Spatially Defined InsP$_3$-Mediated Signaling in Embryonic Stem Cell-Derived Cardiomyocytes

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Abstract

The functional role of inositol 1,4,5-trisphosphate (InsP$_3$) signaling in cardiomyocytes is not entirely understood but it was linked to an increased propensity for triggered activity. The aim of this study was to determine how InsP$_3$ receptors can translate Ca$^{2+}$ release into a depolarization of the plasma membrane and consequently arrhythmic activity. We used embryonic stem cell-derived cardiomyocytes (ESdCs) as a model system since their spontaneous electrical activity depends on InsP$_3$-mediated Ca$^{2+}$ release. [InsP$_3$]$_{i}$ was monitored with the FRET-based InsP$_3$-biosensor FIRE-1 (Fluorescent InsP$_3$ Responsive Element) and heterogeneity in sub-cellular [InsP$_3$]$_{i}$ was achieved by targeted expression of FIRE-1 in the nucleus (FIRE-1nuc) or expression of InsP$_3$ 5-phosphatase (m43) localized to the plasma membrane. Spontaneous activity of ESdCs was monitored simultaneously as cytosolic Ca$^{2+}$ transients (Fluo-4/AM) and action potentials (current clamp). During diastole, the diastolic depolarization was paralleled by an increase of [Ca$^{2+}$]$^{i}$, and spontaneous activity was modulated by [InsP$_3$]$_{i}$. A 3.7% and 1.7% increase of FIRE-1 FRET ratio and 3.0 and 1.5 fold increase in beating frequency was recorded upon stimulation with endothelin-1 (ET-1, 100 nmol/L) or phenylephrine (PE, 10 µmol/L), respectively. Buffering of InsP$_3$ by FIRE-1nuc had no effect on the basal frequency while attenuation of InsP$_3$ signaling throughout the cell (FIRE-1), or at the plasma membrane (m43) resulted in a 53.7% and 54.0% decrease in beating frequency. In m43 expressing cells the response to ET-1 was completely suppressed. Ca$^{2+}$ released from InsP$_3$Rs is more effective than Ca$^{2+}$ released from RyRs to enhance Ik$_{Ca}$. The results support the hypothesis that in ESdCs InsP$_3$Rs form a functional signaling domain with NCX that translates Ca$^{2+}$ release efficiently into a depolarization of the membrane potential.

Introduction

In cardiac muscle the expression of inositol-1,4,5-triphosphate receptors (InsP$_3$R) is most abundant during early development [1,2]. In embryonic as well as neonatal cardiomyocytes the presence of all three InsP$_3$R isoforms has been documented with the most prominent appearance of InsP$_3$R1 and InsP$_3$R2 [3,4]. At embryonic and neonatal stages of differentiation, immunostainings indicate that InsP$_3$R2 pre-dominate locally to the nuclear envelope [4–6]. Receptor mediated G$_{	ext{q}}$-protein stimulation of these cells results in InsP$_3$ production and concomitantly Ca$^{2+}$ release events that occurred mainly at the nuclear envelope [4,7,8]. The functional role of InsP$_3$Rs in the developing myocytes is not well understood, but in the embryonic heart tube, mouse and human embryonic stem cell-derived cardiomyocytes and human iPSC cell-derived cardiomyocytes a role of InsP$_3$R-mediated Ca$^{2+}$ release in the generation of spontaneous electrical activity has been demonstrated [9–12].

