RAPID REPORT

'Eventless' $InsP_3$-dependent SR-Ca^{2+} release affecting atrial Ca^{2+} sparks

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Key points

- Inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$) are functionally expressed in cardiac myocytes.
- The influence of inositol 1,4,5-trisphosphate-induced sarcoplasmic reticulum (SR)-Ca^{2+} release (IP3ICR) on atrial excitation-contraction coupling (ECC) under physiological and pathophysiological conditions remains elusive.
- The present study focuses on local IP3ICR and its functional consequences for ryanodine receptor (RyR) activity and subsequent Ca^{2+}-induced Ca^{2+} release in atrial myocytes.
- Here we show significant SR-Ca^{2+} flux, but eventless SR-Ca^{2+} release through $InsP_3Rs$.
- We suggest a new mechanism based on eventless and highly efficient $InsP_3$-dependent SR-Ca^{2+} flux as a crucial mechanism of functional cross-talk between $InsP_3Rs$ and RyRs, which may be an important factor in the modulation of ECC sensitivity.

Abstract Augmented inositol 1,4,5-trisphosphate receptor ($InsP_3R$) function has been linked to a variety of cardiac pathologies, including cardiac arrhythmia. The contribution of inositol 1,4,5-trisphosphate-induced Ca^{2+} release (IP3ICR) in excitation-contraction coupling (ECC) under physiological conditions, as well as under cellular remodelling, remains controversial. Here we test the hypothesis that local IP3ICR directly affects ryanodine receptor (RyR) function and subsequent Ca^{2+}-induced Ca^{2+} release in atrial myocytes. IP3ICR was evoked by UV-flash photolysis of caged $InsP_3$ under whole-cell configuration of the voltage-clamp technique in atrial myocytes isolated from C57/BL6 mice. Photolytic release of $InsP_3$ was accompanied by a significant increase in the Ca^{2+} release event frequency (4.14 ± 0.72 *vs.* 6.20 ± 0.76 events $(100\ \mu m)^{-1}\ s^{-1}$). These individual photolytically triggered Ca^{2+} release events were identified as Ca^{2+} sparks, which originated from RyR openings. This was verified by Ca^{2+} spark analysis and pharmacological separation between RyR and $InsP_3R$-dependent sarcoplasmic reticulum (SR)-Ca^{2+} release (2-aminoethoxydiphenyl borate, xestospongin C, tetracaine). Significant SR-Ca^{2+} flux but eventless SR-Ca^{2+} release through $InsP_3R$ were characterized using SR-Ca^{2+} leak/SR-Ca^{2+} load measurements. These results strongly support the idea that IP3ICR can effectively modulate RyR openings and Ca^{2+} spark probability. We conclude that eventless and highly efficient $InsP_3$-dependent SR-Ca^{2+} flux is the main mechanism of functional cross-talk between $InsP_3Rs$ and RyRs, which may be an important factor in the modulation of ECC sensitivity.

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Abbreviations 2APB, 2-aminoethoxydiphenyl borate; CICR, Ca^{2+}-induced Ca^{2+} release; CPA, cyclopiazonic acid; ECC, excitation-contraction coupling; ET-1, endothelin-1; FDHM, full-duration at half-maximum amplitude; fullwidth, full width; $InsP_3R$, inositol 1,4,5-trisphosphate receptor; IP3ICR, inositol 1,4,5-trisphosphate-induced Ca^{2+} release; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; TET, tetracaine; Xesto, xestospongin C.

Introduction

In cardiac myocytes two mechanisms for sarcoplasmic reticulum (SR)-Ca^{2+} release have been established, Ca^{2+}-induced Ca^{2+} release (CICR) as the central mechanism for excitation-contraction coupling (ECC) and inositol 1,4,5-trisphosphate-induced Ca^{2+} release (IP3ICR), Ca^{2+} release via inositol 1,4,5-trisphosphate receptor ($InsP_3R$) activation. However, although $InsP_3Rs$ are functionally expressed in cardiac preparations (Mackenzie *et al.* 2004; Zima & Blatter, 2004; Domeier *et al.* 2008; Harzheim *et al.* 2009), their role under physiological as well as under pathophysiological conditions in ECC and CICR is still a matter of an ongoing debate.

It has been suggested that SR-Ca^{2+} release via $InsP_3R$ may effectively modulate ECC in ventricular cardiomyocytes, even though the expression of $InsP_3Rs$ is only a fraction of ryanodine receptor (RyR) expression levels (Lipp *et al.* 2000; Kockskämper *et al.* 2008). These aspects gain even more importance in atrial myocytes, where $InsP_3Rs$ are expressed 3–10 times more compared with ventricle myocytes (Lipp *et al.* 2000; Domeier *et al.* 2008). In addition, this hypothesis is supported by two key observations. First, under pathophysiological conditions, during cellular remodelling, $InsP_3R$ expression is upregulated and may cause delayed after depolarizations, which lead to arrhythmogenicity (Zima & Blatter, 2004; Bootman *et al.* 2007). Second, $InsP_3R$-deficient hearts are largely protected from pro-arrhythmogenic stress (Li *et al.* 2005). Supporting data that encourage a significant contribution of IP3ICR to ECC and CICR are colocalization studies of RyRs and $InsP_3Rs$ (Lipp *et al.* 2000; Mackenzie *et al.* 2002), the microarchitecture of atrial myocytes (Yamasaki *et al.* 1997), and functional studies that have been carried out (Mackenzie *et al.* 2002; Zima & Blatter, 2004; Li *et al.* 2005).

