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Functionally redundant control of cardiac hypertrophic signaling by inositol 1,4,5-trisphosphate receptors

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Abstract

Calcium plays an integral role to many cellular processes including contraction, energy metabolism, gene expression, and cell death. The inositol 1, 4, 5-trisphosphate receptor (IP_3R) is a calcium channel expressed in cardiac tissue. There are three IP $_3$R isoforms encoded by separate genes. In the heart, the IP_3R-2 isoform is reported to being most predominant with regards to expression levels and functional significance. The functional roles of IP_3R-1 and IP_3R-3 in the heart are essentially unexplored despite measureable expression levels. Here we show that all three IP_3Rs isoforms are expressed in both neonatal and adult rat ventricular cardiomyocytes, and in human heart tissue. The three IP_3R proteins are expressed throughout the cardiomyocyte sarcoplasmic reticulum. Using isoform specific siRNA, we found that expression of all three IP $_3$R isoforms are required for hypertrophic signaling downstream of endothelin-1 stimulation. Mechanistically, IP $_3$Rs specifically contribute to activation of the hypertrophic program by mediating the positive inotropic effects of endothelin-1 and leading to downstream activation of nuclear factor of activated T-cells. Our findings highlight previously unidentified functions for IP $_3$R isoforms in the heart with specific implications for hypertrophic signaling in animal models and in human disease.

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Disclosures

None.

Keywords

Inositol Trisphosphate Receptor ($InsP_3R$); Inositol 1,4,5-Trisphosphate (IP_3); Calcium; Cardiac Hypertrophy; Calcium Channel

1. Introduction

In the heart calcium is an essential modulator of a wide variety of cellular functions including cardiomyocyte excitation-contraction coupling (ECC) and gene expression. Cardiomyocyte function can be modulated by neuro-hormonal agonists to accommodate cardiac demand. One example is endothelin-1 (ET-1), which is a potent vasoconstrictor that plays an important role in modulating muscle contractility, vascular tone, cardiomyocyte growth, and survival [1, 2]. Plasma levels of ET-1 are also increased during pathological conditions such as chronic heart failure, myocardial infarction, cardiac hypertrophy and in hypertension [3, 4]. As such, ET-1 has been linked to pathological remodeling of the heart [1, 5]. ET-1 signaling is initiated by ET-1 binding to G-protein coupled receptors at the plasma membrane leading to the activation of phospholipase C (PLC). PLC catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) which leads to increased production of the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. IP_3 then acts as a second messenger that binds inositol 1,4,5-trisphosphate receptors (IP_3Rs), activating IP_3-induced calcium release (IICR).

IP_3Rs are a family of calcium channels involved in a variety of cellular functions. There are three different IP_3R isoforms encoded by separate genes. The three IP_3Rs share a high degree of sequence homology and are found in a variety of tissues including the heart [6, 7]. Cardiac IP_3Rs are implicated in regulating the progression of cardiac hypertrophy [8, 9]. Within the cardiomyocyte, IP_3Rs are known to localize in the dyadic cleft, sarcoplasmic reticulum and at the outer/inner nuclear membrane [1, 8–10]. Several lines of evidence have also implicated nuclear calcium transients as a significant contributor to cardiomyocyte hypertrophy. Nuclear or perinuclear IP_3Rs may promote nuclear-restricted calcium release events that initiate gene transcription [10]. Nuclear calcium transients are involved in the activation of transcription factors such as histone deacetylase 5 (HDAC5) [1, 11]. However the mechanism by which cardiomyocytes can discriminate between calcium signals from ECC and calcium signals that target gene transcription it still unclear, as calcium release events mediated by ECC are also efficiently transmitted to the nuclear matrix. Hypertrophic agents such as ET-1 also increase contractility [9, 12], which may afford a mechanism for decoding IP_3-dependent signals without requiring subcellular compartment-specific IP_3R activation.

The IP_3R-2 isoform is considered the predominant isoform in the heart [13, 14]. However, transgenic IP_3R-2 rodent models have either supported [15, 16], or contradicted [17, 18] the role of IP_3R-2 channel in cardiac hypertrophy. As such, it is still unclear whether IP_3R channels are significant contributors to cardiac physiology and pathologic remodeling such as hypertrophy [19]. It has been shown that all three IP_3R isoforms, at least at the mRNA level, are expressed in the heart of humans and mice [8, 20]. This opens the question of

whether IP $_3$R-1 and -3 are able to functionally compensate for IP$_3$R-2 deficiencies in these models.

We now show that all three IP$_3$R isoforms are expressed in cardiomyocytes and that they are essential for the progression of ventricular hypertrophy induced by ET-1. IP$_3$R-dependent activation of the hypertrophic program was not dependent upon nuclear-specific calcium transients, but instead was mediated by increased contractility induced by ET-1. Lastly, these results were independent of increased IP$_3$R expression both in vitro and in vivo.

