Published in final edited form as:

J Mol Cell Cardiol. 2012 November ; 53(5): 657–667. doi:10.1016/j.yjmcc.2012.08.005.

Ca²⁺ influx through L-type Ca²⁺ channels and transient receptor potential channels activate pathological hypertrophy signaling

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Abstract

Common cardiovascular diseases such as hypertension and myocardial infarction require that myocytes develop greater than normal force to maintain cardiac pump function. This requires increases in [Ca²⁺]. These diseases induce cardiac hypertrophy and increases in [Ca²⁺] are known to be an essential proximal signal for activation of hypertrophic genes. However, the source of “hypertrophic” [Ca²⁺] is not known and is the topic of this study. The role of Ca²⁺ influx through L-type Ca²⁺ channels (LTCC), T-type Ca²⁺ channels (TTCC) and transient receptor potential (TRP) channels on the activation of Calcineurin (Cn) – Nuclear Factor of Activated T cells (NFAT) signaling and myocyte hypertrophy was studied. Neonatal rat (NRVMs) and adult feline (AFVM) ventricular myocytes were infected with an adenovirus containing NFAT-GFP, to determine factors that could induce NFAT nuclear translocation. Four millimolar Ca²⁺ or pacing induced NFAT nuclear translocation. This effect was blocked by Cn inhibitors. In NRVMs Nifedipine (Nif, LTCC antagonist) blocked high Ca²⁺-induced NFAT nuclear translocation while SKF-96365 (TRP channel antagonist) and Nickel (Ni, TTCC antagonist) were less effective. The relative potency of these antagonists against Ca²⁺ induced NFAT nuclear translocation (Nif>SKF-96365>Ni) was similar to their effects on Ca²⁺ transients and the LTCC current. Infection of NRVM with viruses containing TRP channels also activated NFAT-GFP nuclear translocation and caused myocyte hypertrophy. TRP effects were reduced by SKF-96365, but were more effectively antagonized by Nif. These experiments suggest that Ca²⁺ influx through LTCCs is the primary source of Ca²⁺ to activate Cn-NFAT signaling in NRVMs and AFVMs. While TRP channels cause hypertrophy, they appear to do so through a mechanism involving Ca²⁺ entry via LTCCs.

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Disclosures

None.

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Keywords

L-type calcium channel; Hypertrophy; Nuclear factor of activated T cells; Ventricular myocyte; Transient receptor potential channel

1. Introduction

Pathological cardiac stress, such as hypertension and myocardial infarction (MI), causes alterations in cardiac myocyte Ca^{2+} handling[1, 2]. The increase in cytosolic Ca^{2+} activates the calcineurin (Cn)-nuclear factor of activated T cells (NFAT) signaling pathway. Cn-mediated dephosphorylation of NFAT leads to its nuclear translocation where it initiates the expression of genes involved in pathological cardiac hypertrophy. Blocking this pathway has beneficial effects on cardiac structure and function[3–5]. However, the upstream sources of Ca^{2+} that initiate pathological hypertrophy through Cn-NFAT signaling are still not clearly established. Recent studies suggest that Cn-NFAT activation may be independent of the global cardiac Ca^{2+} transient and may take place in signaling microdomains also housing T-type Ca^{2+} channels[6, 7] or transient receptor potential (TRP) channels[8–11]. Other studies have suggested that Cn-NFAT signaling is sensitive to changes in the rate and amplitude of the systolic Ca^{2+} transient[12, 13]. A recent study from our group suggests that L-type Ca^{2+} channels housed in caveolae, away from excitation-contraction coupling proteins are a local microdomain for Ca^{2+} -activation of Cn-NFAT signaling[14].