In contrast to the abundance of InsP$_3$Rs in the early developmental stages, their expression decreases towards adulthood; However, in the adult atrial [13] and ventricular muscle of rat [14], cat [15], and rabbit [16] the expression of InsP$_3$R2 isoforms was demonstrated. In atrial myocytes its distribution is homogeneous throughout the cell, whereas in ventricular myocytes a prevalence in the nuclear envelope (rat) [14] and the dyadic junctions (mouse) [17] was reported. During excitation-contraction coupling in the adult cardiac muscle, Ca$^{2+}$ is released from the sarcoplasmic reticulum mainly through the ryanodine receptor type 2 (RyR2), which is expressed 50 fold higher than InsP$_3$Rs. In contrast InsP$_3$R-mediated signaling has been linked to excitation-transcription coupling. Activation of nuclear InsP$_3$Rs was sufficient for the activation and translocation of the transcription factor HDAC that remained unresponsive to beat-to-beat changes in [Ca$^{2+}$]$^{i}$ [18]. Nevertheless, despite the comparably low expression levels, InsP$_3$Rs play a role in the induction of cardiac arrhythmia. Stimulation of InsP$_3$R-mediated Ca$^{2+}$ release results in increased spark frequency, positive inotropy, and an increase in arrhythmic spontaneous activity in atrial and ventricular myocytes [15,16,18–21]. As indicated by these studies, the amount of InsP$_3$R-mediated Ca$^{2+}$ release appears low and may be more relevant as a facilitator...
of Ca\textsuperscript{2+} release from RyRs thus contributing indirectly to excitation-contraction coupling.

The sub-cellular location of InsP\textsubscript{3}-mediated Ca\textsuperscript{2+} release could critically influence its function. Whereas sub-sarcolemmal Ca\textsuperscript{2+} release can depolarize the membrane by activation of sodium calcium exchange (NCX), Ca\textsuperscript{2+} released at the nuclear envelope might have a higher likelihood to be removed by SERCA [19,21]. The functional differences between spatially distinct Ca\textsuperscript{2+} signaling events are very pronounced in ESDCs. Localized Ca\textsuperscript{2+} release events through RyRs (sparks) can be frequently monitored throughout the ESDC, whereas localized release events through InsP\textsubscript{3}Rs (puffs) are seldom identified [8,9]. Nonetheless, sparks are insufficient to maintain spontaneous activity, whereas InsP\textsubscript{3} mediated Ca\textsuperscript{2+} release can sustain spontaneous activity even after depletion of the RyR operated Ca\textsuperscript{2+} stores or in RyR2 deficient ESDCs [9,22].

We used ESDCs as a model to test the hypothesis that InsP\textsubscript{3}Rs close to the plasma membrane form functional signaling domains with NCX and that, in contrast to cytoplasmic or nuclear InsP\textsubscript{3}Rs, their Ca\textsuperscript{2+} release can be efficiently translated into INCX and a depolarization of the membrane potential (V\textsubscript{m}). For this purpose the FIRE-1nuc coding region was excised with Bgl II and ligated into the ESDC plasmid, whereas localized release events through InsP\textsubscript{3}Rs (puffs) are seldom identified [8,9]. Nonetheless, sparks are insufficient to maintain spontaneous activity, whereas InsP\textsubscript{3} mediated Ca\textsuperscript{2+} release can sustain spontaneous activity even after depletion of the RyR operated Ca\textsuperscript{2+} stores or in RyR2 deficient ESDCs [9,22].

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**Materials and Methods**

The culture of mouse embryonic stem cells (mES) of the cell line CMV (Specialty Media; Phillipsburg, NJ, USA), their differentiation into cardiomyocytes and use for laser scanning confocal microscopy are described in detail elsewhere [9,23].

**FIRE-1 construct**

As previously described [24] the FIRE-1 InsP\textsubscript{3} biosensor was assembled using the InsP\textsubscript{3}-R ligand-binding domain termini fused with enhanced CFP and YFP at the amino and carboxyl termini, respectively. In FIRE-1 transfected COS-1 cells, rat neonatal, adult cat ventricular myocytes and ESDCs (Data S1) FIRE-1 exhibited comparable dynamic range and a 10% increase in donor (CFP) fluorescence upon bleaching of YFP, indicative of FRET [24].

