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β -Adrenergic Receptor Stimulated *Ncx1* Upregulation is Mediated via a CaMKII/AP-1 Signaling Pathway in Adult Cardiomyocytes

Santhosh K. Mani^{1, #}, Erin A. Egan^{1, #}, Benjamin K. Addy¹, Michael Grimm³, Harinath Kasiganesan¹, Thirumagal Thiyagarajan¹, Ludivine Renaud¹, Joan Heller Brown³, Christine B. Kern², and Donald R. Menick^{1, *}

¹Gazes Cardiac Research Institute, Division of Cardiology, Department of Medicine, Medical University of South Carolina, 114 Doughty Street, Charleston, South Carolina 29425

²Gazes Cardiac Research Institute, Department of Cell Biology and Anatomy, Medical University of South Carolina, 114 Doughty Street, Charleston, South Carolina 29425

³Department of Pharmacology, University of California, San Diego, La Jolla, CA 92093

Abstract

The Na^+ - Ca^{2+} exchanger gene (*Ncx1*) is upregulated in hypertrophy and is often found elevated in end-stage heart failure. Studies have shown that the change in its expression contributes to contractile dysfunction. β -adrenergic receptor (β -AR) signaling plays an important role in the regulation of calcium homeostasis in the cardiomyocyte but chronic activation in periods of cardiac stress contribute to heart failure by mechanisms which include *Ncx1* upregulation. Here, using a Ca^{2+} /Calmodulin-Dependent Protein Kinase II ($\text{CaMKII}\delta_c$) null mouse, we demonstrate that β -AR-stimulated *Ncx1* upregulation is dependent on CaMKII. β -AR-stimulated *Ncx1* expression is mediated by activator protein 1 (AP-1) factors and is independent of cAMP-response element-binding protein (CREB) activation. The MAP kinases (ERK1/2, JNK and p38) are not required for AP-1 factor activation. Chromatin immunoprecipitation demonstrates that β -AR stimulation activates the ordered recruitment of JunB homodimers which then are replaced by c-Jun homodimers binding to the proximal AP-1 elements of the endogenous *Ncx1* promoter. In conclusion, this work has provided insight into the intracellular signaling pathways and transcription factors regulating *Ncx1* gene expression in a chronically β -AR-stimulated heart.

Introduction

The Na^+ - Ca^{2+} exchanger (NCX1) is one of the essential regulators of Ca^{2+} homeostasis within cardiomyocytes and is an important regulator of contractility. The exchanger catalyzes the electrogenic exchange of Ca^{2+} and Na^+ across the plasma membrane in either the Ca^{2+} -influx or Ca^{2+} -efflux mode. The exchanger is regulated at the transcriptional level in cardiac hypertrophy, ischemia and failure. There are multiple tissue-specific variants of the *Ncx1* gene resulting from alternative promoter usage (H1, K1, and Br1) and alternative

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*Corresponding author. Mailing address: Gazes Cardiac Research Institute, Medical University of South Carolina, 114 Doughty Street, Box 250773, Charleston, South Carolina 29425 Tel: 843-876-5045 Fax: 843-792-4762, menickd@musc.edu.

#Contributed equally to this work.

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splicing [1-3]. The H1 promoter directs cardiac-specific expression and we have identified many of the *cis* elements and transcription factors that have been demonstrated to be important in both regulation of cardiac expression and induction in response to pressure overload and α -adrenergic receptor stimulation [4, 5]. *Ncx1* is rapidly upregulated at the transcript and protein levels in response to pressure overload [6, 7] and in models of heart failure [8-12]. More importantly, both *Ncx1* mRNA and protein levels are significantly upregulated in human end-stage heart failure [13-16]. The diastolic performance of failing human myocardium correlates inversely with protein levels of NCX1 [17] and upregulation of *Ncx1* alone contributes directly to limiting SR loading and contractile dysfunction [18, 19]. In addition, *Ncx1* gene upregulation results in greater potential for delayed after depolarizations (DADs), which are major initiators of ventricular tachycardia [9, 20].

β -AR activation is common during times of cardiac stress. Initially this leads to increases in heart rate and contractility contributing to increased cardiac output. However, chronic β -AR stimulation leads to changes in cardiac gene expression and eventual heart failure. In congestive heart failure the heart is under intense sympathetic stimulation with very high levels of circulating norepinephrine [21, 22]. The changes in gene expression with chronic β -AR stimulation are the same as what is observed in heart failure [23-25]. Importantly, previous work has shown that *Ncx1* is upregulated at both the transcriptional and protein levels with β -AR stimulation in neonatal rat cardiomyocytes and in the adult rat heart [26, 27] but the signaling pathways and transcription factors that mediate this upregulation have not yet been identified.

The goal of the present study is to determine the mechanism of β -AR-induced upregulation of the cardiac *Ncx1* gene. We demonstrate that the majority of β -AR-stimulated *Ncx1* upregulation is mediated by a Ca^{2+} /calmodulin kinase II (CaMKII) dependent pathway resulting in the ordered recruitment of JunB followed by c-Jun homodimers to the proximal *Ncx1* AP-1 elements.

Materials and Methods

Adult Cardiomyocyte Cell Culture

Adult feline cardiomyocytes were isolated via a hanging heart preparation using enzymatic digestion and cultured by the protocols approved by the Institutional Animal Care and Use Committee as described previously [28]. The cardiomyocytes were plated on culture dishes that were coated with laminin at an initial plating density of 7.5×10^4 cells/ml.