The role of Cn-NFAT signaling in the induction of pathological hypertrophy was first proposed by Molkentin et al.[15]. This study showed that when a constitutively activated Cn was expressed in cardiac myocytes of transgenic mice, pathological cardiac hypertrophy developed and resulted in heart failure. Many studies have since shown that this pathological Cn-NFAT signaling pathway is activated by increases in Ca^{2+} . When the intracellular Ca^{2+} concentration is elevated, Ca^{2+} ions bind to calmodulin (CaM) and the Ca^{2+} /CaM complex activates the protein phosphatase Cn. Activated Cn subsequently dephosphorylates its downstream substrate, NFAT. Dephosphorylated NFAT translocates from the cytosol into the nucleus and activates transcription factors (e.g. GATA4), which initiate hypertrophic gene expression[16]. The source of the Ca^{2+} that activates pathological Cn-NFAT signaling is still not well known and is the topic of this study.

One possible source of Ca^{2+} that activates the Ca^{2+} /CaM-Cn-NFAT pathway in the heart is the high-conductance, voltage-regulated L-type Ca^{2+} channels (LTCC). These channels provide the Ca^{2+} influx that is essential for inducing and regulating myocyte contraction. This Ca^{2+} influx is essential for triggering sarcoplasmic reticulum (SR) Ca^{2+} release (CICR)[17]. There is some evidence that Ca^{2+} entry through the LTCC can induce pathological hypertrophy[18, 19]. In myocytes isolated from animal models of hypertrophy[20–24] or from failing human hearts[25], the L-type Ca^{2+} current density is unchanged or slightly elevated[26]. However, these measurements are made in the absence of the sympathetic agonists that increase Ca^{2+} current and are increased in cardiovascular diseases[27–29]. Therefore, in-vivo, LTCC current is increased in disease and is a possible source of Ca^{2+} to activate pathological signaling[30–34]. Further support for a role for LTCCs comes from studies that have shown beneficial effects of LTCC antagonists on cardiac remodeling in distinct hypertrophy and heart failure models[5]. However there are numerous studies suggesting that other Ca^{2+} influx pathways are the primary source of “hypertrophic” Ca^{2+} . Experiments in the present study support the idea that LTCCs are the primary source of Ca^{2+} to activate Cn-NFAT signaling in NRVMs and AFVMs.

Ca²⁺ can also enter cardiac myocytes through T-type Ca²⁺ channels (TTCC), which are normally expressed throughout cardiac development until the end of the neonatal period[35–37]. TTCC expression decreases after birth with little expression in normal adult ventricular myocytes. However, TTCCs are re-expressed when the heart is subjected to pathological stresses, suggesting a role in cardiac hypertrophy and failure[38–42]. Ca²⁺ influx through TTCC has almost no effect on EC coupling[43]. Cav3.1 (α1G) and Cav3.2 (α1H) are the major TTCC isoforms in the heart[44]. A few studies support the idea that Ca²⁺ influx through TTCCs can activate Cn-NFAT signaling and cause hypertrophy in neonatal cardiac myocytes, when TTCC expression is robust[7]. There are also studies supporting the idea that Ca²⁺ influx through TTCC causes pathological hypertrophy in the adult heart. One study[45] in a mouse model of dilated cardiomyopathy, reported that the R(–)-isoform of efonidipine, a highly selective TTCC antagonist improved survival. Another study[6] showed that pressure overload-induced hypertrophy was suppressed in Cav3.2 knockout (Cav3.2–/–) mice. Angiotensin II-induced cardiac hypertrophy was also reduced in these mice and the activation of NFAT was blunted following pressure overload. However, there are other studies that do not support a role of TTCC as a source of Ca²⁺ for cardiac hypertrophy[43, 46]. Mice overexpressing Cav3.1 and with large TTCC currents did not have cardiac hypertrophy[43]. In fact, when these animals were subjected to pathological stress their hypertrophy was smaller than in wild type animals, suggesting that Ca²⁺ influx through TTCCs is antihypertrophic[46]. The role of Ca²⁺ influx through TTCCs in pathological hypertrophy remains unresolved.

