 hours before harvesting and assaying. In siRNA experiments, three groups were studied (CGB siRNA, negative siRNA and mock treatment). "CGB siRNA"

and “negative siRNA” correspond to cells transfected with CGB specific siRNA[6] or negative (i.e. non-targeting) siRNA, respectively. “Mock” treatment refers to cells that did neither experience treatment with nucleotides nor were they treated with Lipofectamine 2000 but were otherwise treated identically.

Luciferase reporter assay

Cell lysates were generated and luciferase activity was determined using the Luciferase Assay System (Promega, Madison, WI) as described.[1] Luciferase activity was normalized to total protein concentration for each sample as determined by BCA protein assay (Pierce Biotechnology, Rockford, IL). Results are expressed as relative luminescent units per microgram of protein (RLU/µg) normalized to mock treated controls of each culture. Chelation of intracellular Ca^{2+} in NF-κB luciferase reporter experiments was accomplished as described[7] by loading cells with cell permeant acetoxymethyl ester (AM)-derivatives of BAPTA (200 µmol/L) for 1 hour at 37°C. Cells were then washed twice and incubated for another 30 minutes at 37°C to allow complete intracellular de-esterification of the chelator.

BNP ELISA

BNP ELISA experiments with cardiomyocyte or cardiac fibroblast cell culture supernatants and cell lysates were performed 48 hours after siRNA transfection or with supernatants/cell lysates from myocytes/fibroblasts at day 4 after culture from non-transfected cells, respectively. Before any experiment, media was replaced with fresh growth media. To monitor the effects of ANG-II on BNP expression and secretion, cardiomyocytes or fibroblasts were incubated for 4 hours with ANG-II (1 µmol/L). If inhibitors were used, cells were pre-incubated with the inhibitor (identified in the text) for 1 hour followed by a 4 hour incubation period with ANG-II. The following inhibitors and their respective concentrations were used: $InsP_3R$ inhibitor 2-aminoethoxydiphenylborate (2-APB), 25 µmol/L; AT_1-R receptor inhibitor Telmisartan (Telm), 1 µmol/L. Because 2-APB at a concentration of 100 µmol/L also inhibits the mitochondrial permeability transition pore[8] and blocks transient

receptor potential channels (TRPs) in the plasma membrane (Clapham DE, 2007, Clapham Lab Publications, http://clapham.tch.harvard.edu), we used a concentration four times lower. To monitor the effect of CGB knock-down on baseline BNP secretion, cells were incubated for 4 hours before collecting supernatants and/or cell lysates. Collected supernatants and cell lysates were immediately flash frozen in liquid nitrogen and stored at -80°C until ELISA assays were performed. Secretion and intracellular expression of BNP were assayed with the AssayMax Rat BNP-45 (rBNP-45) ELISA Kit (GENTAUR, Brussels, Belgium) using the manufacturer's protocol. Briefly, frozen samples were thawed, diluted, again supplemented with fresh protease inhibitors and added to the wells of the microplate. A new standard curve was prepared for each experimental day. Standard points and samples were prepared and determined as duplicates or triplicates. Absorbance was read using a microplate reader at a wavelength of 450 nm. Absolute rBNP-45 concentrations (ng/ml) were calculated according to the standard curve and normalized to the corresponding values for vehicle treatment or mock transfection, respectively.

Live cell calcium imaging

Cardiomyocytes were kept in growth medium without BrdU for 24 hours before imaging experiments were done. Cells were incubated (30 min at 37°C in 5% CO_2) in Hepes buffer containing the cell permeant Ca^{2+} indicator dyes Mag-Fluo-4/AM (5 µmol/L) or Fura Red/AM (10 µmol/L), respectively, together with 0.1% Pluronic F-127 (Invitrogen, Carlsbad, CA). The Hepes medium contained (in mmol/L): 145 NaCl, 5 KCl, 1 $MgCl_2$, 2.6 $CaCl_2$, 10 Hepes, 5.6 glucose; pH 7.4. Coverslips were mounted in a chamber (Warner Instruments, Hamden, CT) and transferred to a Zeiss LSM 510 NLO laser scanning confocal microscope equipped with Plan-Neofluar 40x/1.3 oil and Plan-Apochromat 63x/1.4 oil immersion objectives (Zeiss, Thornwood, NY). Images were acquired at 0.33 Hz. All imaging experiments were performed with the pinhole set to confocality (1.55 – 3.1 airy units; i.e. 1.5 µm < optical slice < 2.5 µm). Whole cell fluorescence was measured by defining each cell as one region of interest. Fluorescence intensities were quantified as inverse ratios of F_{min}/F_0 (i.e. 1/Mag-Fluo-$4_{Fmin/F0}$