Adenovirus Construction and Cell Infection

We utilized the AdEasy system to generate recombinant adenovirus plasmids [29]. The *1831Ncx1* and *184Ncx1* promoter-luciferase constructs were cloned into the promoterless pAdTrack vector as described [5]. Mutated constructs of the *1831Ncx1* promoter-luciferase construct were generated using QuikChange (Stratagene, La Jolla, CA) site-directed mutagenesis kit. The *1101Ncx1* construct was made by digesting the *1831Ncx1*/pGL2 construct with *Pst* I, deleting out the fragment from the pGL2 multi-cloning site to the *Pst* I site at 1101 in the *Ncx1* promoter. We took advantage of the unique *Xho* I site at position -1113 to engineer the $\Delta 1483-1113Ncx1$. Site-directed mutagenesis was used to engineer a second *Xho* I site at position -1483. *Xho* I digestion allowed for the excision of the -1483, -1229 and the -1121 AP-1 elements. *Hind* III sites were engineered at position -825 and -369 in the *1831Ncx1* and $\Delta 1483-1113Ncx1$ constructs. For both constructs, *Hind* III digestion resulted in the deletion of the -825 through -369 fragment of the *Ncx1* promoter. The deletion of the *Hind* III fragment in *1831Ncx1* produced the $\Delta 825-369Ncx1$ construct in which the -774, -581 and -548 AP-1 elements have been deleted. The deletion of the Hind

III fragment in the $\Delta 1483-1113Ncx1$ construct produced the $\Delta 6AP-1$ construct in which the -1483, -1229, -1121, -774, -581 and -548 AP-1 elements have been eliminated. Site-directed mutagenesis was used to disrupt the -1534 and -965 AP-1 like elements individually and together in the $\Delta 6AP-1$ construct to construct the $-965 AP-1$, $-1534 AP-1$ and $\Delta 8AP-1$ constructs respectively. The -1534 element was changed from (TATGTCA) to (ATTCAA) and the -965 element changed from (CGCGTCA) to (GCTCAA). The entire promoter region of each mutant construct was sequenced to ensure that they contained only the desired point mutations. Homologous recombination was carried out for each of the mutant *Ncx1* promoter constructs by transformation of *Escherichia coli* strain BJ5183 with the *Pme* I digested vector. The recombinant adenoviral DNA was digested with *Pac* I and transfected into HEK-293 cells. Viruses were plaque-purified, amplified and titers determined by the Gazes Adenoviral Core. Cardiomyocytes were infected on day 1 in culture by adding titrated adenovirus to the culture medium at different multiplicity of infection (MOI). After an infection of 8 hours the media was changed and the second adenovirus added if the experiment called for more than one virus. When more than one adenoviral construct was used to infect cells, experiments were carried out to ensure there was no competition for infection between the constructs at the MOIs used. Adult cardiomyocytes infected with MOIs of 1 resulted in the infection and gene transfer to greater than 85% of the plated cells based on GFP.

β -Adrenergic Infusions

Adult *1831Ncx1*-luciferase FVB/N mice [1] or adult *CaMKII δ ^{-/-}* C57BL/6 and *CaMKII δ ^{+/-}* littermates [30], were anesthetized and a small lateral incision was made on the back of the neck. The skin was bluntly dissected to form a pocket into which the micro-osmotic minipump (Alzet model 1003D, Durect Corp. Cupertino, CA) was implanted. The pumps were loaded with isoproterenol dissolved in 0.001 N HCl (calculated to deliver 30 mg/kg/day) or with vehicle alone. At the termination of the experiments the mice were anesthetized, and the heart was then removed and processed for either luciferase analysis or Western blot analysis.

Adult male Sprague-Dawley rats were anesthetized with a ketamine (65 mg/kg) and xylene (10 mg/kg) mixture. A small lateral incision was made on the back of the neck. The skin was bluntly dissected to form a pocket into which the micro-osmotic minipump (Alzet model 1003D, Durect Corp. Cupertino, CA) was implanted. The pumps were loaded with isoproterenol dissolved in 0.001 N HCl (calculated to deliver 3 mg/kg/day) or with vehicle alone. The dose of isoproterenol is sufficient to cause significant upregulation of the “fetal gene program” and result in cardiac hypertrophy [26, 31]. At the termination of the experiments the rats were anesthetized, and the heart was then removed and processed for RNA. All animal experimentation was performed in accordance with the NIH guidelines, and the Institutional Animal Use and Care Committee of the Medical University of South Carolina approved all protocols.

Chromatin Immunoprecipitation Assay

Forty-eight hours after infection with adenovirus constructs and/or β -adrenergic stimulation, cells were treated with 1% formaldehyde for 20 minutes at room temperature with slow rocking. ChIP assay was performed as described in the manufacturer's manual (Millipore, Billerica, MA) with some modifications [5]. Briefly, cells were washed two times with ice cold PBS, scraped and collected by centrifugation at 10,000 rpm for 2 minutes. The cell pellet was suspended in lysis buffer and incubated on ice for 20 minutes. The cell lysate was sonicated 10 times for 10 seconds each and the cell debris spun down. Preliminary data demonstrated that this sonication protocol resulted in the shearing of chromatin to 500-800 bp fragments (data not shown). The sample was pre-cleared and the immunoprecipitation

antibody [c-Jun (H-79), JunB (C-210), JunD (329), Fra1 (R-20), Fra2 (Q-20), from Santa Cruz Biotechnology Inc, Santa Cruz, CA; c-fos from Millipore, Billerica, MA] added to the supernatant and incubated overnight at 4°C. After immunoprecipitation, the eluted protein-DNA complexes were de-crosslinked by heating at 65°C for 4 hours. The DNA was ethanol precipitated and the DNA was suspended in 50 µl 10 mM Tris buffer. The feline *Ncx1* promoter was PCR amplified from the immunoprecipitated and non-immunoprecipitated chromatin using the following *Ncx1* promoter primers: A) proximal domain, sense -152 to -131 (5'-GTGTTGGATGAAGCGGAGAG-3') and antisense -14 to -34 (5'-AACATGGTTTGCATAGCTGGA-3'); B) mid-domain sense -1236 to -1217 (5'-CCATCCTTCCCCTTTTCCTC-3') and antisense -1059 to -1078 (5'-AGCCTTTTGTGTTCCAGTCC-3'); C) distal domain, sense -1673 to -1652 (5'-TGGGTTTGTGTGTGTAGAGT-3') and antisense -1463 to -1484 (5'-CTTCATTTCCCTCCGATACATT-3').