Ca²⁺ can also enter cardiac myocytes through Transient Receptor Potential (TRP) channels. TRP channels are nonselective cation channel subunits responsible for receptor activated Ca²⁺ entry and possibly having a role in store operated Ca²⁺ entry (SOCE)[47]. TRP channels have been grouped into 7 subfamilies[48, 49]. Several studies have reported that the canonical TRP channels (TRPC) are involved in cardiac hypertrophy and heart failure[49]. TRPC have 7 family members and are divided into 2 subgroups based on their primary amino acid sequences and functions. TRPC1, C3–7 are expressed in the heart[50, 51] and TRPC3 expression is up-regulated in multiple rodent models of pathological cardiac hypertrophy[52]. There is some evidence that TRPC promotes cardiomyocyte hypertrophy through activation of Cn-NFAT signaling[9] This effect was blocked by the store operated Ca²⁺ entry (SOCE) antagonist SKF-96365. Small interference RNA-mediated knockdown of TRPC3 and TRPC6 has also been shown to attenuate Ang II-induced NFAT activation and myocyte hypertrophy[10]. Cardiac-specific overexpression of TRPC6 in transgenic mice sensitizes the heart to pathologic hypertrophic signaling, leading to cardiac dysfunction, hypertrophy and increased β-myosin heavy chain expression, which is regulated by Cn-NFAT signaling[11]. Collectively these studies suggest that Ca²⁺ influx through TRP channels can activate pathological hypertrophy signaling.

In the present experiments we tested the respective ability of Ca²⁺ influx through the LTCCs, TTCCs and TRPC channels to activate Cn-NFAT signaling and cause myocyte hypertrophy. NVRMs or AFVMs infected with NFATc3-GFP were used to examine the source of Ca²⁺ to promote NFAT nuclear translocation. Our results suggest that LTCCs and TRPs can supply Ca²⁺ to activate pathological hypertrophy, at least under the conditions of our experiments.

2. Materials and methods

2.1. Cell isolation and culture

Neonatal rat ventricular myocytes (NRVMs) were isolated from 1- to 3-day-old Sprague Dawley rats as described previously[53, 54]. NRVMs were cultured in DMEM with 5% fetal bovine serum for 24 hr. Then the cells were cultured in non-serum media and infected

with an adenovirus encoding NFATc3-GFP to monitor NFAT localization. Addition of 4 mM extracellular Ca^{2+} was used to stimulate Ca^{2+} influx. The L-type Ca^{2+} channel blocker Nifedipine (Nif), T-type Ca^{2+} channel blocker Ni^{2+} and TRP channel blocker (SKF-96365) were used just before addition of Ca^{2+} . AFVMs were isolated and cultured using approaches described in detail previously[14].

2.2. Immunocytochemistry and confocal imaging

Myocytes were cultured on coverslips coated with fibronectin (BD Sciences). After treatment with increased Ca^{2+} for 4 hours, cells were fixed in 4% paraformaldehyde at room temperature for 10 minutes and permeabilized with 0.5% Triton X-100 immediately before labeling with antibodies directed against α -actinin. Staining of α -actinin and DAPI was performed to detect myocytes and location of nuclei. Fixed cells on coverslips were mounted onto slides and observed with a confocal microscope (Nikon). Images were analyzed with EZ-C1 FreeViewer (Nikon) and ImageJ (NIH) software. NFAT localization was quantified as the normalized nucleus/cytoplasm ratio of GFP fluorescence intensity ($\text{NFAT}_{n/c}$).

2.3. Cell surface area measurement

To evaluate ventricular myocyte hypertrophy, cell surface area was measured in NRVMs as described in detail previously[55–57].

2.4. Ca^{2+} current measurement

Measurement of L-type Ca^{2+} current ($I_{\text{Ca-L}}$) was measured as described previously[54]. Briefly, $I_{\text{Ca-L}}$ was measured with whole-cell voltage clamp techniques with pipettes (2–4 M Ω) containing (mM): 130 CsOH, 130 aspartic acid, 10 EGTA, 1 MgCl₂, 10 NMDG, 10 HEPES, 20 TEA-Cl, 5 Tris-ATP, pH 7.2. After 30 seconds of dialysis, cells were perfused with a Na-free, K-free solution containing (mM): 2 CaCl₂, 2 4-AP, 5.4 CsCl, 10 Glucose, 5 HEPES, 1.2 MgCl₂, 150 NMDG, pH 7.4. $I_{\text{Ca-L}}$ was measured from a -50 mV holding potential (TTCC are inactivated). All experiments were performed using the Axopatch 2 voltage-clamp amplifier and Clampex10 software (Molecular Devices).