In atrial myocytes normal Ca^{2+} transient propagation by concentric Ca^{2+} waves, which are not pro-arrhythmogenic, may be facilitated by IP3ICR (Mackenzie *et al.* 2004). Local Ca^{2+} signalling between $InsP_3Rs$/RyRs and vice versa might mediate a functional Ca^{2+} cross-talk between the two types of SR-Ca^{2+} release channels. This was conceptualized for smooth muscle cells, where local RyR Ca^{2+} release events, 'Ca^{2+} sparks', were activated via CICR subsequent to $InsP_3R$ activation (Gordienko & Bolton, 2002).

In atrial cells, RyR is the major Ca^{2+} release channel, organized in clusters and functionally coupled (Niggli & Shirokova, 2007). On a local scale, RyR openings give rise to 'Ca^{2+} sparks', the building blocks of global Ca^{2+} transients in cardiac cells (Cheng *et al.* 1993), while coordinated openings of clustered $InsP_3Rs$ will form 'Ca^{2+} puffs' with distinct properties (Yao *et al.* 1995). Along with microscopic Ca^{2+} release events (Ca^{2+} sparks and Ca^{2+} puffs), eventless Ca^{2+} release has been discovered ('Ca^{2+} quarks', 'Ca^{2+} blips'; Lipp *et al.* 1996; Parker *et al.* 1996; Brochet *et al.* 2011). However, these largely invisible Ca^{2+} release events and concomitant Ca^{2+} fluxes may have a significant impact on Ca^{2+} signalling in subcellular microdomains, and may be involved in the functional cross-talk between RyRs and $InsP_3Rs$, which is still enigmatic.

The present study focuses on local IP3ICR and its functional consequences for RyR activity in atrial myocytes. We examined local IP3ICR triggered by photoactivation of caged $InsP_3$ in isolated atrial myocytes from mice under whole-cell voltage-clamp conditions. This highly specific approach bypasses sarcolemmal membrane receptor activation and subsequent signalling cascades. Surprisingly, we found that although no individual IP3ICR events could be visualized, $InsP_3$ release significantly affected the Ca^{2+} spark probability and fosters that RyR Ca^{2+} release can be modulated by Ca^{2+} release via $InsP_3Rs$ in intracellular microdomains. This suggested a new mechanism based on highly efficient but 'eventless' $InsP_3$-dependent SR-Ca^{2+} release. We conclude that invisible $InsP_3$-dependent SR-Ca^{2+} events are the main mechanism of functional cross-talk between $InsP_3Rs$ and RyRs.

Methods

Atrial myocytes from adult male C57/BL6 mice, obtained from the Central Animal Facility, University Hospital, University of Bern, were freshly isolated by the Langendorff perfusion technique. Hearts were removed after animals were killed by cervical dislocation. The number of animals (N) and myocytes (n) are given in the figure legends. All experiments were performed at room temperature and approved by the State Veterinary Office of Bern, Switzerland, according to Swiss Federal Animal Protection Law. Whole-cell voltage-clamp was combined with confocal Ca^{2+} imaging and UV-flash photolysis. The pipette solution contained (in mmol l^{-1}): CsAsp, 120; Hepes, 10; tetraethylammonium chloride, 20; potassium-ATP, 5; $MgCl_2$, 1; K_5-fluo-3 (Biotium), 0.1; GSH, 2; caged $InsP_3$–6Na, (0.03, 0.06, 0.24; Sichem); pH 7.2 with CsOH. Cells were perfused with external solution containing (in mmol l^{-1}): NaCl, 140; Hepes, 5; $MgCl_2$, 1.1; KCl, 5.4; glucose, 10; $CaCl_2$, 1.8; $BaCl_2$, 0.5; CsCl, 1; pH 7.4 NaOH. Pharmacological experiments

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used 5 μM 2-aminoethoxydiphenyl borate (2APB; Fluka), 5 μM xestospongin C (Xesto; A.G Scientific Inc.), 1 mM tetracaine (TET; Sigma) or 10 mM caffeine (Sigma) added to the external solution. For Ca^{2+} leak/load experiments (Shannon *et al.* 2002), atrial myocytes were loaded with fluo-3 AM (Biotium).

Data are reported as means ± SEM. Where the data were normally distributed, paired or unpaired, where appropriate, Student's *t* test was applied. For not normally distributed data the Wilcoxon Matched-Pairs Signed-Ranks Test was applied. A detailed Materials and Methods section is available in the Online Data Supplement.

Results

Endothelin-1 (ET-1)-induced $InsP_3R$ Ca^{2+} release in atrial myocytes

Figure 1 shows that rapid administration of 100 nM ET-1, an $InsP_3$ pathway activator, caused a significant increase in spontaneous local as well as global Ca^{2+} release events in resting mouse atrial cardiomyocytes. This increase was 2APB ($InsP_3R$ blocker) sensitive (Fig. 1*B*) and was also absent in control (Fig. 1*C*). Observations agreed with previously performed experiments from other groups (Mackenzie *et al.* 2004; Zima & Blatter, 2004; Li *et al.* 2005), that ET-1-mediated IP3ICR could be obtained in various mammalian species including mouse atrial myocytes. ET-1-triggered IP3ICR is based on a complex signalling cascade that involves G-protein-coupled phospholipase C activation followed by diacylglycerol and $InsP_3$ production. To bypass this complex signal transduction pathway, $InsP_3Rs$ were activated by rapid and transient intracellular $InsP_3$ elevation induced by photorelease of $InsP_3$ from caged $InsP_3$.