2. Materials and Methods

2.1. Antibodies, expression constructs, and reagents

Rabbit polyclonal antibody against type-1 IP$_3$R was developed in-house and is specific for the type-1 isoform [21]. The rabbit polyclonal antibody against type-2 IP$_3$R have been described elsewhere [6] and was kindly provided by Dr. Richard Wojcikiewicz (SUNY Upstate). Mouse monoclonal anti-IP$_3$R type-3 was purchased from BD Bioscience. Mouse monoclonal anti-α-actinin and anti-ryanodine receptor antibody was purchased from Sigma-Aldrich. Mouse anti-SERCA2 antibody was from Thermo Fisher and rabbit anti-ANP was purchased from Abcam. Anti-alpha-fodrin was purchased from EMD Millipore. Secondary antibodies conjugated to Alexa-488 and Alexa-555 were from Molecular Probes, and peroxidase-conjugated antibodies were from Jackson ImmunoResearch. Expression constructs 9X NFAT-apha-MHC-Luc was a gift from Jeffery Molkentin (Addgene plasmid # 51941), pGP-CMV-GCaMP6s was a gift from Douglas Kim ([22]; Addgene plasmid # 40753) and Tol2-elavl3-H2B-GCaMP6s was a gift from Misha Ahrens (Addgene plasmid # 59530). Endothelin-1 was purchased from Bachem. Silencer pre-design siRNAs were purchased from Ambion. Fura-2 AM was purchased from Molecular Probes, and the dual luciferase reporter assay kit was from Promega. All other reagents were purchased from Sigma -Aldrich.

2.2. Preparation of primary neonatal cardiomyocytes

Neonatal rat ventricular cardiomyocytes (NRVM) were obtained from 1 to 2 day old Sprague-Dawley rat hearts as previously described, with minor modifications [23]. Cardiomyocytes were plated into fibronectin-coated culture dishes and incubated at 37°C in 5% CO_2 incubator. Two days after plating, media was replaced with 50% Ham's F10 - 50% DMEM culture medium with β-D-arabinofuranoside (ARA-C; 1 μM) to inhibit growth of fibroblasts. NRVMs were transfected using Lipofectamine 3000 following manufacturer's instructions. All experiments were carried out 48 hours after transfection unless otherwise stated. All vertebrate animal procedures were approved by the Animal Welfare Committee (AWC) at UTHealth.

2.3. Preparation of adult cardiomyocytes

Calcium-tolerant adult rat ventricular myocytes (ARVM) were isolated from hearts of wildtype Sprague-Dawley rats (300–320 g) as described by Louch et. al. [24]. Briefly, animals were anesthetized with chloralhydrate (400mg/kg b.w. i.p.) and heparinized (5,000 U/kg b.w.) via direct injection into the vena cava inferior. The hearts were aseptically

removed and directly placed in ice-cold Krebs-Henseleit (KH) buffer (133.5 mM NaCl, 4 mM KCl, 1.2 mM NaH2PO4, 10 mM HEPES, 1.2 mM MgSO4, 10 mM BDM) containing glucose (5.5 mM) before being perfused with a Langendorff preparation. Perfusion (3 min) with KH buffer at 37°C lacking Ca2+ was followed by perfusion with recirculating KH buffer containing 2% BSA (wt/vol), 50 μM Ca2+ and type II collagenase. After 20 minutes of perfusion, hearts were minced, and undigested tissue was separated with a 230 μm mesh sieve. The cell suspension was allowed to settle with gravity within 5 to 7 min, and the cell pellet was re-suspended in KH containing 2% BSA (wt/vol), and calcium was slowly reintroduced to a final concentration of 1 mM. Cardiomyocytes were plated and culture under 5% carbon dioxide.

2.4. Immunofluorescence labeling of isolated cardiomyocytes

Adult and neonatal cardiomyocytes were plated at a density of 300 cells per mm^2 on glass coverslips coated with fibronectin. On day 4 the medium was exchanged and the cells were treated with 100 nM ET-1 for 48 hrs. Adult cardiomyocytes cells were fixed with 4% paraformaldehyde in PBS. Neonatal cardiomyocytes were fixed with 100% cold MeOH. Briefly, cells were incubated with either rabbit polyclonal anti-IP_3R-1 (1:250), rabbit polyclonal anti-IP_3R-2 (1:250), mouse monoclonal anti-IP_3R-3 (1:250), mouse monoclonal anti-α-actinin (1:250), mouse anti-SERCA2 (1:250), or mouse anti-ryanodine2 (1:250), overnight at 4°C. Followed by incubation with secondary antibodies conjugated to Alexa-488 and Alexa-555 for 1 hr.

2.5. Immunofluorescent labeling of human heart failure samples

Disease heart tissue was obtained from patients undergoing heart transplantation due to advanced heart failure. Immediately after explant the tissue is flash frozen with liquid nitrogen for future analyses. Control heart tissue was obtained from organs that were declined for transplantation due to non-cardiac reasons. Frozen left ventricular cardiac tissues were cryo-sectioned onto charged glass slides. The sections were fixed with 4% paraformaldehyde in PBS. Tissue was stained with anti-IP_3R-1 (1:100), anti-IP_3R-2 (1:100), anti-IP_3R-3 (1:100), or anti-α-actinin (1:100), for 1hr at 37°C. Subsequently, slides were washed and incubated with secondary antibodies conjugated to Alexa-488 and Alexa-555 for 1 hr. Total corrected tissue fluorescence was quantified using ImageJ exactly as described previously [25]. All experiments on human samples were approved by the Institutional Review Board (IRB) at the Houston Methodist Institute for Academic Medicine and the McGovern Medical School at UTHealth.

2.6. Cell size determination

NRVM were plated on glass coverslips. Two days after plating cells were transfected with control siRNA or triple IP_3R siRNA. Following transfection cells were treated with ET-1 for 48 hours. After treatment cells were fixed with 4% paraformaldehyde in PBS. Subsequently, coverslips were stained with anti-α-actinin and secondary antibodies conjugated to Alexa-555. For measurement of cell area, at least 30 fields randomly chosen were analyzed in each coverslip. Cardiomyocytes area was measured in captured images using ImageJ software.