2.5. Ca^{2+} transient measurements

Ca^{2+} transients were measured as previously described[58]. Cultured NRVMs were loaded with fluo 4-AM were perfused with normal Tyrode's solution containing 1mM Ca^{2+} . Cells were paced at 0.5Hz and Ca^{2+} transients were recorded using Clampex 10 software.

2.6. Statistics

Data are presented as mean $\pm$ SEM. Unpaired t-test, paired t-test, and one-way ANOVA were performed to detect significance using GraphPad Prism 5.0 (GraphPad Software). $P < 0.05$ was considered significant. “n” is the number of cells examined.

3. Results

3.1. Effects of Ca^{2+} channel antagonists on Ca^{2+} transients

The first series of experiments determined the effects of Nifedipine (a dihydropyridine LTCC antagonist, 10 μM), Ni^{2+} (TTCC antagonist, 50 μM) and SKF-96365 (TRPC blocker, 5 μM) on Ca^{2+} transients of cultured NRVMs (Figure 1). These Ca^{2+} channel antagonists reduced the amplitude of the Ca^{2+} transients by 79%, 33%, and 20%, respectively. These results suggest that all three channel types are present in NRVMs and that LTCCs are the major source of Ca^{2+} to produce the Ca^{2+} transient.

3.2. Effects of Ca^{2+} channel antagonists on Ca^{2+} transients on the L-type Ca^{2+} current

The effects of these three channel blockers on L-type Ca^{2+} current were examined, to ensure that putative TTCC and TRPC channel antagonists did not block the LTCC. Nifedipine blocked the majority of $I_{\text{Ca-L}}$ in cultured NRVMs (Figure 2). Peak $I_{\text{Ca-L}}$ decreased to a low level within 2 min after perfusion with 10 μM Nifedipine. Neither SKF-96365 nor Ni^{2+} had a significant effect on $I_{\text{Ca-L}}$ under our experiment conditions.

3.3. Ca^{2+} induced NFAT nuclear translocation

NFAT translocation from the cytoplasm to the nucleus can be reliably induced by elevation of the extracellular Ca^{2+} concentration in NRVM and AFVMs[59]. We tested the effect of 10 μM Nifedipine, 5 μM SKF-96365, and 50 μM Ni^{2+} on Ca^{2+} induced NFATc3 translocation (Figure 3). Exposure to 4 mM Ca^{2+} for 4 hours significantly increased the ratio of nuclear GFP fluorescence intensity to cytoplasmic GFP fluorescence intensity (NFAT_{n/c} was 9.37 ± 0.55 , $n=240$, in 4 mM Ca^{2+} -treated cells vs. 1.00 ± 0.09 , $n=227$, in control, $P<0.05$). Ca^{2+} induced nuclear NFAT translocation was almost abolished by Nifedipine (NFAT_{n/c} was 1.10 ± 0.12 , $n=19$) and was reduced by SKF-96365 (NFAT_{n/c} was 3.52 ± 0.82 , $n=27$), and Ni^{2+} (NFAT_{n/c} was 5.70 ± 0.69 , $n=26$). Ni^{2+} was the least effective treatment we tested. The relative potency of 10 μM Nifedipine, 5 μM SKF-96365, and 50 μM Ni^{2+} on Ca^{2+} induced NFAT nuclear translocation mirrored the effects of these agents on the Ca^{2+} transient (Figure 1). NFAT nuclear translocation was induced in AFVMs by pacing for 80 min (Supplementary Figure 1). Nifedipine abolished pacing induced NFAT nuclear translocation. Ni^{2+} and SKF-96365 had no effect on pacing induced NFAT nuclear translocation in AFVMs (not shown). TRP channels often require activation through G-protein coupled receptors that lead to diacylglycerol (DAG) production. NFAT was translocated to the nucleus in AFVMs exposed to OAG. This effect was abolished when myocytes were infected with a dominant negative (dn)-TRPC6 adenovirus. Nif also abolished the OAG effect (Supplementary Figure 2). These results suggest that activation of TRPC channels in AFVMs can promote hypertrophic signaling and as in NRVMs there is an obligatory role of Ca^{2+} influx through LTCCs.