Preparation of Cell Lysates

Following treatment, cells were washed twice in sterile filtered cold 1× PBS. Cells were then lysed in 200 µl of Lysis buffer (20mM Tris, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1mM β-glycerol, 2.5mM Na phosphophate, 1% Triton X-100) for Western blot analysis, and for co-immunoprecipitation studies. Reporter Assay Buffer (Promega, Madison WI) was used for luciferase assays. Protease and phosphatase inhibitors were added to these buffers (1:100 dilutions of Phosphatase Inhibitor Cocktail I and II and Protease Inhibitor Cocktail from Sigma-Aldrich, St Louis, MO). The cells were then incubated on ice for 15 minutes, and insoluble material pelleted by centrifugation in a tabletop microcentrifuge at 4°C.

Western Blot Analysis

Protein concentrations were determined by the Bio-Rad protein assay. Cell lysates were subjected to SDS-PAGE, and Western blotting was performed with the appropriate antibodies. Antibodies to CaMKII, P-CaMKII, P-CREB, CREB, P-ERK and ERK were obtained from Cell Signaling Technology Inc (Danver, MA). Antibodies to NCX1 were obtained from Swant (Bellinzona, Switzerland). The proteins were visualized by enhanced chemiluminescence (ECL).

Luciferase and GFP Assay

To determine luciferase activity from adult cardiomyocytes or heart tissue, 5 µl of cell or tissue lysate was added to 50 µl of luciferin mixture (Promega, Madison, WI). Light emission was measured using an Auto Lumat LB 953 luminometer. To measure GFP fluorescence, 50 µl of crude lysate was added to 100 µl of PBS and the sample read in a fluorometer with excitation of 488 nm and emission of 510 nm. Luciferase readings were normalized by GFP expression.

RNA Isolation and Quantitative Real-Time-PCR Analysis

Total RNA was isolated from adult cardiomyocytes with TRIzol (Invitrogen, Carlsbad, CA). The relative amount of mRNA was measured by single-step RT-PCR using the Quantitect SYBR Green RT-PCR kit (Qiagen, Valencia, CA) and real-time PCR instrumentation (iCycler, Bio-rad, Hercules CA). The reverse transcription part of the reaction was performed on dilutions of each RNA sample at 50°C for 45 minutes using *Ncx1* (*Ncx1* sense 5'-CCAGGCAAGGAAGGCAGTCAG 3' and *Ncx1* antisense 5'-CGGAGAATGGTCAGGGCTACAG 3'). The synthesized cDNA was heated for one cycle at 95°C for 15 minutes followed by 35 cycles of real-time PCR consisting of denaturing for 15 seconds at 95°C, annealing for 30 seconds at 60°C, and elongation for 30 seconds at 72°C. The 18S RNA specific primers used were 18S sense 5'-

TATGGTTCCTTTGGTCGCTC-3' and 18S antisense 5'-GGTTGGTTTTGATCTGATAAATG-3'. To verify specificity of the RT-PCR product, a melt curve was generated at the end of each cycle to confirm T_m of the product and to monitor for non-specific products. The specificity of each primer set was further confirmed for the predicted base pair length by running the reaction products on 2% agarose gels. The data are presented as the fold in gene expression normalized to the 18S ribosomal RNA levels and relative to the untreated control. From the C_t values (defined as the threshold cycle at which the SYBR Green fluorescence exceeds background fluorescence), as automatically determined, for both genes the ΔC_t values were calculated as $C_{tNcx1} - C_{t18S}$. These data were analyzed by the following equation:

$$\Delta\Delta C_t = (C_{tNcx1} - C_{t18S})_{\text{isoproterenol treatment}} - (C_{tNcx1} - C_{t18S})_{\text{no treatment}}$$

Statistical Analysis

The results are presented as mean $\pm$ SD. The data were evaluated for significance by performing either an ANOVA or by unpaired t test where appropriate with p values < 0.05 considered significant.

Results

The *Ncx1* promoter is upregulated in response to β -AR stimulation in adult cardiomyocytes

Previous work has shown that *Ncx1* is upregulated at the transcriptional and protein level in the adult rat heart in response to β -AR stimulation [26]. Further, cardiomyocytes isolated from these hearts displayed an increased rate of intracellular Ca^{2+} removal via NCX1. This study did not identify the signaling pathway or transcription factors that mediate *Ncx1* upregulation. Both β_1 -AR and β_2 -AR are expressed in cardiomyocytes with β_1 being the predominant receptor [27]. Importantly, several studies have demonstrated that selective stimulation of these two receptors can result in different physiological responses ([32]; for review). Therefore, we tested whether *Ncx1* is upregulated in adult cardiomyocytes by β -AR stimulation and whether there are any differential effects on the exchanger expression in response to β_1 -AR versus β_2 -AR stimulation.

Adult feline ventricular cardiomyocytes were infected with pAdTrack adenovirus containing a full-length -1831 base pair wild-type *Ncx1* promoter luciferase reporter gene construct (*1831Ncx1*) and CMV-driven GFP [5] at an approximate MOI of 1 for 12 hours. The cells were subjected to stimulation with isoproterenol (0.1 μM) which stimulates both β_1 and β_2 -AR, dobutamine (1 μM) which predominately stimulates the β_1 -AR, or salbutamol (1 μM) which chiefly stimulates the β_2 -AR. After 24 hours of treatment, the cells were lysed, and the luciferase and GFP levels were measured. Figure 1A demonstrates that stimulation with either low levels of β_1 or β_2 -adrenergic agonist is sufficient to induce upregulation of *1831Ncx1*-promoter driven reporter gene expression. To determine if *Ncx1* upregulation is specifically mediated via β -AR, we utilized the β -AR antagonist propranolol. Propranolol treatment did not affect basal *Ncx1* expression, but propranolol completely eliminated both β_1 and β_2 stimulated upregulation of *Ncx1* (Fig 1A).