2.7. Western blotting

Cells were harvested by gently scraping plates with a cell scraper and washing once with cold PBS. Cell lysis buffer (150 mM NaCl, 50 mM Tris-HCl at pH 7.8, 1% Triton X-100 and 1 mM EDTA) was added to the cell pellet. Samples were cleared of insoluble debris by centrifugation at 20,000g at 4°C. Cell lysates were quenched with SDS sample buffer. Samples were resolved by SDS–PAGE and analyzed by western blotting. Where indicated, cardiomyocytes were treated 100 nM ET-1 for 48 hours or six days.

2.8. Calcium imaging

NRVM were plated on fibronectin-coated glass coverslips and were transfected with triple IP $_3$R siRNA targeting rat IP$_3$R-1, IP$_3$R-2 and IP$_3$R-3. The total amount of siRNA transfected was 12.5 pmol per 350,000 cells. Transfected cells were identified by co-transfection with cDNA for YFP (0.25pmol per 350,000 cells). Cells were imaged after 48 hrs. For imaging, cardiomyocytes were incubated with 5 μM Fura-2 AM in imaging solution (1% BSA, 107mM NaCl, 20 mM HEPES, 2.5 mM $MgCl_2$, 7.5 mM KCl, 11.5mM glucose, and 1 mM $CaCl_2$) for 30 min at RT. The solution was replaced with imaging solution without Fura-2 AM for an additional 20 min. Images were acquired using a Nikon TiS inverted microscope as previously described [26]. Responses to 100 nM ET-1 were recorded on YFP only positive cells. In order to specifically look at nuclear calcium transients we used GCaMP6s fused to a sequence encoding human histone H2b at the 5′ end[27]. We sub-cloned H2b-GCAMP6s into the mammalian expression vector pcDNA 3.1(+).NRVM were transfected with different siRNAs (IP $_3$R-1, IP$_3$R-2 or IP$_3$R-3) and with H2b-GCAMP6s two days after plating. Cells were loaded with Fura-2 AM in imaging solution 48 hrs after transfection. Responses to 100nM ET-1 were acquired at 1 Hz during continuous recording. Oscillation frequency was determined manually. An oscillation was counted when the Fura-2 ratio rose 10% above the baseline ratio. Similar to Fura-2, an oscillation was counted when GCaMP6s fluorescence rose above 5% from baseline fluorescence.

2.9. NFAT luciferase

For assessment of NFAT activation cells were co-transfected with 9xNFAT-TATA luciferase plasmid [28] and pRL-TK control vector (2:1). All experiments were performed 48 hours after transfection. Cells were harvested and cell extracts were assayed using dual luciferase reporter assay as specified by manufacturer's protocol (Promega). All data are shown as mean ± SEM, with statistical significance determined at $p < 0.01$ vs control using an unpaired Student's t-test.

3. Results

3.1 Expression of IP$_3$R protein in neonatal and adult ventricular cardiomyocytes

Previous studies have indicated that IP $_3$R-2 mRNA is predominant compared to other IP $_3$R isoforms in ventricular cardiomyocytes [13, 14]. Using highly specific antibodies we analyzed the protein expression and localization of the three IP $_3$R isoforms in primary neonatal and adult rat cardiomyocytes. In neonatal ventricular cardiomyocytes all three IP $_3$R isoforms were expressed throughout the cell (Fig. 1A–C, second column; Supplemental

Fig. 1). Expression was more prominent in the perinuclear region consistent with previous reports [1]. Similarly, all three IP_3R isoforms are expressed throughout the cell in adult rat cardiomyocytes (Fig. 1D–F, second column). In contrast to neonatal ventricular cardiomyocytes, IP_3R expression in adult cardiomyocytes was more equally distributed throughout the cell with no obvious concentration in the perinuclear region. This expression pattern overlaps with SERCA2 localization indicating is it present throughout the sarcoplasmic reticulum in both neonatal and adult cardiomyocytes (Fig. 2A, C). As expected, IP_3R localization was distinct from the localization with RYR ([1]; Fig. 2B, D; Supplemental Fig. 2). These results indicate that in addition to IP_3R-2, IP_3R isoforms type 1 and 3 are expressed in rat neonatal and adult ventricular cardiomyocytes.

3.2 Altered contractility induced by ET-1 is attenuated by decreasing IP_3R expression

ET-1 is known act as a positive inotropic agent in cardiomyocytes [9, 12]. We monitored intracellular calcium in spontaneously contracting neonatal ventricular cardiomyocytes treated with ET-1. As shown in Fig. 3A (control bars) there is an increase in the calcium oscillation frequency in neonatal cardiomyocytes stimulated with 100 nM ET-1. Previous studies using overexpression of an IP_3 5′-phosphatase to inhibit IP_3 signaling suggested that IP_3R channels do not play a role in this response to ET-1 [1]. To evaluate the role of IP_3R channels in the process more specifically, we used siRNA knockdown of each individual IP_3R isoform. Western blot analysis confirmed efficient and specific knockdown of each of the three IP_3R isoforms (Supplemental Fig. S3). Knockdown of individual IP_3R isoforms did not have significant effects on the increased oscillation frequency induced by ET-1 stimulation (Fig. 3A). Next we used double knockdowns (IP_3R 1/2, 2/3 and 1/3) and determined their effect on the response to ET-1. Similar to single knockdowns, in double knockdowns there is no significant effects on the increased oscillation frequency induced by ET-1 stimulation. Triple knockdown of all three IP_3R isoforms completely suppressed the response of cardiomyocytes to ET-1 (Fig. 3A). Thus, the increased contractility in response to ET-1 requires IP_3R activity, and furthermore all three IP_3Rs are expressed and play a functionally redundant role in the response to ET-1 stimulation.