3.4. CsA and FK506 inhibited Ca^{2+} -induced NFAT translocation (Figure 4)

Our hypothesis is that Ca^{2+} -induced NFAT nuclear translocation in our preparations results from Ca^{2+} mediated activation of Cn. To verify the role of Cn in this process NRVMs were pretreated with either cyclosporine A (CsA) or FK506 (known Cn inhibitors) before increasing bath $[\text{Ca}^{2+}]$. Both CsA and FK506 completely inhibited (and actually reduced) NFAT nuclear translocation induced by elevated Ca^{2+} concentration (NFAT_{n/c} was 0.41 ± 0.02 , $n=55$, in CsA treated cells and 0.48 ± 0.01 , $n=98$, in FK506 treated cells vs. 8.07 ± 0.79 , $n=115$, in NRVMs with Ca^{2+} treatment, $P<0.05$). These observations support the hypothesis that in this system elevation of bath $[\text{Ca}^{2+}]$ activates Cn to induce translocation of NFAT from the cytoplasm to the nucleus.

3.5 TRPC overexpression increases cell surface area

The experiments with the TRP antagonist SKF-96365 suggest that Ca^{2+} influx through TRP channels can activate Cn-NFAT signaling. To further explore this idea NRVM were infected with Ad-TRPC3 (Figure 5). TRPC3 increased cell surface area (1.86 ± 0.06 , $n=57$, in Ad-TRPC3 infected cells vs. 1.00 ± 0.04 , $n=57$, in control, $P<0.05$). SKF-96365 (1.30 ± 0.05 , $n=57$), and FK506 (1.10 ± 0.04 , $n=50$), blocked the TRPC3 induced myocyte hypertrophy. Interestingly, Nif also blocked NRVM hypertrophy induced by TRPC3 (1.17 ± 0.05 , $n=74$, in Nif treated cells vs. 1.86 ± 0.06 , $n=57$, in Ad-TRPC3 infected cells, $P<0.05$). Ni^{2+} (1.74 ± 0.08 , $n=44$) had no significant effect on TRPC3 induced myocyte hypertrophy. Elevation of

bath $[Ca^{2+}]$ and Ad-TRPC3 also increased ANP abundance, consistent with the activation of hypertrophic gene expression (Supplementary Figure 3).

3.6. Ca^{2+} influx through TRPC3 induces NFAT translocation (Figure 6)

Infection of NRVMs with ad-TRPC3 induced NFAT nuclear translocation without elevation of extracellular Ca^{2+} (NFAT_{n/c} was 5.51 ± 0.48 , $n=153$, in Ad-TRPC3 infected cells vs. 1.00 ± 0.09 , $n=149$, in control, $P<0.05$). Elevating bath $[Ca^{2+}]$ induced further NFAT translocation in TRPC3 infected NRVMs (NFAT_{n/c} was 9.23 ± 1.03 , $n=59$). Infection of NRVMs with Ad-dnTRPC6 inhibited most of the NFAT nuclear translocation induced by elevated bath $[Ca^{2+}]$ (NFAT_{n/c} was 2.60 ± 0.45 , $n=127$, in Ad-dnTRPC6 infected-cells with Ca^{2+} treatment vs. 8.66 ± 0.98 , $n=80$, in Ca^{2+} treated cells, $P<0.05$).