Ncx1 is upregulated in response to β -AR stimulation *in vivo*

We have used a transgenic mouse model to demonstrate that the 1831 bp *Ncx1HI* (*1831Ncx1*) promoter directs cardiac-specific expression of the exchanger in both development and in the adult, and is sufficient for the upregulation of *Ncx1* in response to pressure overload [1, 5]. We utilized the *1831Ncx1* transgenic mouse to ensure that our data

from adult cultured cardiomyocytes was consistent with an *in vivo* model of β -AR stimulation. The *1831Ncx1* transgenic mouse was treated with isoproterenol infusion via a micro-osmotic pump. After 3 days the hearts were removed and luciferase activity was recorded. β -AR stimulation resulted in a 1.7 fold increase over control animals (Fig 1B). This is similar to the upregulation we detected after 48 hours of pressure overload via transaortic constriction [1]. Finally, we wanted to ensure that the experiments performed with the *Ncx1* promoter-reporter gene construct were consistent with what is occurring with the endogenous *Ncx1* transcript. Here rats were administered isoproterenol via a micro-osmotic pump for 72 hours. Real-time PCR revealed that endogenous *Ncx1* when normalized to 18S rRNA was upregulated 1.9 fold with β -AR stimulation when compared to control animals (Fig. 1C).

cAMP activation of PKA is sufficient but not required for β -AR-stimulated *Ncx1* upregulation

β -AR stimulation can activate multiple signaling pathways in the adult cardiomyocyte. Many of the β -AR responses are mediated through cAMP activation of PKA. To determine the role of PKA in *Ncx1* gene regulation, adult cardiomyocytes were treated for 48 hours with either forskolin, which activates adenylyl cyclase. Treatment with 10 μ M forskolin upregulates *Ncx1* promoter-driven reporter gene expression by ~ 9 fold when compared to control levels (Fig. 2A). Therefore, cAMP activation of PKA is sufficient for *Ncx1* upregulation in adult cardiomyocytes. In order to determine if PKA activation is required for β -AR-stimulated changes in *Ncx1* promoter activity, cardiomyocytes were treated for 24 hours with dobutamine and/or the PKA inhibitor H-89. H-89 treatment alone did not alter basal *Ncx1* expression levels but only partially inhibited dobutamine-stimulated *Ncx1* upregulation (approximately 38%; Fig. 2B). H-89 at the same dose completely inhibited β -AR-stimulated PKA activation (data not shown).

Role of CaMKII

Recent studies have shown that CaMKII is required for β -AR stimulated changes in atrial natriuretic peptide (ANP), α -myosin heavy chain (α MHC), β -myosin heavy chain (β -MHC) and cardiac ankyrin repeat protein expression [25, 33]. The positive inotropic and lusitropic effects resulting from sustained β -AR stimulation have been shown to be primarily mediated by CaMKII [34]. Persistent β -AR stimulation of cardiomyocytes overexpressing β_1 -AR results in apoptosis, which is also mediated by CaMKII [35]. CaMKII is an important downstream target of Ca^{2+} in the *in vivo* hypertrophic signaling pathways and has been implicated in the regulation of many of the changes in gene expression seen in hypertrophy and heart failure. Therefore, we determined whether CaMKII mediates the β -AR-stimulated upregulation of *Ncx1*. Inhibition of CaMKII by KN93 dramatically repressed β -AR-mediated upregulation of *Ncx1* whereas the inactive analogue of this molecule, KN92 had no effect (Fig. 3A). Further, KN93 treatment resulted in the repressed *Ncx1* expression in control cells. Consistent with our finding, Western analysis shows a significant, robust autophosphorylation of CaMKII T287 with β -AR stimulation. CaMKII autophosphorylation is inhibited with KN93 treatment (Fig. 3B). There does appear to be a trace amount of activated CaMKII present in the control cells that may account for why KN93 treatment of control cells repressed *Ncx1* expression. We detected no significant difference in CaMKII protein levels under any of the conditions.

CaMKII δ deletion prevents β -AR stimulated *Ncx1* upregulation

In order to confirm that CaMKII is required for β -AR stimulated NCX1 upregulation, we conducted studies in mice lacking the predominate cardiac CaMKII isoform, CaMKII δ [30]. Mice were treated with isoproterenol (30mg/kg/d) and assessed after 3d. As expected, NCX1 protein was significantly upregulated in the isoproterenol treated WT littermates

when compared to vehicle treated WT mice. Strikingly, however, there is no NCX1 upregulation seen in the isoproterenol treated CaMKII^{-/-} mice when compared to vehicle treated controls (Fig. 4). Western blots of representative mouse ventricles show NCX1 levels for each of the treatments and demonstrate that no CaMKII δ is expressed in the KO mice.

β -AR-stimulated *Ncx1* expression is independent of CREB activation

Activation of transcription by β -AR stimulation is classically mediated through AP-1 and/or CREB transcription factors. Although the feline *Ncx1* promoter does not contain any consensus AP-1 elements, it contains eight AP-1-like motifs at -1534, -1422, -1229, -1121, -965, -774, -581 and at -546 (Fig 5A). The *Ncx1* promoter contains one conserved CRE (CGTCA) at -965 and three CRE-like elements at -790 (CGTCT), -581 (GGTCA) and -522 (CGTCT). The difference between CRE and AP-1 consensus sequences is only one nucleotide (CRE: TGACGTCA, AP-1: TGA(C/G)TCA), which allows for cross talk between AP-1 and CREB transcription factors [36, 37]. The *Ncx1* -965 and -581 are overlapping CRE/AP-1 elements. Activation of CREB by phosphorylation of S113 was initially thought to be dependent on PKA [38]. More recent work has shown that it can be activated via several kinases including p90RSK [39], Akt [40], PKC [41] and CaMKII [42]. In order to determine if CREB was required for *Ncx1* upregulation, we examined whether CREB phosphorylation was inhibited by KN93. Figure 5B shows that CREB is phosphorylated by β -AR stimulation in adult cardiomyocytes. But importantly, KN93 treatment, which completely inhibits β -AR-stimulated *Ncx1* upregulation, does not affect CREB phosphorylation. Using three primer sets (Fig 5C) that cover the conserved CRE at -965 and the three CRE-like elements at -790, -581 and -522, chromatin immunoprecipitation demonstrated that CREB is not associated with the *Ncx1* promoter in control or β -AR-stimulated cardiomyocytes (Fig. 5C). Therefore activated CREB is not required for *Ncx1* upregulation in feline adult cardiomyocytes.