$InsP_3$-induced Ca^{2+} release triggered by UV-flash photolysis of caged $InsP_3$

Atrial myocytes were voltage-clamped in the whole-cell configuration of the patch-clamp technique and held at –80 mV. Caged $InsP_3$ in combination with the Ca^{2+} indicator fluo-3 were dialysed into the cell through the patch pipette. A SR-Ca^{2+} loading protocol based on L-type Ca^{2+} current activation elicited by depolarization steps from –80 mV to 0 mV (up to 10 times) was applied to ensure comparable SR-Ca^{2+} content, before line scan images were recorded (Fig. 2*A*). Under these conditions spontaneous Ca^{2+} event activity of about 3.7 ± 0.92 events $(100\ \mu m)^{-1}\ s^{-1}$ was observed.

In order to evoke IP3ICR in a highly specific manner, global UV-flashes were applied to photorelease $InsP_3$ in the entire cytosol, which was followed by an increase of local Ca^{2+} event frequency triggered in a dose-dependent manner (Fig. 2*C*). The number of Ca^{2+} release events observed after photorelease of $InsP_3$ was normalized to the number of spontaneous Ca^{2+} releases counted before $InsP_3$ uncaging. Caged $InsP_3$ concentrations of 30 μM and 60 μM caused a slight but not significant increase in the frequency of Ca^{2+} release events (30 μM: 2.73 ± 0.51 to 3.06 ± 0.71 events $(100\ \mu m)^{-1}\ s^{-1}$; 60 μM: 4.03 ± 0.73 to 4.20 ± 0.58 events $(100\ \mu m)^{-1}\ s^{-1}$). However, photolysis of 240 μM caged $InsP_3$ triggered a significant ($P = 0.02$) increase in frequency of Ca^{2+} release events from 4.14 ± 0.72 to 6.20 ± 0.76 events $(100\ \mu m)^{-1}\ s^{-1}$ (Fig. 2*B* and *C*). It is to be mentioned that the effective $InsP_3$ concentration after UV-flash application is orders of magnitude lower but in proportion to the loading concentration of the caged compound in the pipette solution (see online data supplement). Figure 2*C* shows control experiments lacking the caged compound, indicating that UV-flash application alone had no effect on the frequency of events. We expected that at least part of the total number of photolytically triggered Ca^{2+} events was based on IP3ICR (e.g. Ca^{2+} 'puffs'). Although Ca^{2+} sparks show similarities with Ca^{2+} puffs, it has been shown that Ca^{2+} puffs exhibit significantly distinct spatio-temporal properties that are different from Ca^{2+} sparks (Cheng *et al.* 1993; Yao *et al.* 1995; Tovey *et al.* 2001; Niggli & Shirokova, 2007). At maximal event activity (240 μM caged $InsP_3$), local Ca^{2+} release events were analysed in more detail for amplitude, full-duration at half-maximum amplitude (FDHM), full-width at half-maximum amplitude (FWHM), full duration, full width (fullwidth) and rise time (Picht *et al.* 2007). Cells were divided into two groups: one group showed little (<1.3 events $(100\ \mu m)^{-1}\ s^{-1}$) increase (frequency: 0.98 ± 0.08 events $(100\ \mu m)^{-1}\ s^{-1}$); a second group showed >1.3 events $(100\ \mu m)^{-1}\ s^{-1}$ increase in Ca^{2+} release events (frequency: 3.24 ± 0.55 events $(100\ \mu m)^{-1}\ s^{-1}$) after photolytic $InsP_3R$ activation. Ca^{2+} release events obtained in both groups showed no significant difference in their temporal characteristics. The fullwidth was significantly larger in the group of cells that showed little increase in the frequency of Ca^{2+} release events, from 3.80 ± 0.29 to 4.51 ± 0.38 μm (Fig. 2*D*), an indication for contribution of IP3ICR in Ca^{2+} spark formation. Nevertheless, Ca^{2+} event analysis did not show the anticipated two classes of local Ca^{2+} release events (i.e. Ca^{2+} sparks and Ca^{2+} puffs). The obtained parameters of Ca^{2+} events were comparable to those reported for Ca^{2+} sparks (Niggli & Shirokova, 2007) and are summarized in Supplementary Table 1 in the online material.

However, control measurements revealed photolytically triggered local $InsP_3$ Ca^{2+} events (Ca^{2+} puffs) in neonatal rat cardiomyocytes. In other words, experimental conditions can be assumed to be appropriate for detecting microscopic subcellular Ca^{2+} events including Ca^{2+} puffs (see Supplementary Fig. 2S).

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While photorelease of $InsP_3$ lead to an increased Ca^{2+} spark frequency (Fig. 2), the precise mechanism by which this occurred still remained unclear. Therefore, pharmacological interventions were used to distinguish between Ca^{2+} sparks and Ca^{2+} puffs by selective inhibition of either RyRs or $InsP_3$Rs. To inhibit IP3ICR, cells were incubated for at least 20 min in 5 μM Xesto, or 5 μM 2APB was acutely applied. TET (1 mM) was applied to inhibit RyR Ca^{2+} release (Fig. 3*A*). Photolytic increase of Ca^{2+} release events was inhibited by 2APB and Xesto, without affecting the spontaneous Ca^{2+} release event activity.

Surprisingly, in the presence of TET a complete inhibition of all Ca^{2+} release events (Fig. 3*B* and *C*) was observed. This observation is in full agreement with the Ca^{2+} event analysis given in Fig. 2, showing that all provoked SR-Ca^{2+} release events can be classified as Ca^{2+} sparks. Taken together, photolytically activated IP3ICR efficiently facilitates RyR Ca^{2+} release.