**FIRE-1nuc construction**

The FIRE-1 indicator was targeted to the nucleus by insertion into a triplet tandem of the SV40 large T-antigen nls using the following oligonucleotides: (sense: GCTCGAGATCCAAAAAAGAGAGAAGCCCTTTCTTGATCGAGTTTCTTCTTCTCTTCTTCTTTTGAGATCCACCTGTCTTCTCTCTTCTTCTTTTGAGATCGACCCAC and antisense: GCTCGAGATCCAAAAGAGAGAAGCTACCTTTCTCTCTTTTGAGATCCACCTGTCTTCTCTCTTCTTTTGAGATCGACCCAC) (kindly provided by Dr. Elizabeth A. Woodcock, Baker Heart Research Institute, Melbourne, Victoria, Australia) by PCR using the following primers (sense: GGGGTCGACCTACCTATACAGAGAC and antisense: GGGGTCGACCTACCTATACAGAGAC). The PCR product was digested with Sal I, ligated into similarly digested pCMV-5 vector, and expression was verified in COS-1 cells by transient transfection and Western blotting with anti-FIRE antibody (Affinity BioReagents) (Figure S2). The FLAG-tagged m43 coding region was excised with Sal I and ligated into Sal I digested pShuttle-CMV vector (Stratagene; La Jolla, CA) for adenoviral production.

**FIRE-1nuc and m43 adenovirus production**

The adenoviruses were created using the commercially available AdEasyTM XL adenoviral vector system kit (Stratagene; La Jolla, CA). Briefly, the bacterial cell line BJ5183-AD-1, pre-transformed with the plasmid pAdEasy-1 was used for in vivo homologous recombination with either pShuttle-CMV-m43 or pShuttle-CMV-FIRE-1nuc. The pAdEasy-1-m43 or pAdEasy-1-FIRE-1nuc insert containing plasmids were separately transfected into DH5α and produced in bulk. Purified pAdEasy-1-m43 or pAdEasy-1-FIRE-1nuc was used to transfect AD-293 cells for virus amplification. Both viruses were plaque-purified, amplified, CaCl\textsubscript{2} gradient-purified, and stored at −80°C.

**Adenoviral transduction and FRET measurements**

24 hours post plating, dissociated ESDCs were transduced with recombinant replication-deficient adenovirus carrying sequence for either the InsP\textsubscript{3} biosensor FIRE-1 [24], FIRE-1nuc [24] sequence plus 3 tandem nuclear localization signals [3 tandem-DPKKKRRK], or FLAG tagged m43 phosphatase [25]. After overnight incubation at a multiplicity of infection (MOI) of 1–10 the media was replaced. Changes in fluorescence resonance energy transfer (FRET) between the cyan fluorescent protein (CFP) and the yellow fluorescent protein (YFP) were measured by laser scanning confocal microscopy. CFP was excited with a 440 nm diode laser. CFP and YFP emissions were measured at 488 (F\textsubscript{CFP}) and >560 nm (F\textsubscript{YFP}), respectively. Changes in InsP\textsubscript{3} activity are defined as the relative change in the background corrected ratio of F\textsubscript{YFP}/F\textsubscript{CFP}. To obtain a reliable reproducible readout for the changes induced by the pharmacological agents, the fluorescence was determined after 3 min of superfusion. The change was then quantified as the average fluorescence over the time period of 5 min. The experiments were conducted at room temperature. FRET between CFP and YFP was confirmed by photobleaching of the acceptor molecule (YFP; Figure S1).

**Chemicals and statistics**

Endothelin-1 (ET-1), phenylephrine (PE), and caffeine were diluted in H\textsubscript{2}O, 2-aminoethoxydiphenyl borate (2-APB), U73122 and U73343 were dissolved in dimethylsulphoxide (DMSO) and further diluted >1,000 fold for experiments. All chemicals were purchased from Sigma. Results are presented as mean ± SEM and n represents the number of experiments. Statistical differences between two groups were analyzed by student’s t-test and considered significant at P<0.05. Multiple comparisons were performed by analysis of variance (ANOVA) and significant differences between the groups were identified with the Tukey HSD Test indicating significance at P<0.05. A detailed description of
the confocal imaging, electrophysiological recordings, and immunocytochemistry can be found in Data S1.