β -AR-induced *Ncx1* expression is mediated via the AP-1 elements

Many studies have shown that CaMKII mediates AP-1 transactivation of gene expression [43-45]. All of the *Ncx1* promoter AP-1-like elements are distal to the minimal 184 bp that we have demonstrated to be sufficient for the correct spatial temporal pattern of NCX1 expression in the developing and adult heart [5]. We first used the minimal 184*Ncx1* promoter that has no AP-1 elements to test whether they are required for β -AR-stimulated *Ncx1* upregulation. As expected, cardiomyocytes infected with the 184*Ncx1* promoter show no β -AR-stimulated upregulation (Data not shown). Deletion of the distal 721 bp of the *Ncx1* promoter, eliminating the distal AP-1 elements, -1534 AP-1, -1422 AP-1, -1229 AP-1, and -1121 AP-1 ($\Delta 1101$ *Ncx1*) did not prevent β -AR-stimulated upregulation (Fig. 6). A second construct in which -1422 AP-1, -1229 AP-1, and -1121 AP-1 were deleted ($\Delta 1483-1113$ *Ncx1*) also did not prevent β -AR-stimulated upregulation. Deletion of the proximal AP-1 elements, -774 AP-1, -581 AP-1, and -546 AP-1 ($\Delta 825-369$ *Ncx1*) also had no significant affect on β -AR-stimulated upregulation. When 6 of the 8 AP-1 elements are eliminated ($\Delta 6$ AP-1 construct, which eliminates -1422 AP-1, -1229 AP-1, -1121 AP-1, -774 AP-1, -581 AP-1, -546 AP-1) the level of β -AR-stimulated *Ncx1* upregulation is slightly lower. Using the $\Delta 6$ AP-1 construct, we mutated each of the remaining AP-1 elements individually resulting in two adenovirus constructs with only one AP-1 element (-965 AP-1, and -1534 AP-1) respectively and together resulting in one construct in which all the AP-1 elements have been mutated or deleted ($\Delta 8$ AP-1). Importantly, both constructs containing only one of the eight AP-1 elements (-965 AP-1 and -1534 AP-1) were upregulated by β -AR stimulation. Only when all eight AP-1 elements were deleted or mutated ($\Delta 8$ AP-1) was the majority of the β -AR-induced *Ncx1* upregulation prevented. Taken together, our data suggest that although no specific AP-1 element is required, a single

AP-1 element (either -965 AP-1 or -1534 AP-1) is sufficient for the majority of the β -AR-stimulated upregulation.

CaMKII activation of AP-1 is independent of MAP kinase pathways

AP-1 is composed of either homodimers of the Jun family (c-Jun, JunB and JunD) or heterodimers of the Fos and Jun families (c-Fos, Fra-1, Fra-2 and FosB). CaMKII activation leads to phosphorylation of ERK1/2, the subsequent upregulation of c-Fos and an increase in the transactivation of genes regulated by AP-1. ERK1/2 is activated in many cell types in response to β -AR stimulation and regulates the upregulation of c-Fos [46]. We used the MEK1 inhibitor UO126 and overexpression of the ERK phosphatase, MKP3 to determine if β -AR-stimulated-ERK1/2 activation is required for *Ncx1* upregulation. Treatment of adult cardiomyocytes with either UO126 or the overexpression of MKP3 does not affect β -AR-stimulated *Ncx1* upregulation (Fig. 7A). Importantly, treatment with UO126 and MKP3 overexpression prevented β -AR-stimulated-ERK1/2 activation. We have shown that the stress activated kinase, p38 plays an important role in *Ncx1* upregulation after α -AR stimulation [4]. p38 is activated in cardiac hypertrophy [47] and multiple studies have demonstrated that both α and β -AR-stimulated pathways activate p38 in cardiomyocytes [48-50]. Therefore, we examined whether p38 is important for mediating β -AR-stimulated *Ncx1* upregulation. As expected, p38 is activated by β -AR stimulation of adult cardiomyocytes (Fig 7B). Treatment with the p38 inhibitor, SB 203580 did not significantly alter basal or dobutamine-stimulated *Ncx1* upregulation. Further, overexpression of a dominant negative p38 (DNp38) [4] did not inhibit β -AR-stimulated *Ncx1* upregulation. Importantly, overexpression of DNp38 or SB 203580 treatment inhibited β -AR-stimulated-p38 activation (Fig 7B).

Following α and β -AR stimulation, the N-terminal Jun kinases, JNK1/2 are activated leading to the phosphorylation of c-Jun on S63 and S73 located in the transactivation domain. Surprisingly, treatment of adult cardiomyocytes with the JNK inhibitor SP00125 alone induced *Ncx1* upregulation. Further, SP00125 treatment did not inhibit but slightly increased the β -AR-stimulated *Ncx1* upregulation (Fig 7C). Taken together our results demonstrate one of two possibilities. Either CaMKII mediated AP-1 transactivation is independent of ERK1/2, p38 and JNK1/2 in adult cardiomyocytes, or activation of AP-1 is not required for β -AR-induced *Ncx1* expression. Therefore, we directly tested whether the *Ncx1* AP-1-like elements mediated β -AR-stimulated upregulation.