3.3 Attenuating IP_3R expression inhibits ET-1 induced NFAT activation and hypertrophy

At the cellular level cardiac hypertrophy is characterized by activation of the hypertrophic transcriptional program by NFAT ultimately leading to increased cardiomyocyte cell size. ET-1 is one of the best described neuro-hormonal factors known to induce cardiomyocyte hypertrophy [10, 16]. ET-1 also induces hypertrophic remodeling of neonatal rat ventricular cardiomyocytes [1, 29], and thus we used this well-characterized model to assess the role of IP_3Rs in hypertrophic signaling. As mentioned previously, IP_3R mRNA levels are altered during hypertrophy [7, 13], however the effect on IP_3R protein levels is not clear. In order to assess whether IP_3R protein expression is altered during ET-1 induced hypertrophy, we treated neonatal cardiomyocytes with ET-1 for 48 hours and 6 days. Using this paradigm, protein expression of all three IP_3R isoforms is unchanged compared to non-treated controls (Fig. 3B–C) despite robust induction of the hypertrophic marker protein atrial natriuretic peptide (ANP; Fig. 3D).

Next we determined whether IP $_3$R expression is essential for activation of the hypertrophic program downstream of ET-1 stimulation. Treatment of cells with ET-1 led to significant NFAT activation within 12 hours as determined by a luciferase reporter assay (Fig. 3E). Triple knockdown of all three IP_3R isoforms completely abrogated NFAT activation in response to ET-1 stimulation (Fig. 3E), a finding consistent with our observation that triple IP $_3$R knockout suppresses the increased calcium oscillation frequency in response to ET-1 (Fig. 3A). Treatment of cardiomyocytes with ET-1 leads to an increase in cell size, the cardinal feature of hypertrophy ([1, 5]; Fig. 3F). Triple knockdown of all three IP_3R isoforms completely abrogates the increased cell size induced by to ET-1 treatment as shown in Fig. 3F and Supplemental Fig. S5. Thus, all three IP_3Rs contribute to signaling downstream of ET-1 in cardiomyocytes and are essential for activation of the hypertrophic program induced by ET-1 *in vitro*.

It has also been shown that IP_3R mRNA levels are altered in both animal models [9, 17] and in the human failing heart [8]. To determine whether IP_3R expression levels are altered *in vivo*, we compared IP_3R expression levels in heart tissue samples from three patients diagnosed with dilated cardiomyopathy to biopsies of left ventricular heart tissue obtained from three healthy human subjects (non-failing hearts). Patients diagnosed with dilated cardiomyopathy were male, 60±3.5 years old, with a reduced ejection fraction of 20%, and with an increased left ventricular end diastolic diameter (LVEDD) and left ventricular pressure (LVP, 1.10±0.44) as assessed by transthoracic echocardiography (Supplemental Table 1). Protein expression of all three IP $_3$R isoforms could be readily detected in the three human heart samples. We found no significant differences in the protein expression level of IP_3R isoforms in human failing hearts relative to control (Fig. 4 and Supplementary Fig. S4).

3.4 Calcium release induced by ET-1 is not restricted to the nuclear compartment

Rhythmic contraction of the heart is mediated by intracellular RyR calcium channels in a process termed calcium-induced calcium release (CICR). An important question is how IP_3R-mediated calcium transients could be “decoded” in the constant background of CICR in the beating heart. One prevalent model is that nuclear-compartment specific IP $_3$R calcium transients mediate gene expression in response to hypertrophic agents [10]. However, as CICR calcium transients also diffuse into the nucleus the specific mechanism is not clear. Furthermore, NFAT is a cytosolic protein in the inactive state, and translocates to the nucleus in response to elevations in cytosolic calcium [5]. To resolve these discrepancies, we used the genetically encoded calcium indicator GCaMP6s targeted to the nucleus in order to measure nuclear calcium transients unambiguously (Fig. 5A–B). Using the spectrally separated indicator Fura-2 to measure cytosolic calcium, we were able to quantify calcium release in both nuclear and cytosolic compartments with high specificity. As shown in Fig. 5C–D, calcium release was detectable in both compartments of spontaneously beating cardiomyocytes. After ET-1 stimulation there is an increase in the oscillation frequency consistent with the positive inotropic effects of ET-1 (Fig. 5C–D), which we showed is absolutely dependent upon IP $_3$R expression (Fig. 3A). Visual observation of Fura-2 versus H2b-GCaMP6s traces revealed essentially overlapping plots (Fig. 5C–D), however the H2b-GCaMP6s signal had lower resolving power at higher oscillation frequencies. Expression of cytosolic GCaMP6s revealed that this was due to a buffering effect of the indicator, not an

intrinsic property of the nuclear matrix (Fig. S6). In order to compare the cytosolic versus nuclear oscillations more quantitatively, we calculated the oscillation frequency over the course of the experiment in 20 second bins in both the cytosolic and nuclear compartments (Fig. 5E). Data points above the line in Fig. 5E would indicate a higher frequency in the nucleus (thus highlighting nuclear only events). Conversely, data points below the line are representative of events that occur in the cytosol but were not detectable in the nucleus. Presented in this way, we were unable to visualize any “nuclear only” release event either before or after ET-1 stimulation. We did, however, note a few “cytosol only” events at various frequencies. This may be due to either a cytosol restricted transient or insufficient sensitivity of the GCaMP6s sensor. Regardless, if there are nuclear restricted calcium transients they are either rare or low amplitude events. The results presented herein are also entirely consistent with the large body of evidence indicating that NFAT is activated by changes in calcium release frequency, not amplitude [30–32].