and 1/Fura $Red_{Fmin/F0}$) with F_0 being the averaged fluorescence intensity obtained during the first 20 seconds of recording before drugs were applied. Ca^{2+} release velocity was determined by calculating the ratio of the change in fluorescence intensity upon ANG-II stimulation and the time elapsed ($\Delta y_{Fmax(local)-Fmin(local)}/\Delta x_{tmin-tmax}$). In representative traces, the fluorescence intensity ratio F/F_0 was plotted as a function of time. To study $InsP_3$ mediated Ca^{2+} release from internal stores we used the low-affinity ($K_{D,Ca2+}$ = 22 µmol/L) Ca^{2+} indicator dye Mag-Fluo-4, which is only bright where Ca^{2+} is high such as in the sarcoplasmic reticulum (SR) and nuclear envelope (NE). Differential loading of Mag-Fluo-4 into internal stores was verified by (i) a reticular pattern of Mag-Fluo-4 fluorescence (supplementary figure 1a), (ii) KCl induced depolarization of cardiomyocytes causing Ca^{2+} induced Ca^{2+} release (CICR) which resulted in a rapid drop of fluorescence intensity followed by a recovery to baseline (supplementary figure 1b) and (iii) by challenging cardiomyocytes with caffeine (5 mmol/L), a well known ryanodine receptor (RyR) agonist[9] (supplementary figure 1c). Depolarization also served as viability control and enabled us to distinguish between excitable cardiomyocytes and contaminating fibroblasts. Cells that did not respond to depolarization ($F_{max(local)}/F_{min(local)}$ < 1.5) were either damaged myocytes or fibroblasts and therefore excluded from evaluation. For depolarization in Ca^{2+} free imaging solution (0 Ca^{2+} plus 1 mmol/L EGTA in extracellular solution), KCl (50 mmol/L) was added together with $CaCl_2$ (3.6 mmol/L) to facilitate CICR. In imaging experiments with cells expressing DsRed (co-transfection), the high-affinity Ca^{2+} indicator Fura Red was used to assess cytosolic Ca^{2+} changes. This replacement of Mag-Fluo-4 was necessary due to an overlap of Mag-Fluo-4 and DsRed excitation and emission spectra that caused substantial quenching of Mag-Fluo-4 fluorescence by DsRed. Substantial quenching was not observed for the combination of DsRed with Fura Red.

Immunoblotting

Immunoblotting was performed as described.[10] Briefly, cell lysates were prepared using radioimmuno precipitation assay (RIPA) lysis buffer. Protein samples were separated by

SDS-PAGE followed by electrophoretic transfer onto polyvinylidene fluoride (PVDF) membranes for 3 hours at 80 V (4°C). Membranes were incubated with primary antibodies overnight (4°C). Primary antibodies against the following proteins and their respective dilutions were used: CGB, 1:1.000 (BD Bioscience, Franklin Lakes, NJ); $InsP_3R$-1, $InsP_3R$-2 and $InsP_3R$-3;[10] β-actin, 1:2.500; GAPDH-HRP, 1:2.000 (both Abcam, Cambridge, MA); Serca 2 ATPase, 1:500; cardiac RyR 1:500 (both Affinity BioReagents, Golden, CO). Incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (Bio-Rad, Hercules, CA) was done for 1 hour at room temperature. The bands were visualized using SuperSignal West Dura chemiluminescent detection reagent. β-actin and GAPDH served as loading controls. Protein expression was normalized to loading control and quantified by scanning densitometry using UN-SCAN-IT software (Silk Scientific Corporation, Orem, UT).