Results
In our previous study we demonstrated that InsP3-mediated Ca\(^{2+}\) release plays a critical role in the generation of spontaneous activity in ESdCs [9]. To determine whether the changes in [Ca\(^{2+}\)]\(i\) correlate with changes in membrane voltage (V\(_m\)) we recorded action potentials (APs) in Fho-4/AM loaded ESdCs with the perforated patch technique. As shown in Fig. 1, changes in [Ca\(^{2+}\)]\(i\) closely correlated with changes in V\(_m\) showing a clear increase in basal [Ca\(^{2+}\)]\(i\) during the diastolic depolarization. This increase in [Ca\(^{2+}\)]\(i\) was spatially homogeneous and did not correlate with a specific location inside the cell e.g. the nuclear envelope [26] or sub-sarcolemmal space [27].

To determine the location of InsP3Rs in ESdCs, cells were stained with antibodies against InsP3Rs type-1 and type-2. As shown in Fig. 2, ESdCs stained positive for both InsP3R isoforms. Pronounced perinuclear staining was identified, together with extensive endoplasmic reticulum staining throughout the cell that extended to the plasma membrane. This localization pattern suggests that InsP3R-mediated Ca\(^{2+}\) release is not restricted to the nuclear envelope.

ESdCs express RyRs and caffeine induced Ca\(^{2+}\) transients have been recorded already when the cells first develop spontaneous activity [9,28]. To evaluate whether RyRs and InsP3Rs control different functional pools of Ca\(^{2+}\) stored in the SR we superfused ESdCs with ET-1 (100 nmol/L) after the caffeine sensitive stores were depleted (caffeine: 10 mmol/L; Fig. 3A). The refilling of the stores was prevented by caffeine in the extracellular Ca\(^{2+}\) solution. After recovery from the caffeine induced Ca\(^{2+}\) release, ET-1 induced a small but significant increase in basal [Ca\(^{2+}\)]\(i\) (Fig. 3BC). The ET-1 induced change indicates the presence of an InsP3R regulated SR Ca\(^{2+}\) pool in ESdCs that is independent of caffeine-sensitive stores. This is consistent with the fact that ESdCs maintain their spontaneous activity when RyR sensitive stores are depleted by caffeine [9].

To determine if stimulation of InsP3Rs can influence NCX activity we measured I\(_{\text{NCX}}\) during the superfusion of ESdCs with ET-1 (100 nmol/L; for details on the voltage protocol see Data S1). A significant increase of I\(_{\text{NCX}}\) was determined in the presence of ET-1 (Fig. 4AD). This ET-1 induced increase, was inhibited by the InsP3R blocker 2-APB (2 μmol/L; Fig. 4BD). To determine whether the effect of ET-1 depended on an overall increase in [Ca\(^{2+}\)]\(i\), we measured I\(_{\text{NCX}}\) in ESdCs superfused with 100 μmol/L caffeine. At this concentration caffeine increases the open probability of RyRs and leads to increased diastolic [Ca\(^{2+}\)]\(i\). Caffeine and ET-1 both increased basal [Ca\(^{2+}\)]\(i\), to a similar extent (Fig. 4E). However, the frequency of spontaneous Ca\(^{2+}\)-transients was only increased during ET-1 superfusion while it remained unchanged in the presence of caffeine (Fig. 4F). Consistent with this, I\(_{\text{NCX}}\) remained unchanged following 3 min of superfusion with caffeine (Fig. 4CD). These findings support that InsP3R dependent Ca\(^{2+}\) release more efficiently enhances I\(_{\text{NCX}}\) activity.

To determine the spatial organization of InsP3 signaling in ESdCs we transduced cells with an adenosine expressing FIRE-1 (24 h). FIRE-1 exhibits an increase in the fluorescence ratio (F\(_{560}/F_{488}\)) upon binding of InsP3 [24]. When InsP3 production in ESdCs was stimulated by ET-1 (100 nmol/L; for details on the voltage protocol see Data S1) a positive chronotropic effect was determined in the frequency of spontaneous Ca\(^{2+}\) transients (Fig. 5AB and CD, respectively) with a 3.0±1.1 fold (from 0.13±0.03 Hz to 0.32±0.06 Hz; n = 4) and a 1.5±0.3 fold (from 0.5±0.12 Hz to 0.65±0.1 Hz; n = 5) fold increase in the frequency after 3 minutes of superfusion, respectively. In FIRE-1 expressing ESdCs the same superfusion protocol was applied. When the fluorescence was integrated over the entire width of the cell, an ET-1 or PE induced increase in the FRET ratio (F\(_{560}/F_{488}\)) was determined that reached a steady state after about 2.5 min of superfusion. For ET-1, a 3.7±0.6% change (Fig. 5E & 6F; n = 4) in the FRET ratio was determined while the change for PE amounted to 1.7±0.03% (steady state after 2.5 min of superfusion).