SR-Ca^{2+} load and invisible $InsP_3$R Ca^{2+} release (SR-Ca^{2+} leak)

Ca^{2+} spark probability strongly depends on luminal Ca^{2+} ($[Ca^{2+}]_{SR}$). RyRs and $InsP_3$Rs may share, at least in part, the same luminal Ca^{2+} pool, suggesting that $[Ca^{2+}]_{SR}$ may have a potential role in the functional cross-talk of both Ca^{2+} release channels, which was examined using caffeine-induced Ca^{2+} transients. $InsP_3$R block (Fig. 3*D*) did not significantly affect the SR-Ca^{2+} content (2APB: 1 ± 0.18 to 1.09 ± 0.08 a.u., $P = 0.8$; and Xesto: from 1.13 ± 0.07 to 0.78 ± 0.09 a.u., $P = 0.07$), whereas TET caused a significant increase in the SR-Ca^{2+} content from 1 ± 0.07 to 1.4 ± 0.1 a.u., $P = 0.02$, presumably by suppressing Ca^{2+} leak (Shannon *et al.* 2002). Besides the SR-Ca^{2+} content, SR-Ca^{2+} leak is an important determinant for Ca^{2+} spark frequency. Indirect evidence that $InsP_3$R stimulation may play a role in SR-Ca^{2+} leak came from the observation that in the presence of TET subsequent $InsP_3$ release caused a slow but significant increase in background fluorescence, which was not seen in the presence of 2APB (Fig. 3*E*). This result suggests that 'eventless' Ca^{2+} flux through the $InsP_3$R, which cannot be resolved as local Ca^{2+} release events, may be more pronounced in atrial cardiomyocytes than previously expected.

In Fig. 4 we addressed the question whether $InsP_3$Rs may contribute to the SR-Ca^{2+} leak and thus affect the Ca^{2+} spark appearance by performing a modified SR-Ca^{2+} leak protocol introduced by Shannon *et al.* (2002). Atrial myocytes were field stimulated for 45 s at 1 Hz, followed by a rapid solution switch from 1.8 mM extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) to a nominally $[Ca^{2+}]_o$ free and $[Na^+]_o$ free solution. After 10 s, 10 μM cyclopiazonic acid (CPA; a potent SERCA blocker) was added and finally, 30 s later, the SR-Ca^{2+} content was measured with rapid caffeine application (Fig. 4*A*). Atrial myocytes were divided into two groups. The control group was recorded under conditions given above, the second group was pre-incubated with 100 nM ET-1 for at least 120 s before CPA was applied (Fig. 4*A*). CPA causes an inhibition of the SERCA function. Hence, in combination with the blocked Na^+/Ca^{2+} exchanger function, any Ca^{2+} leaking from the SR is trapped in the cytoplasm and mirrored by an increase in the cytosolic Ca^{2+} concentration $[Ca^{2+}]_i$. This increase was measured and defined as SR-Ca^{2+} leak.