3.7 Nifedipine blocks NFAT translocation induced by TRPC3 overexpression

As shown above, Nifedipine inhibited the hypertrophy induced by expression of TRPC3 in NRVMs. The next experiments defined the effects of $10\mu M$ Nifedipine, $5\mu M$ SKF-96365, and $50\mu M$ Ni^{2+} on TRPC3 induced NFAT nuclear translocation (Figure 7). FK506 completely blocked NFATc3 translocation induced by TRPC3, documenting the essential role of Cn in this process. SKF-96365 blocked some but not all of the NFATc3 translocation in TRPC3 infected myocytes. Ni^{2+} had no effect on TRPC3 overexpression-induced NFATc3 translocation. Nif completely blocked TRPC3 induced nuclear NFAT translocation. These findings support a scenario in which TRP channels influence LTCCs to activate Cn-NFAT signaling. The nature of this effect remains to be defined. We did find that TRPC3 infected cells had increased LTCC protein abundance (Supplementary Figure 3) and amplitude of the LTCC current (Supplementary Figure 4).

3.8 TRPC3 expression alters the Ca^{2+} transient

The results above suggest that TRP channels might influence the number and/or behavior of LTCCs to provide the Ca^{2+} that induces cardiac myocyte hypertrophy. If so, there might also be an effect on the global $[Ca^{2+}]$ transient that initiates contraction. To explore this idea Ca^{2+} transients were measured in NRVMs infected with Ad-TRPC3 (Figure 8). The peak of the Ca^{2+} transient was not significantly different between control and Ad-TRPC3-infected cells (B). However, Time to half decay was significantly prolonged in NRVMs infected with Ad-TRPC3 as compared with control (C). These results are consistent with an interaction of TRPC3 and LTCCs that leads to activation of Cn-NFAT signaling.

4. Discussion

The present study explored the respective roles of Ca^{2+} influx through L- and T-type Ca^{2+} channels and TRP channels in the activation of the Cn-NFAT signaling. Activation of this signaling pathway is involved in the induction of pathological cardiac hypertrophy[60]. The major findings of this study are that Ca^{2+} influx through the LTCC is the major source of Ca^{2+} to activate Cn-NFAT signaling, at least in the preparations used in the current experiments. Ca^{2+} influx through TRP channels also appeared to be able to activate this signaling cascade, while Ca^{2+} influx through TTCC was least able to induce myocyte hypertrophy. The most novel aspect of the study is those findings that suggest an interaction between TRP and LTCC channels to activate Cn-NFAT signaling and induce myocyte hypertrophy. The nature of this interaction deserves additional study.

Cytoplasmic $[Ca^{2+}]$ during diastole falls to about 150nM (when the cells are relaxed and the heart is filling with blood) and is rapidly increased during each heartbeat to induce contraction. Changes in the amplitude and duration of the Ca^{2+} transient largely determine the strength (contractility) of the heartbeat[2]. In cardiac diseases such as hypertension and

after myocardial infarction, the myocytes must beat more forcefully to maintain cardiac output against a pathologically elevated afterload. This requires an increase in cytosolic Ca^{2+} . In these diseases, cardiac myocytes undergo “pathological” hypertrophy and this is associated with cardiac dysfunction, arrhythmias and sudden arrhythmogenic death[61]. It is known that the persistent increase in myocyte Ca^{2+} in the diseased heart is linked to pathological hypertrophy, but the actual source of Ca^{2+} is not clearly defined[1]. Most recent studies suggest that the global Ca^{2+} transient that initiates contraction is not the source of Ca^{2+} to activate Cn-NFAT signaling and cardiac hypertrophy. Maybe the two best examples of this are the phospholamban knock out mouse[62] and the TTCC, $\alpha 1\text{g}$, overexpression mouse[43]. Both of these mice have hypercontractile hearts with a persistent increase in the amplitude of the systolic Ca^{2+} transient. However, there is no pathological hypertrophy in these mice. In fact, the $\alpha 1\text{g}$ mouse appears to be protected from hypertrophy. These studies suggest that the amplitude of the global Ca^{2+} transient is not the activator of Cn-NFAT signaling and suggest that a local source of Ca^{2+} might be responsible for activation of this signaling cascade. Our study explored three potential Ca^{2+} influx pathways as the source of “hypertrophic Ca^{2+} ”.