Figure 1. Interplay between spontaneous APs and [Ca\(^{2+}\)]\(i\) in ESdCs. Confocal line scan (A) and corresponding F/F\(_0\) plot (B) in a 17 day old ESdC with simultaneous measurement of changes in V\(_m\). (C). Spontaneous action potentials (APs) are recorded that correlate in time with Ca\(^{2+}\) transients. D: Superposition of Ca\(^{2+}\) transients and APs clearly show an increase in [Ca\(^{2+}\)]\(i\), in the late phase of the diastolic depolarization.

Figure 2. InsP3 receptor isoform expression in ESdCs. Dissociated ESdCs (d10–11) were stained for α-actinin (A, D), InsP3R1 (B) and InsP3R2 (E, F). C, F: Superposition of α-actinin and InsP3R staining.

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in FRET ratio with U73122 were due to a decrease in basal $[\text{Ca}^{2+}]_i$ we used 3 alternative approaches to reduce basal $[\text{Ca}^{2+}]_i$ and spontaneous activity in ESdCs. During superfusion of ESdCs with either 2-APB (Fig. 6C), Ca $^{2+}$-free solution (Fig. 6D) or BAPTA-AM (Fig. 6E) the fluorescent ratio F560/F488 was determined. We had previously demonstrated that these interventions attenuate ESdCs spontaneous activity and reduce basal $[\text{Ca}^{2+}]_i$ by 18.1 $\pm$ 6.9% (n = 2) and 18.6 $\pm$ 0.36% (n = 2), and 27.05 $\pm$ 1.5% (n = 3), respectively [9]. Under none of the conditions, (Ca $^{2+}$-free, n = 3; 2-APB: 2 $\mu$mol/L, n = 3; or BAPTA/AM: 5 $\mu$mol/L, n = 3).

**Figure 3.** InsP$_3$R controlled Ca$^{2+}$ stores are in part functionally separated from RYR controlled stores. A: $F/F_0$ plot of $[\text{Ca}^{2+}]_i$ in a spontaneously active ESdC after superfusion of the cell with tyrode solution supplemented with 4 mmol/L Ca$^{2+}$. After depletion of ryanodine receptor operated Ca$^{2+}$ stores by 10 mmol/L caffeine, ET-1 (100 nmol/L) induced an increase in basal $[\text{Ca}^{2+}]_i$. B: Magnification of the section of the $F/F_0$ plot indicated by the box. C: Bar graph illustrating the increase in basal Ca$^{2+}$ induced by ET-1 in the presence of caffeine (n = 6; *: P<0.05).

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**Figure 4.** InsP$_3$R induced Ca$^{2+}$ release stimulates NCX activity. Current voltage plots for $i_{\text{NCX}}$ recorded in ESdCs under control conditions and after 3 min superfusion with either A: ET-1 (100 nmol/L; n = 8), B: ET-1 (100 nmol/L)+2-APB (2 $\mu$mol/L; n = 5), or C: caffeine (Caff: 100 $\mu$mol/L; n = 7).

Currents are corrected for the nickel (5 mmol/L) insensitive background. D: Normalized $i_{\text{NCX}}$ recorded at −80 mV. $i_{\text{NCX}}$ recorded on superfusion with ET-1 was significantly different from Caff and ET-1+2-APB. E: Normalized change in basal $[\text{Ca}^{2+}]_i$. Significant increase of basal $[\text{Ca}^{2+}]_i$ was observed upon superfusion with Caff and ET-1. F: ESdCs beating frequency after 3 min superfusion with ET-1 (n = 5) or caffeine (n = 4) respectively. *: paired t-test P<0.005; #: one way ANOVA P<0.05.