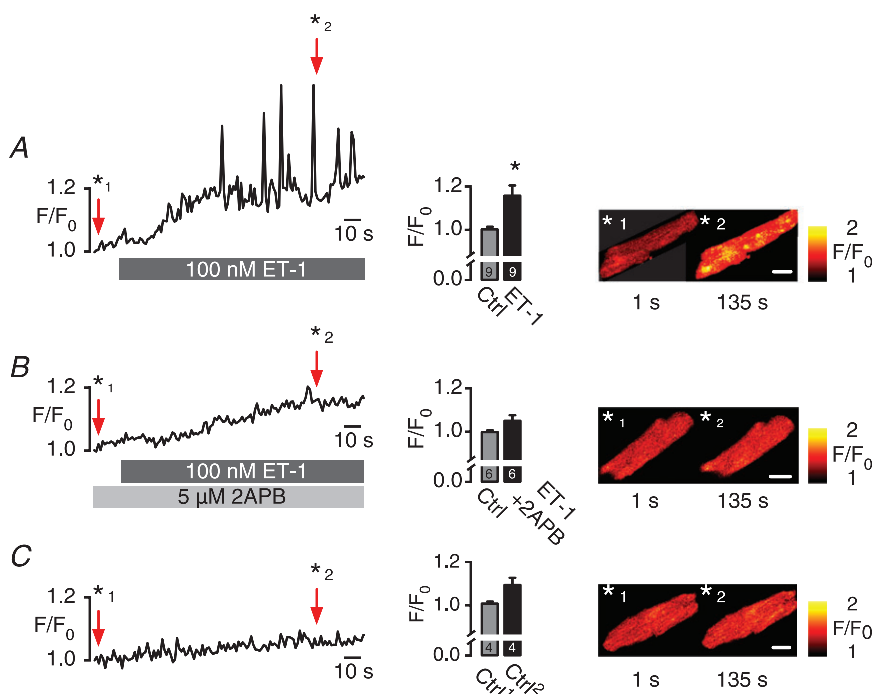

Figure 1. Endothelin-1 (ET-1)-induced $InsP_3$R Ca^{2+} release in atrial myocytes
Time series of $InsP_3$-induced Ca^{2+} release events expressed as F/F_0 of fluo-3 fluorescence obtained in atrial myocytes. Cells were loaded with 5 μM fluo-3 AM (Biotium) for 20 min and left for de-esterification for 15 min before time series were collected in resting cells. *A*, the $InsP_3$ pathway was stimulated with 100 nM ET-1 causing a substantial increase in SR-Ca^{2+} release event activity. *B*, inhibition of ET-1-triggered Ca^{2+} events with 5 μM 2-aminoethoxydiphenyl borate (2APB). *C*, spontaneous Ca^{2+} event activity under control conditions. SR-Ca^{2+} load was measured at the end of each experiment and found to be not significantly different in the various conditions (Ctrl: 3.45 ± 0.28 F/F_0; ET-1: 3.89 ± 0.48 F/F_0; 2APB + ET-1: 3.84 ± 0.53 F/F_0). Bar graphs represent averaged F/F_0 of the first 20 s (Ctrl) and 160–200 s; scale bars represent 10 μm, $N = 3$, $n = 4$–9.

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When CPA was added in the presence of ET-1 there was a significant increase in SR-Ca^{2+} leak $\Delta[Ca^{2+}]_i$) compared with the leak in control (0.16 ± 0.03 to 0.38 ± 0.09 $\Delta F/F_0$, $P = 0.02$). The SR-Ca^{2+} leak/SR-Ca^{2+} load relationship increased accordingly, although not significantly ($\Delta[Ca^{2+}]_i$/SR-Ca^{2+} load: 0.047 ± 0.007 to 0.086 ± 0.020, $P = 0.09$). This may be due to the additional Ca^{2+} influx induced by ET-1 (He *et al.* 2000), causing an increased SR-Ca^{2+} content (3.30 ± 0.24 to 4.53 ± 0.26 F/F_0, $P = 0.001$). However, reduced SERCA activity unmasked a substantial contribution of InsP$_3$R activity to the total SR-Ca^{2+} leak (Fig. 4), which was not seen before. Taken together, eventless InsP$_3$R-Ca^{2+} release flux is part of SR-Ca^{2+} leak and facilitates SR-Ca^{2+} spark probability.

Discussion

The physiological role of atrial myocytes is to ensure ventricular blood refilling especially under conditions of physical stress and activity. This involves stimulation by direct autonomic nervous system innervation, neuro-humoral effects and interactions with hormones released by the vasculature that boost ECC on a cellular level. Similar to ventricular myocytes, the fundamental mechanism for ECC in atrial myocytes is driven by CICR. However, the contribution of IP3ICR in initiation, propagation and amplification of local and global SR-Ca^{2+} release and thus CICR in atrial myocytes remains controversial (Blatter *et al.* 2003; Mackenzie *et al.* 2004).

Our results obtained in atrial myocytes showed significant alterations in Ca^{2+} sparks (width and frequency) during InsP$_3$R activation. This emphasizes that local Ca^{2+} release from InsP$_3$Rs may play a significant role in the modulation of Ca^{2+}-dependent RyR activity in atrial myocytes. Our approach, based on the combination of different biophysical methods, revealed some discrepancies between InsP$_3$R activation and the appearance of local SR-Ca^{2+} release events, such as apparent invisible, 'eventless' Ca^{2+} release although InsP$_3$R activation occurred. From these discrepancies we derived a novel conclusion regarding functional cross-talk