L-type Ca^{2+} channels are the major pathway for Ca^{2+} entry in the heart. These channels activate with the depolarization during each action potential and the resultant Ca^{2+} entry elevates submembrane $[\text{Ca}^{2+}]$ within an EC coupling microdomain (the couplon) where it induces opening of the SR Ca^{2+} release channel (RyR) to initiate SR Ca^{2+} release[63]. The role of Ca^{2+} influx through these channels in the activation of pathological hypertrophy is not clear. As an example, Ca^{2+} influx through LTCCs increases with exercise or pregnancy, but this does not initiate pathological hypertrophy[60]. However, there are many studies that show that pharmacological reduction of Ca^{2+} entry through LTCCs in animals or cells reduces pathological hypertrophy[3, 5]. Recently we have shown that a subpopulation of LTCCs in caveolae signaling microdomains can activate Cn-NFAT signaling[14]. In the present studies we show that LTCCs are a major source of Ca^{2+} to activate Cn-NFAT signaling. Interestingly, TRP channels appear to activate Cn-NFAT signaling by a mechanism that involves the LTCCs (see below). Our studies cannot determine if the LTCCs that provide the Ca^{2+} to activate Cn-NFAT signaling are independent of those that initiate contraction. This topic deserves additional study. We did find that the relative potency of LTCC, TTCC and TRP channel antagonists on contraction was similar to their ability to block Cn-NFAT signaling. However, this does not mean that the same channels that are involved in EC coupling activate Cn-NFAT signaling.

T-type Ca^{2+} channels were the least effective activators of Cn-NFAT signaling in the present studies. These results support findings we have made in mice that overexpress these channels, in which Ca^{2+} influx through these channels appears to be antihypertrophic and cardioprotective[43]. Why others[6, 7] have found an important role for these channels in the induction of pathological hypertrophy is unclear.

TRP channel expression is increased in hearts with pathological stress and their presence has been linked to the induction and maintenance of pathological hypertrophy[8, 64]. The mechanism by which these channels induce pathological hypertrophy is unclear, at least to us, but is thought to involve Ca^{2+} influx through the TRP channels[8–10]. Our studies show that these TRP channels can induce activation of Cn-NFAT signaling. Two independent observations in our studies support an interaction between TRP and LTCC channels to activate Cn-NFAT signaling. First, TRPC3-induced Cn-NFAT activation (Figure 7) and myocyte hypertrophy (Figure 6) were both entirely blocked by selective LTCC blockers. Secondly, infecting myocytes with dnTRPC6 significantly reduced Ca^{2+} -induced activation of Cn-NFAT (Figure 6) that was shown to be the result of Ca^{2+} entry through LTCCs (Figure 3). Collectively our results suggest that TRP channels modulate the activity/

abundance of the LTCCs, and it is the Ca^{2+} influx through TRP-modified LTCCs that activates Cn-NFAT signaling. This idea will require substantial additional testing.

5. Conclusions

In summary, our results suggest that Ca^{2+} influx through the LTCC is the major source of Ca^{2+} to activate the signaling cascade that is known to cause pathological hypertrophy in NRVM and AFVMs. TTCCs were not a major source of hypertrophic Ca^{2+} . The most surprising aspect of this study is the finding that gain and loss of function experiments with TRP channels induce/block hypertrophic signaling through an LTCC dependent mechanism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Elevated Ca^{2+} and pacing induced NFAT nuclear translocation.
- Nifedipine inhibited Ca^{2+} transient, LTCC current, and NFAT translocation.
- Nifedipine blocked TRPc3 induced NFAT translocation and hypertrophy.
- Ca^{2+} influx through LTCC is primary source of Ca^{2+} to activate Cn-NFAT signaling
- Hypertrophy resulting from TRP channel is involved in Ca^{2+} entry via LTCC.

Figure 1.