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1 µmol/L, n = 2) was a change in the FRET ratio (F560/F488) measured indicating that changes in FIRE-1 did not depend on changes in [Ca\textsuperscript{2+}]\textsubscript{i} or spontaneous activity. FIRE-1 transduction changed the spontaneous activity in ESdCs. The overall number of spontaneously active cells was reduced and in beating cells the frequency of spontaneous Ca\textsuperscript{2+} transients was attenuated (FIRE-1: 0.43 ± 0.09 Hz; n = 5) com-

![Figure 5](image)

**Figure 5.** Agonist induced InsP\textsubscript{3} increase regulates ESdC beating frequency. Line scan and corresponding F/F\textsubscript{0} plots from spontaneously active ESdCs in Ctrl conditions (A, C) and after superfusion with either B: ET-1 (100 nM) or D: PE (10 µmol/L). Superfusion of FIRE-1 expressing cells with E: ET-1 (n = 4) or F: PE (n = 2) induced an increase in the fluorescence ratio F560/F488 indicating an increase in [InsP\textsubscript{3}]\textsubscript{i}. doi:10.1371/journal.pone.0083715.g005

![Figure 6](image)

**Figure 6.** Changes in [IP\textsubscript{3}] are independent of [Ca\textsubscript{2+}]\textsubscript{i}. Fluorescence ratio F560/F488 measured in FIRE-1 expressing ESdCs superfused with A: the PLC inhibitor, U73122 (1 µmol/L; n = 5), B: its inactive analog, U73343 (1 µmol/L; n = 2), C: the InsP\textsubscript{3}R blocker, 2-APB (2 µmol/L; n = 2), D: Ca\textsuperscript{2+} -free solution (n = 2) or E: BAPTA-AM. F: The normalized changes of F\textsubscript{560}/F\textsubscript{488} recorded in ET-1, PE or U73122. #: one way ANOVA P<0.05. doi:10.1371/journal.pone.0083715.g006

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pared to non-transduced cells (Control: 0.93±0.1 Hz; n = 11) of the same age (day 16). The results indicate that the InsP$_3$ buffer capacity of FIRE-1 in ESdCs reduces the beating frequency. To determine if frequency regulation through InsP$_3$-mediated Ca$^{2+}$-release depends on defined InsP$_3$ signaling domains we employed cells expressing m43 or FIRE-1nuc 24 hours post adenoviral transduction. The distribu-
tion of m43 was determined through immunoblotting of fractionated whole cell lysate from COS-1 cells expressing FLAG-tagged m43 (Fig. 2) and immunostaining of transduced cat atrial myocytes (Fig. 3D) and ESdCs (Fig. 7E). Immunoblotting clearly localizes m43 in the membrane fraction of the cell lysate and immunostainings show a preferential localization of m43 at the plasma membrane of atrial myocytes and ESdCs 24 hours post adenoviral transduction. The distribution is in agreement with previous findings of Vasilevski et al. (2000) [25].

To determine how localized suppression of InsP$_3$ signaling effects the spontaneous activity of ESdCs we measured [Ca$^{2+}$]$_i$ in cells expressing m43 or FIRE-1nuc 24 hours post adenoviral transduction. Figure 8A shows spontaneous whole cell Ca$^{2+}$-transients in an ESdC expressing FIRE-1nuc. Non-transduced 14 days old cells obtained from the same isolation served as control. Control ESdCs and ESdCs transduced with FIRE-1nuc exhibited no significant difference in their Ca$^{2+}$ transient frequency (0.51±0.05 Hz; n = 7 and 0.44±0.02 Hz; n = 6, respectively; Fig. 8C). Upon stimulation with ET-1 the frequency of spontaneous Ca$^{2+}$-transients increased 58±9% (n = 3) in control and 24±1% (n = 4; P<0.05) in FIRE-1nuc transduced ESdCs. The data indicate that the ET-1 induced positive chronotropic effect persists when InsP$_3$R is buffered in the nucleus of ESdCs. In contrast, cells transduced with m43 exhibited a significantly reduced beating frequency in comparison to control and FIRE-1nuc cells (54±9% of control; n = 5; P<0.05; Fig. 8BC) and an ET-1 induced positive chronotropic effect was not observed (Fig. 8C). The data support the hypothesis that membrane delineated inhibition of InsP$_3$ signaling can efficiently modulate the spontaneous activity of ESdCs.