4. Discussion

IP_3Rs likely play a key role in the progression of hypertrophy [1, 9, 16]. However, the majority of the studies on the role of IP $_3$Rs in hypertrophy have focused solely on IP_3R-2, despite the fact that the other two isoforms are also expressed in the heart [8, 17, 20]. We now show that all three IP_3R channels are expressed at readily detectable levels in cardiomyocytes in both rodent and human heart. Furthermore, all three channels contribute to calcium release and activation of the hypertrophic program in a functionally redundant manner. Lastly, our findings indicate that IP_3Rs contribute to activation of the hypertrophic program after ET-1 stimulation primarily by mediating the increase in calcium release frequency/contractility. We do not find evidence, at least in this model, for nuclear-specific calcium events.

Our finding that IP_3Rs play functionally redundant roles in ventricular cardiomyocytes may resolve some controversies regarding transgenic models of IP $_3$R function in the heart. Previously, the IP_3R-2 was thought to be the predominant isoform expressed in the heart. Consequently, most of the studies that focus on the role of IP $_3$R in the heart have focused solely on IP_3R-2 function [13, 33]. However, global genetic knockout of the IP_3R-2 in mice does not cause any significant difference in the hypertrophic response in pressure overload or dilated cardiomyopathy mouse models [17]. However, the potential involvement of IP_3R-1 and IP_3R-3 activity has not been investigated further. Another group has shown that global knockout of IP_3R-2 eliminates the positive inotropic effects of endothelin-1 (ET-1) in the atria and protects against arrhythmias [15]. Overexpression of the ligand binding domain of the IP $_3$R inhibits signaling through the channel by buffering IP $_3$ levels (so called “IP_3 sponge” [34]). Targeted inducible overexpression of the IP $_3$ sponge in the heart inhibited hypertrophy in response to isoproterenol and angiotensin-II [16]. As the IP_3 sponge would inhibit signaling through all three IP_3R channels, these findings are consistent with our results suggesting that all three IP $_3$Rs can contribute to hypertrophic signaling in cardiomyocytes. Similarly, another study using overexpression of an IP_3 5′-phosphatase (presumably INPP5A) demonstrated that IP_3 signaling is required for the hypertrophic effects of ET-1 in neonatal rat ventricular myocytes [1]. Interestingly, overexpression of this enzyme did not inhibit the increased beating frequency in response to ET-1 treatment [1].

One possible explanation for this discrepancy with our results in Fig. 3 is that this enzyme is effective in buffering long-term elevation of IP_3 levels (such as during chronic exposure to ET-1) but is not as effective at metabolizing IP_3 after acute exposure to agonist. This explanation is consistent with other studies indicating that IP_3 5′-phosphatases becomes the dominant mediator of IP $_3$ metabolism only at high IP_3 concentrations [35, 36].

The subcellular localization and precise aspects of how IP $_3$Rs regulate spatio-temporal aspects of calcium release in the contracting myocyte is an area of debate. In particular, how IP $_3$R signals are decoded to regulate processes such as transcriptional activation in the beating cardiomyocyte is unclear. IP_3Rs are known to be primarily localized at the ER/SR membranes in most cells, however in the heart it is thought to be concentrated at the nuclear and perinuclear membranes, where it is thought to play a key role in gene transcription [1, 10, 11, 37]. Nuclear localized IP $_3$R would thus facilitate spatially restricted calcium transients to the nuclear matrix. It has been shown that IP $_3$ and ET-1 can trigger nuclear calcium sparks and nuclear localized calcium transients [10, 37–39], however it is unclear whether these transients originate from the cytosol/perinuclear area. We show now that all three IP_3Rs are expressed throughout the SR of the cardiomyocyte in both isolated rat cardiomyocytes and human tissue. Using nuclear-localized GCaMP6s to unambiguously monitor nuclear calcium, our results simultaneously imaging nuclear and cytosolic calcium indicate that nuclear-only calcium transients, if present, are an exceedingly rare events. This is consistent with a study by Nakao et. al. using chelators and targeted parvalbumin which concluded that nuclear calcium signals in cardiomyocytes after electrical stimulation originate in the cytoplasm [29]. This report also compared cytosolic and nuclear release events induced by the IP_3-coupled agonist IGF-1. Nuclear release events were of higher amplitude compared to cytosolic events as measured with the genetically encoded calcium sensor GECO1 [29]. The ability of nuclear and cytosolic calcium chelators to block these events were not investigated, so it is unclear whether IGF-1 can specifically stimulate nuclear or perinuclear calcium release via the IP_3R. Similar to our results Nakao et. al. also observed that the IP_3R is mainly localized at the perinuclear membrane in NRVMs where it interacts with NCS-1 [29]. Thus, a possible explanation for the difference in calcium peak amplitude observed in the nucleus after IGF-1-induced calcium release is that perinuclear localization of the IP_3R may allow calcium to diffuse rapidly into the nucleus. Consistent with the results of Nakao et. al. we have also observed that agonist-induced calcium release is able to induce cytosolic calcium oscillations (Fig. 5) which is relevant to the activation of different key transcription factors. Further work will be necessary to evaluate the possibility that IGF-1, ET-1, or other IP_3-coupled agonists can activate a pool of nuclear or perinuclear receptors to elicit nuclear-specific calcium transients.