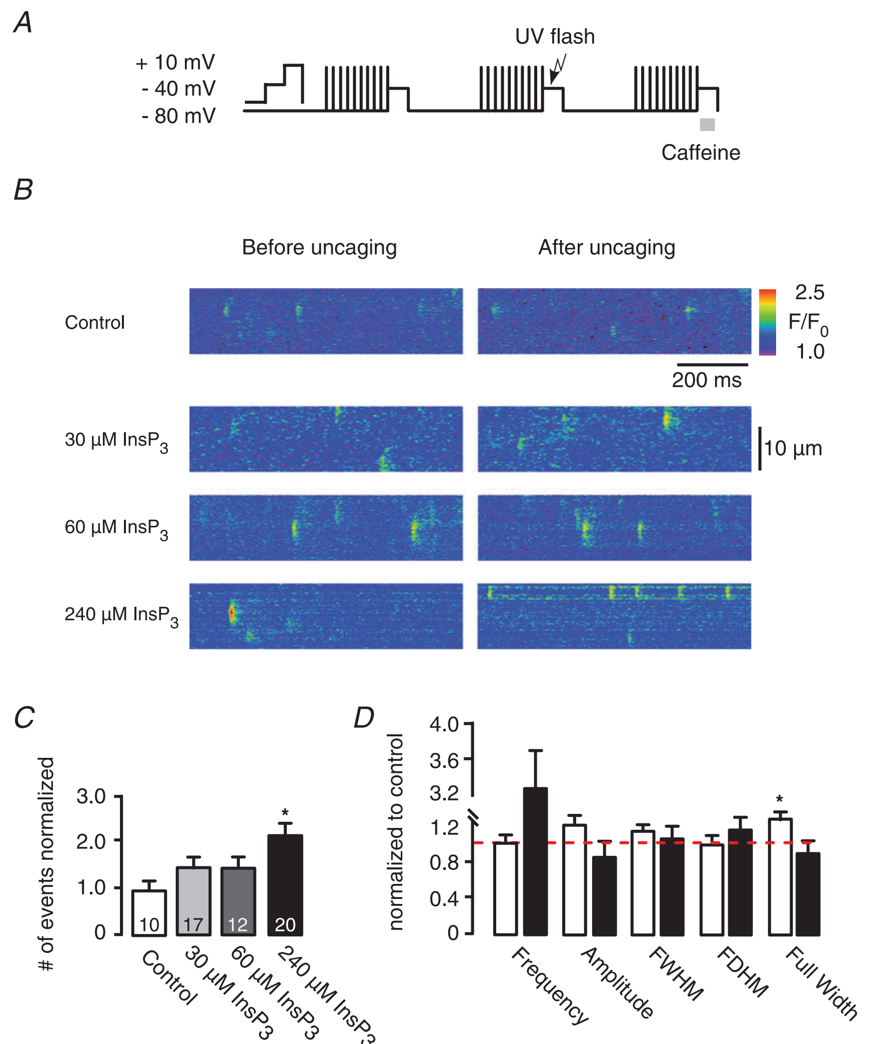

Figure 2. Inositol 1,4,5-trisphosphate (InsP$_3$)-induced Ca^{2+} release by UV-flash photolysis of caged InsP$_3$ in atrial myocytes
A, SR-Ca^{2+} loading protocol performed by $I_{Ca,L}$ (10 times). *B*, representative line scan images of voltage-clamped atrial myocytes in whole-cell configuration before and after UV-flash photolysis in control and with 30, 60, 240 μM caged InsP$_3$. *C*, the frequency of Ca^{2+} release events counted after UV-flash photolysis normalized to the spontaneous Ca^{2+} release events. *D*, averaged spatio-temporal Ca^{2+} spark properties after uncaging of InsP$_3$ (240 μM) normalized to control. Cells were divided into two groups, white bars represent cells that showed little (<1.3) increase in Ca^{2+} release frequency (0.98 ± 0.08 events (100 μm)$^{-1}$ s^{-1}), black bars represent cells that showed >1.3 increase in Ca^{2+} release frequency (3.24 ± 0.55 events (100 μm)$^{-1}$ s^{-1}). Amplitude ($\Delta F/F_0$), full-width at half-maximum amplitude (FWHM; μm), full-duration at half-maximum amplitude (FDHM; ms) showed no significant difference after flash photolysis in both groups (see Supplementary Table 1). Full width (μm) showed a significant increase (from 3.80 ± 0.3 μm to 4.50 ± 0.4 μm) in cells with low Ca^{2+} event frequency in response to photolysis. $^*P < 0.05$ *vs.* control, $N = 6$–7, $n = 6$–17, mean $\pm$ SEM.

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between RyRs and $InsP_3Rs$ in atrial myocytes based on highly efficient but 'eventless' IP3ICR.

Identification of Ca^{2+} sparks

We analysed individual Ca^{2+} release events triggered by photorelease of $InsP_3$. RyRs Ca^{2+} sparks are characterized by amplitudes of up to 2 $\Delta F/F_0$, widths $\approx 2\ \mu m$, FDHM ≈ 30 ms, whereas $InsP_3$ Ca^{2+} puffs are characterized by smaller amplitudes, increased widths $\approx 6\ \mu m$ and a prolonged FDHM of about 100–600 ms (Zima & Blatter, 2004; Niggli & Shirokova, 2007; Cheng & Lederer, 2008). Our spark analysis revealed Ca^{2+} sparks but did not show a second distinct class of Ca^{2+} release events that could be *ad hoc* assigned to Ca^{2+} puffs, respectively. In addition, selective pharmacological inhibition of either $InsP_3Rs$ or RyRs let us conclude that the detected $InsP_3$-triggered Ca^{2+} release events can be classified as Ca^{2+} sparks. However,