**Discussion**

In the present study we demonstrate that a basal production of InsP$_3$ maintains spontaneous activity in ESdCs by regulating Ca$^{2+}$-release from a SR Ca$^{2+}$ pool that is functionally independent from RyR-mediated Ca$^{2+}$-release. In addition we show that while InsP$_3$ production changes [Ca$^{2+}$]$_i$ throughout the cytoplasm, the InsP$_3$Rs and RyRs localize to and deplete respective other InsP$_3$ or caffeine sensitive store [36]. In ESdCs the InsP$_3$R1 maintained spontaneous activity in embryonic cardiomyocytes which was suppressed with introduction of antisense cDNA of InsP$_3$R1 [10].

**InsP$_3$Rs in developing cardiomyocytes**

All three InsP$_3$R subtypes -1, -2, and -3 are expressed in undifferentiated ES cells [30] and embryonic cardiomyocytes [1,3,31] where InsP$_3$R1 is most prevalent in the nuclear envelope [3,10]. We have identified InsP$_3$R1 and InsP$_3$R2 in ESdCs (see Fig. 2BE) with a sub-cellular distribution comparable to that in neonatal myocytes [4]. Previous studies also suggested that InsP$_3$R1 maintains spontaneous activity in embryonic cardiomyocytes which was suppressed with introduction of antisense cDNA of InsP$_3$R1 [10].

**Ca$^{2+}$ release from the sarcoplasmic reticulum by InsP$_3$Rs**

The SR is a continuous network [32] where Ca$^{2+}$ can redistribute [18,33–35]. InsP$_3$Rs and RyR$_3$ localize to and deplete the same SR network in rabbit ventricular myocytes [33]. Interestingly our data demonstrate that InsP$_3$R-mediated Ca$^{2+}$ release can still be induced when RyR$_3$-controlled Ca$^{2+}$ stores were depleted by caffeine. This supports the hypothesis that InsP$_3$R signaling domains are functionally isolated and not immediately affected by RyR$_3$-controlled Ca$^{2+}$ store depletion. A similar finding was described in colonic smooth muscle cells where in an interconnected SR network, Ca$^{2+}$ release from RyR$_3$ or InsP$_3$R controlled stores could be demonstrated after depletion of the respective other InsP$_3$ or caffeine sensitive store [36]. In ESdCs the size of this functionally independent InsP$_3$ sensitive Ca$^{2+}$ store remains to be determined but as demonstrated, it is sufficient to maintain spontaneous activity of ESdCs [9].
Spatial Differences in InsP₃ Signaling

Role of InsP₃Rs for the generation of spontaneous activity

We and others have demonstrated that Ca²⁺ release plays a dominant role in the generation of spontaneous Ca²⁺ transients in mouse [3,7,9,10,37] and human embryonic cardiomyocytes [11,26]. The transients coincide with changes in Vₘ, and the late phase of the diastolic depolarization is accompanied by an increase in [Ca²⁺]; (Fig, 1). A similar increase in [Ca²⁺] was described in cat latent pacemaker cells and cat and rabbit sinus nodal cells [27,38] where sub-sarcoplasmic Ca²⁺ release from RyRs is proposed to enhance a depolarization of Vₘ, by activation of NCX.