JunB and c-Jun bind sequentially to the proximal AP-1 elements of the *Ncx1* promoter

In order to determine if any AP-1 factors, bind to specific *Ncx1* AP-1-like motifs and investigate the dynamics of β -AR-stimulated *Ncx1* upregulation, we performed ChIP. After the prescribed dobutamine treatment time, cardiomyocytes were fixed in 1% paraformaldehyde and then the chromatin sheared by sonication. ChIP assays were performed to study the recruitment of c-Jun, JunB, JunD, c-Fos, Fra1 and Fra2 to AP-1 sites on the endogenous *Ncx1* promoter in response to β -AR stimulation. Three sets of primers covering the distal, mid and proximal *Ncx1* promoter were used (Fig. 8A). As expected, no AP-1 factor was bound to the *Ncx1* promoter in untreated cells. In order to assess the recruitment of transcription factors to the *Ncx1* promoter in response to β -AR stimulation, we examined the kinetics of transcription factor recruitment. Figure 8B demonstrates that JunB is recruited to the endogenous *Ncx1* promoter within 30 min of β -AR stimulation. Interestingly, it is only detected in the proximal domain of the promoter. JunB dissociates from the promoter somewhere between 30 and 60 minutes after β -AR stimulation. c-Jun is first detected 2 hours after β -AR stimulation and remains associated with the *Ncx1* proximal promoter out to 4-6 hours after stimulation. Importantly, we did not detect JunD, c-Fos, Fra1 or Fra2 associated with any region of the *Ncx1* promoter under any of the conditions (data

not shown). Because shearing results in DNA fragments around 500-800 bp, the data are consistent with the sequential binding of JunB and c-Jun to either the -546 AP-1 or -581 AP-1 element. The -1236 to -1078 primer set would have detected binding if JunB or c-Jun were associated with the -774 AP-1.

CaMKII inhibition prevents c-Jun binding to the proximal AP-1 element in the *Ncx1* promoter

Our work has demonstrated that inhibition of CaMKII prevents *Ncx1* upregulation in response to β -AR stimulation and that AP-1 elements are required for β -AR-stimulated *Ncx1* upregulation. We carried out ChIP analysis to examine whether inhibition of CaMKII with KN93 blocked the binding of c-Jun with the *Ncx1* promoter. Significantly, treatment of adult cardiomyocytes with KN93 prevented the recruitment of c-Jun to the proximal AP-1 elements of the *Ncx1* promoter in response to β -AR stimulation (Fig. 9).

Discussion

There are three major findings in this study. First, the β -AR-stimulated upregulation of *Ncx1* is dependent on CaMKII activation in the adult heart. Second, the majority of β -AR-stimulated *Ncx1* upregulation is mediated via AP-1 elements. Finally, ChIP analysis demonstrates that β -AR stimulation activates an ordered recruitment of JunB, which then is replaced by c-Jun binding to the proximal AP-1 elements of the endogenous *Ncx1* promoter. Our results show for the first time the signaling pathway, transcription factors and promoter elements required for the upregulation of *Ncx1* expression in response to β -AR stimulation in adult cardiomyocytes.

β -AR activation is one of the most important pathways regulating E-C coupling in the heart. β -AR stimulation results in increased amplitude and rate of cardiomyocyte $[Ca]_i$ at each beat resulting in increased contractility. CaMKII is activated by β -AR stimulation in response to the increase in level and frequency of calcium transients (for review;[51]). There are four CaMKII isoforms, α , β , γ , and δ . CaMKII δ is the predominant form in the heart and the α and β isoforms are expressed only in nerve tissue [52]. CaMKII phosphorylates several downstream targets in common with cAMP-activated PKA including the ryanodine receptor, phospholamban, and L-type Ca^{2+} channel complex, and thus also plays an important role in regulating E-C coupling in the heart [51, 53-55]. Recently CaMKII has been shown to phosphorylate Na^+ channels which may contribute to arrhythmogenesis in heart failure [56]. CaMKII is activated in hypertrophy and has been shown to induce dilated cardiomyopathy and heart failure [57, 58]. In addition to acutely modulating calcium influx, SR calcium release and uptake, chronic activation of CaMKII results in the induction of the fetal gene program, which is expressed in cardiac hypertrophy and failure. It is significant that forskolin treatment, which is downstream of $G\beta\gamma$ -regulated effectors is sufficient to stimulate *Ncx1* upregulation. These data suggest that cAMP is sufficient for mediating CaMKII activation in adult cardiomyocytes. Our findings that β -AR-stimulated *Ncx1* upregulation is dependent on CaMKII further illuminates the important role CaMKII has in the chronic dysregulation of cardiac Ca^{2+} homeostasis and E-C coupling. Transgenic overexpression of the cytosolic splice variant, CaMKII δ_c , induces severe heart failure associated with SR Ca^{2+} leak and reduced SR Ca^{2+} content [57]. Correspondingly, the upregulation of *Ncx1* contributes directly to limiting SR loading, contractile dysfunction and greater potential for delayed after depolarizations which lead to ventricular tachycardia [9, 18-20]. Further, numerous studies of human and animal models of heart failure demonstrate that diastolic performance in the failing heart, correlates inversely with protein levels of NCX1 [17, 20, 59]. Importantly, inhibition of CaMKII activity prevents cardiac arrhythmias and suppresses after depolarizations [60].

β -AR-stimulated changes in cardiomyocyte gene expression are classically mediated by the cAMP-responsive element binding protein (CREB) and activating-protein-1 (AP-1) transcription factors which bind respectively to CRE and AP-1 promoter elements. CREB is activated by phosphorylation of S133 by either PKA or CaMKII resulting in CREB-stimulated transcription via CRE and AP-1 sites on select promoters [61]. As we anticipated, CREB's S133 is phosphorylated with β -AR-stimulation in adult cardiomyocytes. The difference between CRE and AP-1 consensus sequences is only one nucleotide (CRE: TGACGTCA; AP-1: TGA(C/G)TCA) and as expected, crosstalk between CREB and AP-1 transcription factors does occur [36, 62]. Because β -AR-stimulated, CaMKII-dependent *Ncx1* upregulation was seen via AP-1 elements, CREB could be responsible. But, KN93 treatment, which inhibits all CaMK's and *Ncx1* upregulation had no effect on CREB S-133 phosphorylation levels. Conclusively, ChIP analysis demonstrated that CREB does not bind to the proximal 2 Kb of the endogenous *Ncx1* promoter. Therefore, CREB does not appear to play a role in β -AR-stimulated *Ncx1* upregulation.