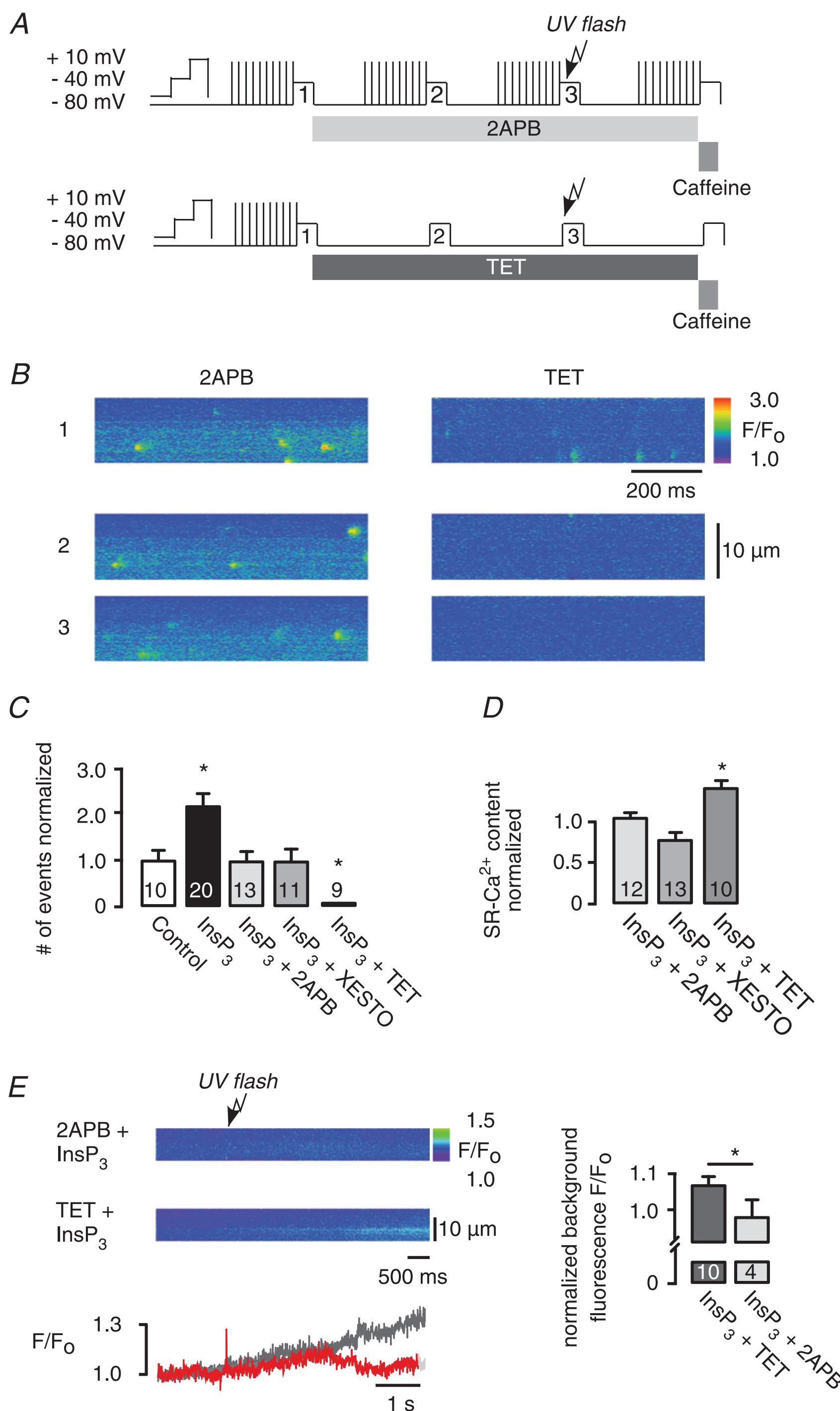

Figure 3. Identification of inositol 1,4,5-trisphosphate ($InsP_3$)-evoked Ca^{2+} sparks
A, experimental protocol, sarcoplasmic reticulum (SR)-Ca^{2+} loading protocol performed by $I_{Ca,L}$ (10 times); atrial myocytes were voltage-clamped under whole-cell conditions, the pipette solution contained 240 μM caged $InsP_3$; line scans are numbered 1–3: (1) control; (2) pharmacological interventions; and (3) pharmacological interventions combined with photolytically released $InsP_3$. Modified protocols were used for the application of 2-aminoethoxydiphenyl borate (2APB) and tetracaine (TET). *B*, representative data showing line scan images of fluo-3 fluorescence under whole-cell conditions taken at selected time points: (1) control; (2) in the presence of 2APB or TET; (3) pharmacology and photolytic release of $InsP_3$. *C*, frequency of Ca^{2+} release events evoked by uncaging of caged $InsP_3$ normalized to control (spontaneous frequency in rest). The increase in Ca^{2+} spark frequency was inhibited by the application of either 2APB or xestospongin (Xesto; $InsP_3R$ inhibitors) and by TET (RyR inhibitor). *D*, SR-Ca^{2+} content measured by rapid caffeine (10 mM) application. *E*, change in background fluo-3 fluorescence F/F_0 after $InsP_3$ uncaging, in the presence of TET (dark grey) or 2APB (red). Fluorescence F/F_0 was normalized to background fluorescence before $InsP_3$ release (right). Background fluorescence increase after UV-flash photolysis of caged $InsP_3$ in the presence of TET was blocked by 2APB. $^*P < 0.05$ *vs.* control, $N = 2–5$; $n = 6–13$, mean ± SEM.

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the absence of photolytically triggered IP3ICR events was surprising.

Functional cross-talk of InsP$_3$R and RyR2 via invisible SR-Ca^{2+} release

Despite the evidence that IP3ICR occurs in cardiac cells (Zima & Blatter, 2004), reports of elementary Ca^{2+} signals arising from InsP$_3$R openings have been rare, which may be due to the Ca^{2+} spark dominance. In atrial myocytes, InsP$_3$Rs are colocalized with RyRs, preferentially in the subsarcolemmal space and around the nucleus (Lipp *et al.* 2000; Mackenzie *et al.* 2002). The expression of InsP$_3$Rs is known to be regulated in a highly dynamic manner, notably during development and cellular remodelling in pathophysiological situations (Janowski *et al.* 2006).