In latent pacemaker and sinus nodal cells the Ca²⁺ release that initiates the diastolic depolarization originates at the nuclear envelope [7,8,39]. This was supported by the nuclear localization of InsP₃Rs and the demonstration of peri-nuclear InsP₃,R-mediated Ca²⁺ release [7,8,40]. While most of the InsP₃ synthesis occurs at the plasma membrane PLCs and InsP₃ production are also described within the nuclear envelope [41]. In our experiments nuclear InsP₃ buffering through FIRE-1nuc had no significant effect on Ca²⁺ transient frequency thus excluding a major contribution of nuclear PLCs to spontaneous activity.

In cardiac myocytes, stimulation of InsP₃,R-mediated Ca²⁺ release by ET-1 can induce spontaneous arrhythmic Ca²⁺ transients although RyRs outnumber InsP₃Rs by 50:1 [19,20,42]. Differences in InsP₃,R to RyR signaling are also reflected in our data where ET-1 but not caffeine has a positive chronotropic effect (Fig. 4F) in ESdCs. The efficient translation of InsP₃,R-mediated Ca²⁺ release into a depolarization of Vₘ, could depend on the localization of InsP₃,R close to the plasma membrane or within a functional signaling domain. A close apposition was demonstrated in rat atrial myocytes [43], and proposed in rat ventricular myocytes [19]. Data from Harzheim et al. (2009) [20] show that in hypertrophic rat ventricular myocytes InsP₃Rs predominantly increase in the cytoplasm and correlate with enhanced ET-1 induced arrhythmic activity. Our data demonstrate that over-expression of the InsP₃ 5-phosphatase m43 [25,29] in the plasma-membrane [44] decreased ESdCs beating frequency and abolished ET-1 induced positive chronotropy (Fig. 8C). This is consistent with previous results from neonatal cardiomyocytes where m43 reduced the InsP₃ response after z-adrenergic stimulation [25] and supports that the InsP₃ production and the InsP₃,R-mediated Ca²⁺ release relevant to spontaneous activity occurs at the plasma membrane.

In addition to a preferred sub-sarcolemmal location of InsP₃,Rs, the formation of a specialized signaling domain could explain the efficient translation of Ca²⁺ release into changes of Vₘ. Signaling domains between InsP₃,Rs and the effector proteins NCX or the Ca²⁺ activated chloride channel have been demonstrated. The adaptor protein ankyrin [45] that binds to NCX and InsP₃,R [46] could form a potential linker that maintains a close spatial and functional proximity between the proteins. Recent data show that decreased levels of ankyrin attenuate sinus node activity [47,48]; so future experiments will have to reveal how ankyrin loss changes the functional coupling between InsP₃,R mediated Ca²⁺ release and Iₐ(CX).

Conclusion

In the current study we demonstrate that spontaneous activity in ESdCs depends on sub-sarcolemmal signaling domains of InsP₃,R and NCX that allow an efficient translation of InsP₃,R-mediated Ca²⁺ release into a depolarization of the plasma membrane. While the InsP₃ signaling domain described around the nucleus of adult and neonatal ventricular myocytes might enable excitation-transcription coupling, sub-sarcolemmal InsP₃ signaling has significant impact on cellular excitability and arrhythmogenicity. The data indicate that pathological changes in cardiac muscle cells might not only depend on the level of InsP₃,R expression but more critically on their location within the myocytes.

Supporting Information

Data S1 Supporting information.

Figure S1 A. Fluorescent images of an ESdC taken at >560 nm (top) and 488 nm (bottom) before (left) and after bleaching (right).

B. Bar graphs display the change in CFP (right) and decrease of
YFP (right) fluorescence after photobleaching (hatched bar, n = 3). The results are comparable to bleaching experiments in FIRE-1 expressing COS-1 cells [12].

Figure S2 Western blot of the cytoplasmic (soluble) and membrane fraction (pellet) of M43 transfected COS cells. Blots probed with the anti-tag antibody show positive M43 immunostaining only in the membrane fraction.

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Author Contributions

Conceived and designed the experiments: NK, JTM, DW, KB. Analyzed the data: NK, JTM, DW, GAM, LAB, KB. Performed the experiments: NK JTM DW KB. Wrote the paper: KB NK, JTM.

Spatial Differences in InsP3 Signaling