Nifedipine, SKF-96365, and Ni^{2+} have distinct effect on Ca^{2+} transients. Ca^{2+} transients were measured in NRVMs perfused with normal Tyrode's solution (Ctrl) before and after exposure to solutions containing either 10 μ M Nifedipine (Nif, A), 5 μ M SKF-96365 (B), or 50 μ M Ni^{2+} (C). Peak Ca^{2+} was reduced 79%, 33%, and 20% by these treatments, respectively (D). * indicates a significant treatment effect versus control. # indicates significant difference versus Nif. $P < 0.05$. Numbers in bars represent the number of cells studied.

Figure 2.

Nifedipine blocked L-type Ca^{2+} current. Ten micromolar Nif reduced L-type Ca^{2+} current by 64.03% (A, B). SKF-96365 ($5\mu M$) and Ni^{2+} ($50\mu M$) had no significant effect on peak L-type Ca^{2+} current. Nif significantly inhibited peak I_{Ca-L} currents (C). * indicates significant difference as compared with control. $P < 0.05$.

Figure 3. Ca²⁺ channel inhibitors blocked Ca²⁺ influx induced NFATc3 nuclear translocation. NRVMs were infected with Ad-NFATc3-GFP and exposed to vehicle (Ctrl, A), 4mM Ca²⁺ (B), 4mM Ca²⁺ with 10μM Nifedipine (C), 4mM Ca²⁺ with 5μM SKF-96365 (D), and 4mM Ca²⁺ with 50μM Ni²⁺ (E), respectively, for 4 hours. The nuclear GFP fluorescence intensity to cytoplasmic GFP fluorescence intensity ratio was significantly higher in NRVMs exposed to 4mM Ca²⁺ than in control (F). * indicates a significant difference versus control. # indicates a significant difference versus elevated Ca²⁺ treated NRVMs. *P* < 0.05. Scale bar equals 50 microns.

Figure 4. Calcineurin inhibitors blocked Ca²⁺-induced NFAT translocation. CsA (2 μ M) and FK506 (2 μ M) both abolished Ca²⁺-induced NFAT nuclear translocation. * indicates a significant difference versus control. # indicates a significant difference versus Ca²⁺ treated myocytes. $P < 0.05$. Scale bar equals 50 microns.

Figure 5.

TRPC3 induced NRVM hypertrophy (A, B, G). Nifedipine (10μM, C, G) and SKF-96365 (5μM, D, H) caused a significant reduction in TRPC3 induced hypertrophy while Ni²⁺ (50μM, E, G) had no effect. FK506 (2μM, F, G) inhibited TRPC3 induced hypertrophy. Cells were stained for α-actinin (red) and DAPI (blue) to identify myocytes. Cell surface areas were measured using ImageJ. * indicates a significant difference versus control. # indicates a significant difference versus Ad-TRPC3. $P < 0.05$. Scale bar equals 50μm.

Figure 6.

TRPC3 induced NFAT-GFP nuclear translocation (A, B, G). NRVMs were infected with Ad-NFATc3-GFP alone, Ad-NFATc3-GFP and Ad-TRPC3, and Ad-NFATc3-GFP and Ad-dnTRPC6 (A–G), respectively. Elevated extracellular Ca²⁺ induced NFAT translocation in control cells and increased NFAT nuclear translocation in TRPC3 cells (A, B, D, E, G). dnTRPC6 reduced Ca²⁺ induced NFAT nuclear translocation (C, F, G). * indicates a significant difference versus control. # indicates a significant difference versus Ca²⁺ treatment. $P < 0.05$.

Figure 7. Nifedipine blocked NFAT translocation induced by TRPC3 overexpression (A, B, C, G). SKF-96365 (5 μ M, D) reduced TRPC3-induced NFAT nuclear translocation but was less effective than Nif. Ni²⁺ (50 μ M, E) had no significant effect on TRPC3 induced NFAT translocation. FK506 (2 μ M, F) eliminated TRPC3 induced NFAT translocation. * indicates a significant difference versus control. # indicates a significant difference versus TRPC3. $P < 0.05$.

Figure 8.

TRPC3 prolonged the Ca²⁺ transient (A). The peak of Ca²⁺ transient was not significantly altered in TRPC3-infected cells (B). The time to half decay was significantly prolonged in NRVMs infected with Ad-TRPC3 versus controls (C).