GCaMP6s has been shown to be more sensitive to calcium than other genetically encoded calcium indicators and synthetic calcium dyes such as Fura-2 [22, 40]. One limitation of GCaMP6s is the relatively slow kinetics which could preclude the possibility of imaging fast calcium release events [40]. However, using cytosolic GCaMP6s we can detect cytosolic events not detected by Fura-2 (Fig. S6). The superior sensitivity of GCaMP6s have been used to detect cytosolic and nuclear restricted calcium transients [27]. However, it remains entirely possible that there are low amplitude calcium release events in the nucleus which are below the detection capability of GCaMP6s. As it is highly likely that more sensitive

genetically encoded calcium indicators will be developed, this possibility can be tested in future studies. Another limitation of our study is the use of neonatal rat ventricular cardiomyocytes (NRVMs) as a model of hypertrophy. Although they display many characteristics of hypertrophy in vitro, they do not precisely reflect the response of adult cardiomyocytes in vitro or in vivo. Neonatal and adult cardiomyocytes are well known to have differences in calcium-handling properties due to obvious differences in T-tubule/SR morphology and physiology. Interestingly we found that the perinuclear localization of the IP_3R was much less prominent in adult cardiomyocytes when compared to NRVMs. This is strongly suggestive that the spatio-temporal pattern of calcium release in adult cardiomyocytes would be very different compared to NRVMs. Indeed, this has been intensively investigated (reviewed in [41]), however the remodeling of IP_3R signaling in the developing myocyte is still at an early stage of understanding [42]. Thus it will be important in future studies to compare the nuclear versus cytosolic release events due to IP_3R activation in adult cardiomyocytes. It would also be interesting to examine in vivo models such as cardiac-specific knockout of each of the three IP_3Rs in rodent models of heart failure. Finally, it has also become increasingly apparent that epigenetics plays a major role in cardiac hypertrophy, and this likely contributes to widespread transcriptional changes leading to fetal reprogramming [43–45]. It would also be of interest in future studies to examine how calcium/IP_3 signaling pathways are regulated by epigenetic mechanism

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- All three IP_3R isoforms are expressed in rat ventricular cardiomyocytes and in human heart tissue.
- All three IP3R isoforms are required for ET-1 induced hypertrophic signaling.
- Calcium release induced by ET-1 is not restricted to the nuclear compartment in rat ventricular cardiomyocytes.

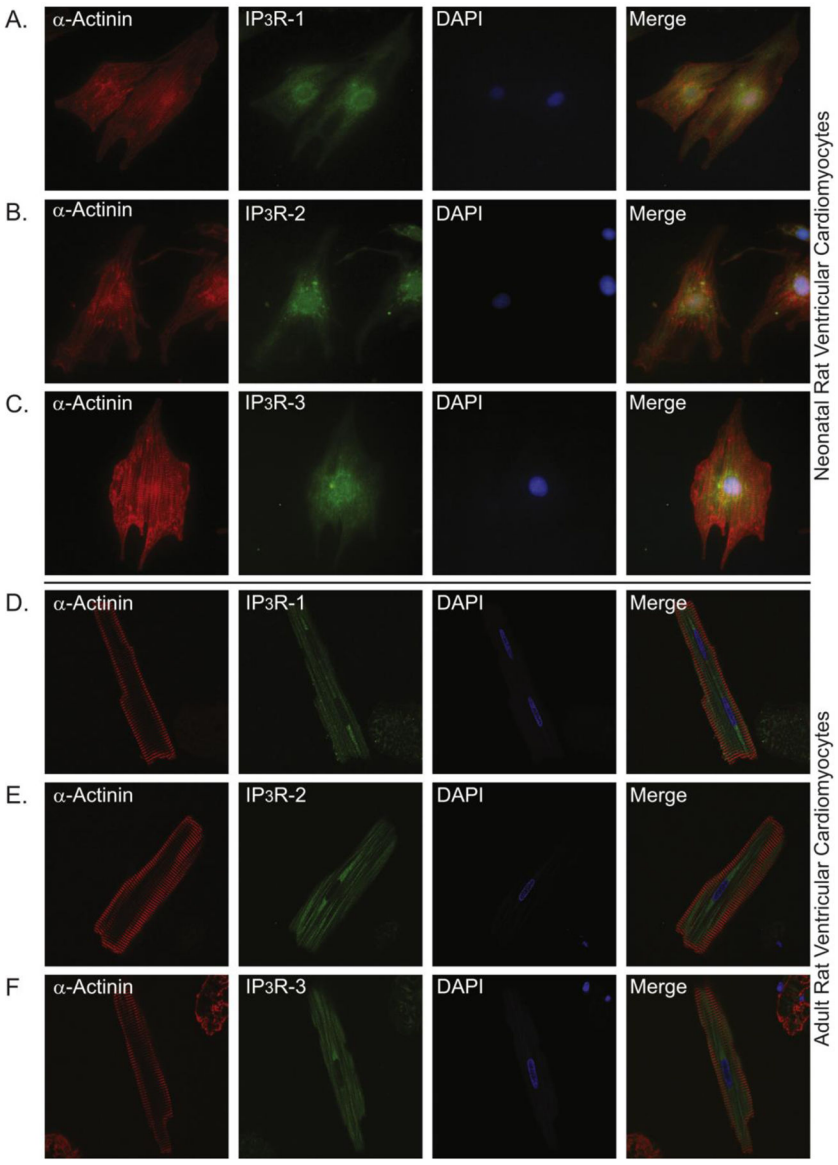

Fig. 1. Expression and distribution IP_3R isoforms in primary neonatal and adult ventricular cardiomyocytes

Immunofluorescence staining of neonatal (rows A–C) and adult (rows D–F) ventricular cardiomyocytes. Column 1 is stained with α-actinin to label sarcomeres. Column 2 is stained with indicated IP_3R antibodies. Column 3 is DAPI staining of the nucleus. Column 4 is the merged images of each row. See also Supplemental Fig. 1 for larger versions of the merged images.