References

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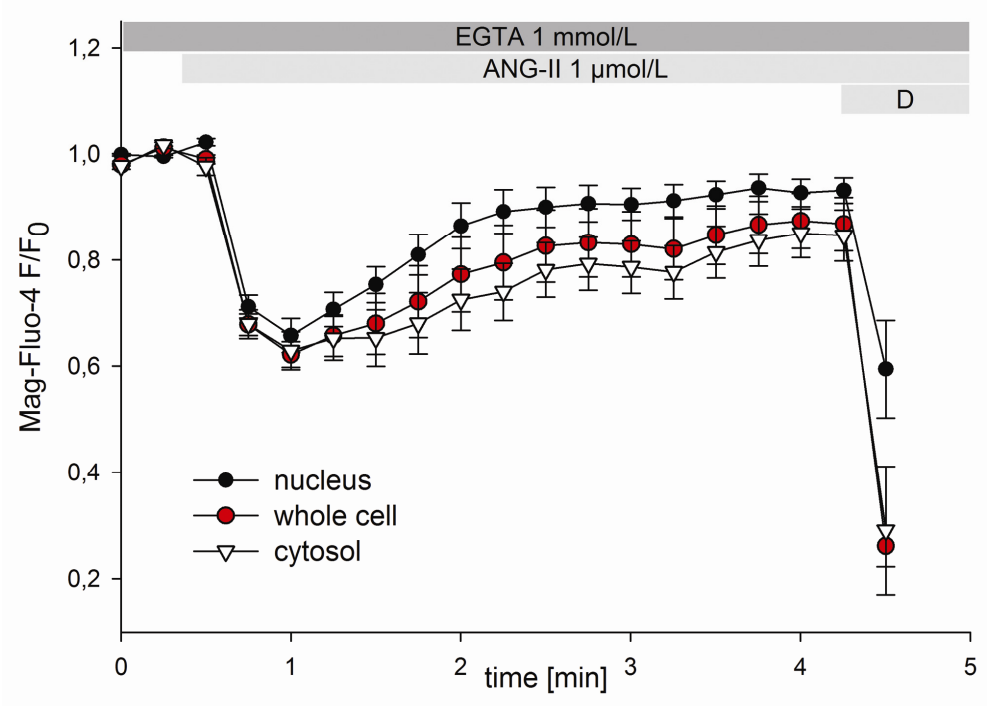
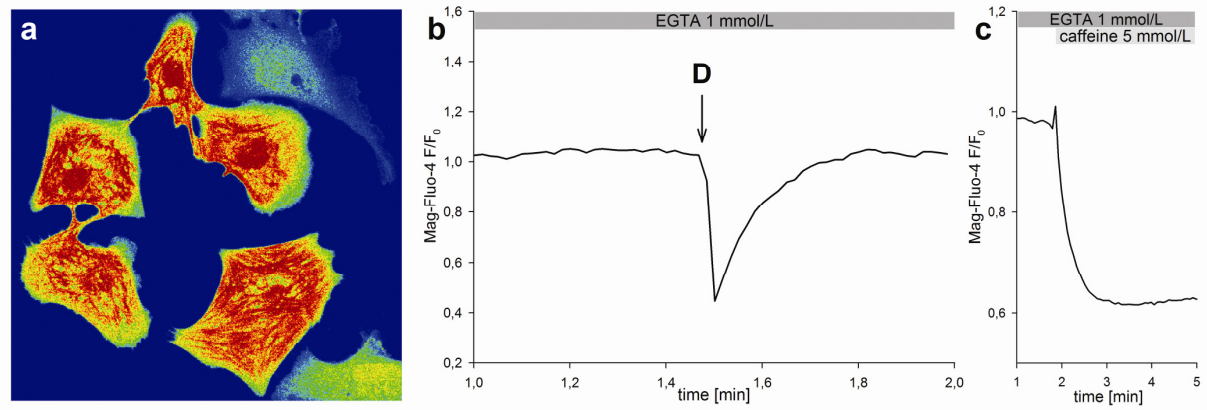
Supplementary Figures



Supplementary Figure 1. Whole cell F/F_0 is between the nuclear and cytosolic signal

Changes in sarcoplasmic reticulum (SR) and nuclear envelope (NE) Ca^{2+}-content in response to ANG-II (1 µmol/L) were monitored using the low-affinity ($K_{d,Ca2+}$ = 22 µmol/L) fluorescent Ca^{2+}-indicator dye Mag-Fluo-4/AM. Fluorescence was measured by defining regions of interests (ROIs) and quantified in relation to baseline fluorescence (F/F_0). The 6 cardiomyocytes shown in the representative pseudo-coloured image in figure 2d of the original manuscript were each assigned 3 distinct ROIs (nucleus, cytosol and whole cell) and changes in the fluorescence intensity (F/F_0) upon ANG-II stimulation were monitored separately. Data are presented as mean ± SEM; n=6 for each ROI. These results show that nucleus, cytosol and whole cell F/F_0 show similar changes in Ca^{2+} and that the nuclear signal does not dominate and does not behave differently from other components. These data show that whole cell F/F_0 is between the nuclear and cytosolic signal and therefore provides a representative read-out for our study.



Supplementary Figure 2. Loading of Mag-Fluo-4 into the cardiomyocyte ER and NE

(a) Reticular pattern of Mag-Fluo-4 fluorescence indicates loading of Mag-Fluo-4 into internal stores. Note faint staining of cardiac fibroblasts. (b) Cardiomyocytes loaded with Mag-Fluo-4 respond to depolarization ("D") with a rapid drop in fluorescence intensity followed by subsequent recovery as it would be expected for Ca^{2+} release and re-uptake into internal stores. (c) Cardiomyocytes challenged with caffeine (5 mmol/L), a well known RyR agonist,[9] show a rapid drop in Mag-Fluo-4 fluorescence due to Ca^{2+} release from internal stores.