The members of the AP-1 family, (c-Jun, JunB, JunD c-Fos, Fra-1, Fra-2 and FosB), interact with the promoter elements on many genes either as AP-1 homo- or heterodimers or as heterodimers via their leucine zipper domain with other transcription factors (including CREB/AT, NF- κ B and NFAT) and coactivators (including CBP, PCAF and p300) [63, 64]. The composition of the AP-1 homo- or heterodimer bound to the promoter may affect which transcription factors and coactivators are recruited. Although, *in vitro* promoter analysis showed that β -AR induction was not lost until all eight AP-1 elements had been eliminated, ChIP analysis indicated that JunB and c-Jun preferentially bind to one or both of the two proximal AP-1-like elements (-581, -546) on the endogenous promoter. We postulate that our ChIP data best represents the *in vivo* mechanism but it is clear that any of the other AP-1 like elements are sufficient for mediating *Ncx1* upregulation. Our kinetic evaluation of AP-1 binding to the *Ncx1* promoter shows the ordered and sequential recruitment of JunB homodimers (30 minutes β -AR stimulation) that dissociate before c-Jun homodimers are recruited (after 2 hours of β -AR stimulation). Stimulation of AP-1 activity by β -AR agonist, 12-O-tetradecanoyl phorbol-13-acetate, and growth factors usually proceed through two steps: first, activation of the basally expressed JunB and c-Jun by phosphorylation and second by the activated c-Jun subsequently inducing its own transcription forming a post auto-regulatory loop. It appears that the upregulation of c-Jun may be required for it to replace JunB on the *Ncx1* promoter. Importantly, KN-93, which inhibits β -AR stimulated *Ncx1* upregulation also prevents the association of c-Jun with the endogenous *Ncx1* promoter. It is significant to note that our ChIP analysis indicates that c-Jun is associated with the *Ncx1* from 1-4 hr after β -AR stimulation. Our data suggests that for initial upregulation, JunB and c-Jun binding to the *Ncx1* promoter is required. However additional transcription factor binding may be requisite to sustain *Ncx1* upregulation. We are currently examining what other transcription factors may be recruited with or subsequent to JunB and c-Jun, which may be responsible for this sustained upregulation.

c-Jun and JunB are known to be phosphorylated primarily by the Jun N-terminal kinases (JNKs) [63]. Interestingly, our data shows that in adult cardiomyocytes c-Jun activation by CaMKII appears to be independent of JNK. The nuclear CaMKII δ_B splice variant, shuttles into the nucleus where it is known to phosphorylate nuclear factors such as HDAC4 at serine 467 and serine 632 [65]. Although it is possible that CaMKII may directly phosphorylate JunB and c-Jun, our data does not discriminate whether it is direct or whether intermediate signaling factors are required.

In summary, this work adds to the list of downstream adverse affects of chronic β -AR stimulation events, mediated by the activation of CaMKII. Chronic CaMKII activation induces fetal gene expression, which contributes to contractile dysfunction and cardiac

failure. Our data also demonstrates that chronic CaMKII activation leads to the upregulation of *Ncx1*, which is known to directly contribute to limiting SR Ca^{2+} loading, contractile dysfunction and thus a greater potential for ventricular tachycardia [9, 18-20]. In addition this work is the first to identify the transcription factors, promoter elements and the sequential dynamics of transcription factor recruitment through which this is mediated. These new insights into the regulation of *Ncx1* expression may add to our general understanding of the highly dynamic nature of cardiac gene expression.

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Figure 1. Stimulation of either β_1 -AR or β_2 -AR agonist is sufficient to induce the upregulation of *Ncx1*

A) Freshly isolated adult feline cardiomyocytes were infected with adenovirus expressing *1831Ncx1* promoter-luciferase reporter construct (MOI 1.5). Cells were treated with or without β_1 and β_2 -AR agonist 0.1 μ M isoproterenol (Iso), β_1 -AR agonist 1 μ M dobutamine (dob) or β_2 -AR agonist 1 μ M salbutamol (Sal) for 24 hours. Cardiomyocytes were also pretreated with propranolol (Prop) for 30 minutes and then subjected to β_1 -AR and β_2 -AR agonist treatment for 24 hours. Cells were lysed in reporter buffer and luciferase activity was determined. The values are expressed as relative luciferase activity normalized to GFP levels (RLU/GFP). * $p < 0.001$ when compared to control, (experiments performed in duplicate from 6 independent cell isolations) # $p < 0.001$ when compared to β -AR stimulations, (experiments performed in duplicate from 6 independent cell isolations). **B)** Isoproterenol (30 mg/kg/day) or vehicle control was continuously infused into *1831Ncx1* promoter-luciferase transgenic mice. After 72 hours, the mice were euthanized and luciferase activity was determined as described in the methods. Relative luciferase units were normalized to protein levels (RLU/Protein). Values are the mean $\pm$ S.E.M (n=4 mice for each condition). * $p < 0.05$ when compared to control. **C)** Isoproterenol (3.0 mg/kg/day) or vehicle control was continuously infused into adult male rats by osmotic pump. After 72 hours of β -adrenergic infusion, the rats were euthanized and total RNA isolated from the hearts. Relative endogenous *Ncx1* transcript levels were quantified by real time RT-PCR. Relative *Ncx1* mRNA levels were extrapolated from a standard curve and were normalized to 18S rRNA. Values are the mean $\pm$ S.E.M (n=4 rats for each condition). * $p < 0.01$ when compared to control.

Figure 2. PKA activation is not required for *Ncx1* upregulation

Adult cardiomyocytes were infected with *183/Ncx1* promoter-luciferase adenovirus (MOI 1.5). **A)** Cells were subsequently treated with 1 μ M dobutamine or 10 μ M forskolin for 48 hours. **B)** Infected cardiomyocytes were treated with or without 1 μ M dobutamine for 24 hours and/or PKA inhibitor H-89 (20 μ M). Cells were lysed in reporter buffer and luciferase activity was determined. The values are expressed as relative luciferase activity normalized to GFP levels (RLU/GFP). Values are the mean $\pm$ S.E.M (experiments performed in triplicate from 3 independent cell isolations). * $p < 0.05$ when compared to control and # $p < 0.05$ when compared to dobutamine treated.