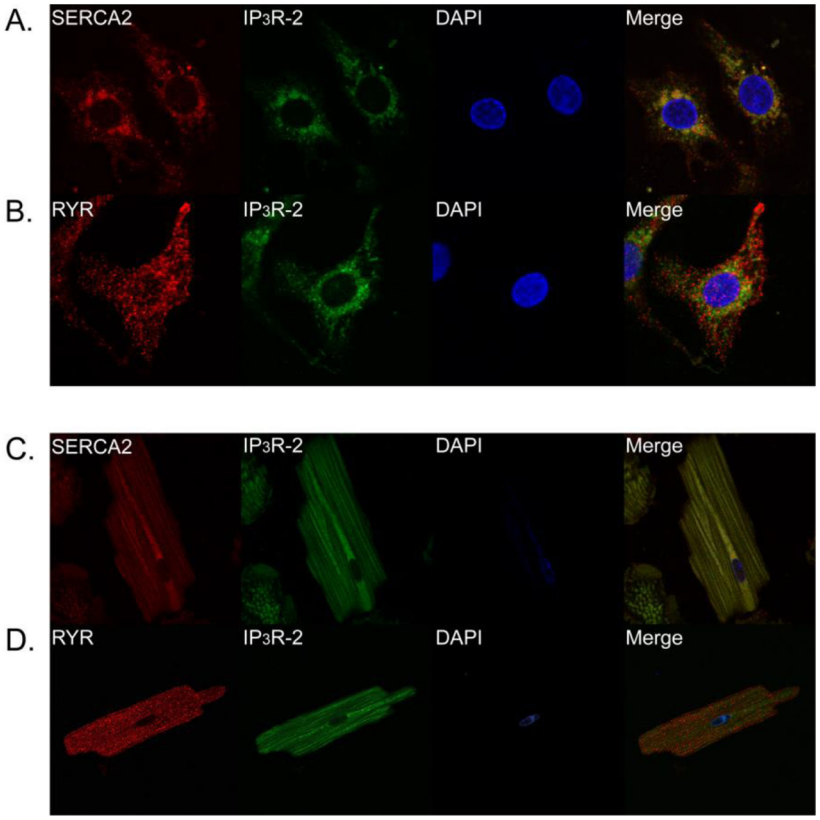

Fig. 2. Expression and distribution of IP3R, SERCA2, and RYR in primary ventricular cardiomyocytes

Immunofluorescence staining of neonatal ventricular cardiomyocytes stained with SERCA2 and IP3R-2 (A); RYR2 and IP3R-2 (B). Immunofluorescence staining of adult ventricular cardiomyocytes with SERCA2 and IP3R-2 (C); RYR2 and IP3R-2 (D). See also Supplemental Fig. 2 for larger versions of the merged images.

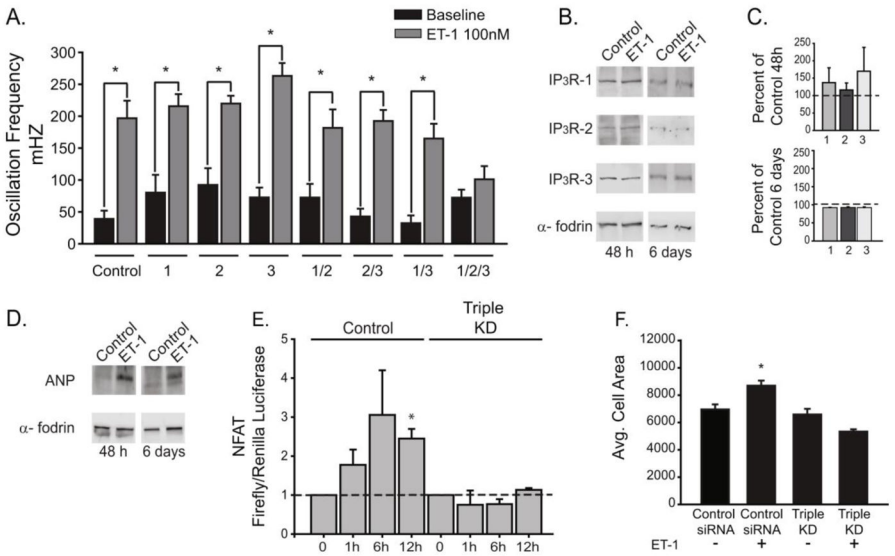

Fig. 3. All three IP$_3$R isoforms contribute to hypertrophic signaling by ET-1

(A) Neonatal cardiomyocytes were transfected with control or IP $_3$R siRNA as indicated. Cells were treated with ET-1 and oscillation frequency was quantified from at least 4 separate experiments (control n=6, 1 n=6, 2 n=7, 3 n=6, 1/2 n=6, 2/3 n=5, 1/3 n=5 and 1/2/3 n=4). (B). Western blot of indicated proteins after 48 hours or 6 days treatment with 100 nM ET-1. (C). Quantification of IP $_3$R levels after ET-1 treatment for 48 hours or 6 days expressed as percent of control. (D) Atrial natriuretic peptide (ANP) levels after 48 hours and 6 days treatment with ET-1. Blotting with alpha-fodrin was used as control. (E) NFAT activity using luciferase reporter construct after ET-1 treatment for the indicated times in control siRNA and triple IP$_3$R siRNA transfected cardiomyocytes. (F) Average cell area before and after 48 hours treatment with ET-1. Neonatal cardiomyocytes were transfected with control siRNA and triple IP $_3$R siRNA. All data are shown as mean ± SEM, *P < 0.01 vs control.

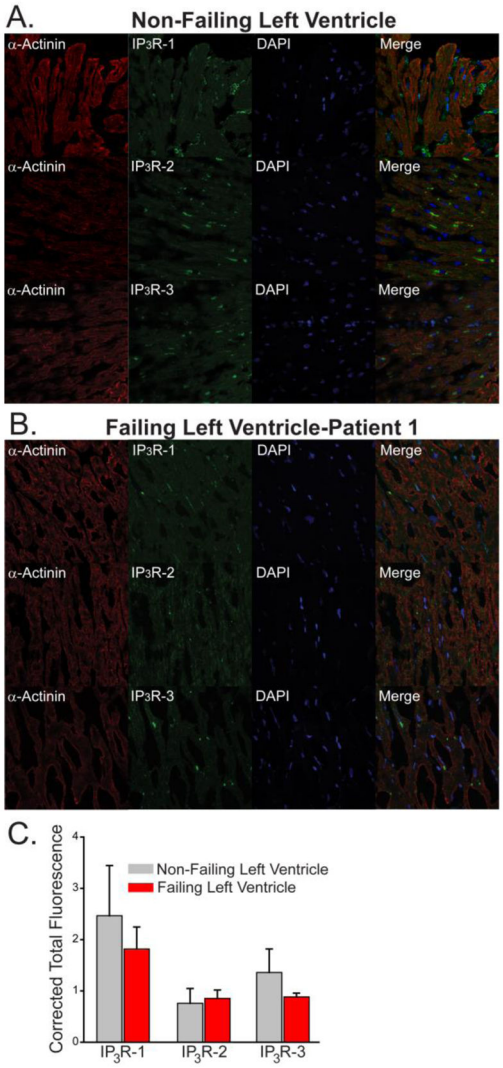

Fig. 4. Expression of IP_3R isoforms in non-failing and end stage heart failure samples

Immunofluorescence staining of non-failing human left ventricle (A). Left ventricular heart failure patient 1(B). Column 1 is stained with α-actinin to label sarcomeres. Column 2 is stained with indicated IP $_3$R antibodies. Column 3 is DAPI staining of the nucleus. Column 4 is the merged images of each row. (C) Quantified immunofluorescence from three control and three failing heart samples (see Supplemental Fig. S4 for additional staining).

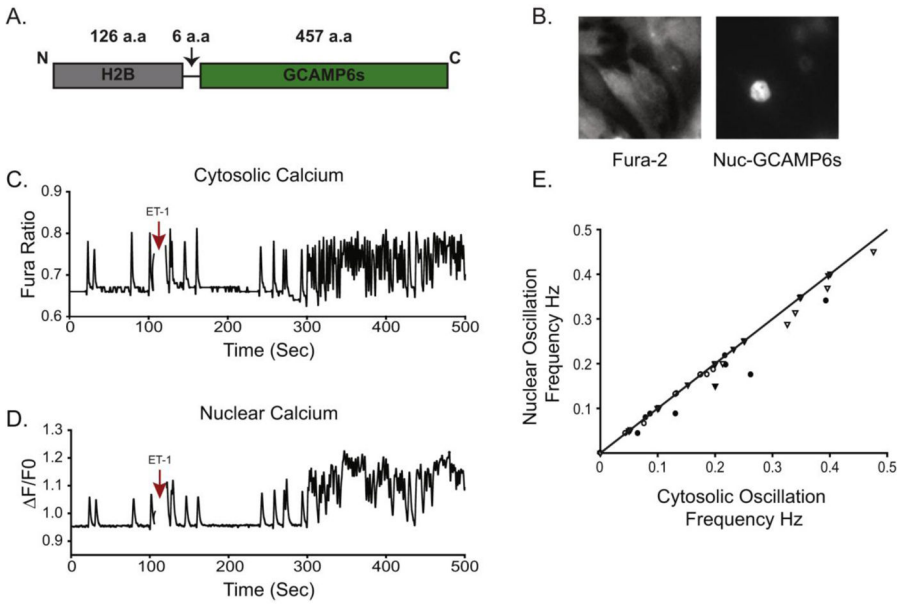

Fig. 5. Nuclear and cytosolic calcium in response to ET-1 stimulation

(A) Schematic cartoon of H2B-GCaMP6s. (B) Representative image of neonatal cardiomyocytes expressing H2B-GCaMP6s loaded with Fura-2 AM. (C) Single cell imaging of a neonatal cardiomyocyte treated with 100 nM ET-1 at indicated time. (D) H2b-GCaMP6s signal in the same cell as in C. (E) Plot of cytosolic vs. nuclear oscillation frequency. Each symbol represents a single coverslip averaging 5–10 cells for a total of four separate coverslips. Frequency data was quantified from 20 seconds bins.