Figure 3. CaMKII mediates β -AR-stimulated *Ncx1* expression

A) Isolated adult cardiomyocytes were infected with the *1831Ncx1* adenovirus (MOI 1.5). Infected cardiomyocytes were treated with or without 1 μ M dobutamine for 24 hours and/or CaMKII inhibitor KN93 (10 μ M) or analog to KN93, KN92 (10 μ M) as indicated. Cells were lysed in reporter buffer and luciferase activity was determined. The values are expressed as relative luciferase activity normalized to GFP levels (RLU/GFP). * $p < 0.05$ when compared to control and # $p < 0.05$ when compared to dobutamine treated (experiments performed in duplicate from 6 independent cell isolations). **B)** Cell lysates from each condition were run on separate SDS-PAGE gels followed by Western blotting with antibodies against either phospho-T287 CaMKII or CaMKII. The graph represents normalized P-CaMKII levels determined by Western analysis from four separate experiments. Values are the mean $\pm$ S.E.M., * $p < 0.05$ when compared to control and # $p < 0.05$ when compared to dobutamine treated.

Figure 4. CaMKII is required for β -AR-stimulated *Ncx1* expression

β -AR agonist isoproterenol (30 mg/kg/day) or vehicle was injected by IP to wild-type and CaMKII $\delta^{-/-}$ mice for 72 hour. NCX levels (Upper panel) and CaMKII δ expression levels (lower panel) were measured by Western blot. The graph represents the NCX1 protein level normalized to total protein. Values are the mean $\pm$ S.E.M, n=4 mice for each group * $p < 0.008$ versus wild-type).

Figure 5. CREB does not mediate β -AR-stimulated *Ncx1* expression

A) Depicts the AP-1 and CRE binding elements in the *Ncx1* promoter. **B)** Cell lysate samples for each condition were run on separate SDS-PAGE gels followed by Western blotting with antibodies against either phospho-S113 CREB or CREB, (experiments performed from 4 independent cell isolations). **C)** Diagram of *Ncx1* promoter depicts the presence of CRE elements and the proximal, middle (mid) and distal primers used for ChIP. **D)** ChIP shows that CREB does not associate with *Ncx1* promoter. Freshly isolated adult cardiomyocytes were treated with dobutamine (1 μ M) and/or KN93 (10 μ M) for 2 hr. Following crosslinking and sonication, cell extracts were immunoprecipitated with CREB antibody. A negative control using rabbit IgG as the precipitating antibody was run to demonstrate the specificity of the ChIP assay. Immune complexes were eluted, decrosslinked and then analyzed by PCR with primer sets specific for the proximal, mid and distal regions of the *Ncx1* promoter. Input chromatin was subjected to PCR as a positive control (experiments performed from 3 independent cell isolations).

Figure 6. Identification of AP1 sites mediating β -AR-stimulated *Ncx1* upregulation

Schematic of *Ncx1* promoter illustrating the AP1 consensus sites, the deletions and site-specific point mutations used to evaluate the role of AP-1 elements in β -AR-stimulated upregulation of *Ncx1* (**Upper panel**). Adenoviral constructs for these mutations were made and used to infect freshly isolated adult cardiomyocytes at a MOI of 1.5. Cells were stimulated with or without 1 μ M dobutamine for 24 hours (**Lower panel**). Cells were lysed in reporter buffer and luciferase activity was determined. The values are expressed as relative luciferase activity normalized to GFP levels (RLU/GFP) * $p < 0.001$ when compared to control, # $p < 0.001$ when compared to β -AR stimulations (experiments performed in triplicate from 4 independent cell isolations).

Figure 7. MAP Kinases are not required for β -AR-stimulated upregulation of *Ncx1*

Isolated adult cardiomyocytes were infected with the *1831Ncx1* adenovirus (MOI 1.5). Cells were then incubated for 24 hours with 1 μ M dobutamine in the presence or absence of: **A)** the MEK inhibitor 5 μ M U0126 or co-infected with MKP3 adenovirus (MOI 4), **B)** the p38 inhibitor 10 μ M SB202190 (SB) or co-infected with DNp38 adenovirus (MOI 4), **C)** the JNK inhibitor 10 μ M SP600125 (SP). Cells from each treatment were lysed in reporter buffer and luciferase activity was determined. The values are expressed as relative luciferase activity normalized to GFP levels (RLU/GFP) * $p < 0.05$ when compared to control, (experiments performed in duplicate from 4 independent cell isolations in each case).

Figure 8. β -AR-stimulation initiates binding of AP1 transcription factors c-Jun and JunB to the endogenous *Ncx1* proximal promoter

A) Diagram of *Ncx1* promoter depicts the presence of AP1 elements and the proximal, middle (mid) and distal primers used for ChIP. **B)** Freshly isolated adult cardiomyocytes were treated with dobutamine (1 μ M) for times indicated. Following crosslinking and sonication, cell extracts were immunoprecipitated with JunB, and c-Jun antibodies. A negative control using rabbit IgG as the precipitating antibody was run to demonstrate the specificity of the ChIP assay. Immune complexes were eluted, decrosslinked and then analyzed by PCR with primer sets specific for the proximal, mid and distal regions of the *Ncx1* promoter. Input chromatin was subjected to PCR as a positive control (experiments performed from 7 different cell isolations).

Figure 9. Inhibition of CaMKII blunts c-Jun binding of the *Ncx1* promoter

ChIP assays were performed from primary adult feline cardiomyocytes in the presence or absence of CaMK inhibitor KN93 (10 μ M) and/or dobutamine (1 μ M) for 2 hours using c-Jun antibody. PCR was performed with *Ncx1* proximal promoter primers. Input chromatin was subjected to PCR to control for variations in immunoprecipitation starting material. Rabbit IgG was used as a negative control for nonspecific binding, (experiments performed from 4 different cell isolations).