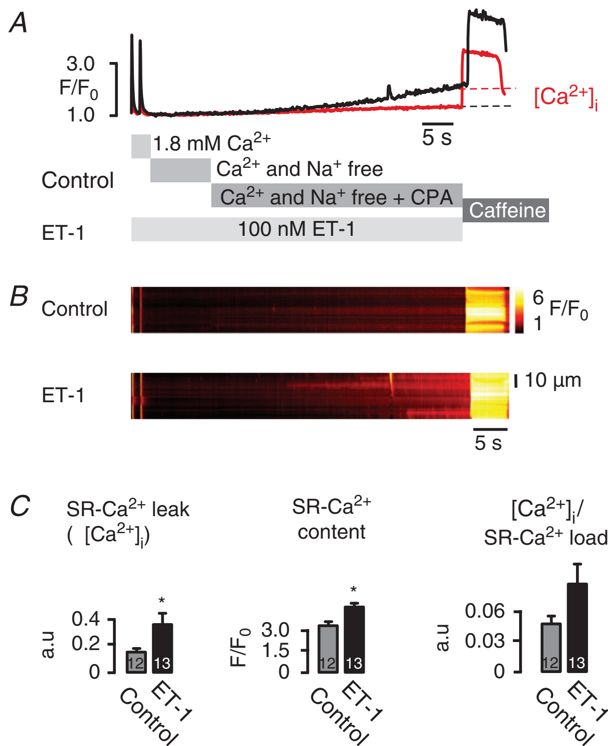

Figure 4. Invisible InsP$_3$-dependent sarcoplasmic reticulum (SR)-Ca^{2+} release unmasked by SR-Ca^{2+} leak-load relationship
A, representative data showing averaged F/F_0 of fluo-3 fluorescence changes (control: red; endothelin-1 (ET-1): black) and *B*, corresponding line scan images of fluo-3 fluorescence recorded in atrial myocytes. Cells were field stimulated for at least 45 s at 1 Hz before line scan images were recorded. After pre-pulse protocol, superfusion was rapidly switched from 1.8 mM $[Ca^{2+}]_o$ to a nominally $[Ca^{2+}]_o$- and $[Na^+]_o$-free extracellular solution for 15 s, then 10 μM cyclopiazonic acid (CPA) was added for 20 s, before 10 mM caffeine was applied. The protocol was repeated in the presence of 100 nM ET-1 after pre-incubation with 100 nM ET-1 for at least 120 s. The increase in background fluorescence when CPA was applied represents SR-Ca^{2+} leak ($\Delta[Ca^{2+}]_i$). *C*, averaged data, SR-Ca^{2+} leak ($\Delta[Ca^{2+}]_i$), SR-Ca^{2+} content and SR-Ca^{2+} leak/SR-Ca^{2+} load relationship ($\Delta[Ca^{2+}]_i$/SR-Ca^{2+} load) compared with control (no InsP$_3$ pathway stimulation). In the presence of ET-1 there was a significant increase in the SR-Ca^{2+} leak ($\Delta[Ca^{2+}]_i$) when CPA was added, unmasking a substantial contribution of the InsP$_3$R opening to the total SR-Ca^{2+} leak. $^*P < 0.02$–009 *vs.* control, $N = 3$; $n = 12$–13, mean $\pm$ SEM.

RyRs are arranged in functional arrays (10–300 RyRs) and subject to stochastic cluster assembly processes (Baddeley *et al.* 2009). The synchronized openings of clustered RyRs result in local Ca^{2+} sparks. Under resting conditions, in diastole, spontaneous Ca^{2+} sparks reflect the finite open probability of RyRs, which is controlled by $[Ca^{2+}]_i$, luminal Ca^{2+} ($[Ca^{2+}]_{SR}$), phosphorylation state and numerous other factors (Eisner *et al.* 1998). A comparable configuration of spatially segregated clusters of 10–100 InsP$_3$Rs is believed to be responsible for the generation of 'Ca^{2+} puffs', activated by InsP$_3$ and Ca^{2+} (Foskett *et al.* 2007), but direct ultrastructural evidence is yet missing (Shuai *et al.* 2007). In addition, there are species differences in human/rat/rabbit *versus* mouse in the InsP$_3$R cluster dynamics and microarchitecture that shape the InsP$_3$ Ca^{2+} event characteristics (Diambra & Marchant, 2011). It has been suggested that InsP$_3$Rs are mobile in the SR membrane and can form clusters upon stimulation (Pantazaka & Taylor, 2011). There is also evidence that InsP$_3$R Ca^{2+} release sites represent pre-established, stable clusters of InsP$_3$Rs (Smith *et al.* 2009). Under this view, brief exposure to InsP$_3$ should trigger Ca^{2+} puffs, which was not the case in our study. We suggest that InsP$_3$Rs in intact mouse atrial myocytes may not form sufficient cluster size to facilitate Ca^{2+} puffs due to a low level of InsP$_3$R expression and/or intercluster distance. InsP$_3$Rs may rather be distributed near RyRs to facilitate Ca^{2+} sparks. This view is supported by our immunohistochemistry study, showing that InsP$_3$Rs seem to be rather homogeneously distributed (Supplementary Fig. 3S). According to our data, expression of functional InsP$_3$Rs in atrial myocytes may not necessarily lead to the formation of distinguished Ca^{2+} puffs.

Thus, how can the increase in Ca^{2+} spark frequency after InsP$_3$ photorelease be explained? The prevalent view that Ca^{2+} sparks and Ca^{2+} puffs are elementary events becomes more comprehensive, as for both types of local Ca^{2+} release events, even smaller elementary Ca^{2+} release events have been confirmed. Compared with Ca^{2+} sparks, openings of single RyRs may result in smaller but more frequent events ('Ca^{2+} quarks'; Lipp *et al.* 1996; Brochet *et al.* 2011). At the same time, similar to Ca^{2+} quarks, InsP$_3$R-dependent elementary Ca^{2+} release events arising from single InsP$_3$R openings, termed 'Ca^{2+} blips', have been discovered (Parker *et al.* 1996). Opposed to triggered events, which are experimentally easy to detect, 'blips' and 'quarks' are largely invisible because these Ca^{2+} events are below detection threshold (Sobie *et al.* 2002; Williams *et al.* 2011). This is due to the low signal-to-noise

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ratio given by fluorescent Ca^{2+} measurements. The absence of visible Ca^{2+} puffs in our measurements, together with the observed increase in spark frequency mediated by $InsP_3Rs$, suggests a significant contribution of 'invisible' or 'eventless' IP3ICR to the occurrence of Ca^{2+} sparks (and possible Ca^{2+} wave propagation). We propose that these eventless $InsP_3R$ openings may be responsible for microdomain $[Ca^{2+}]_i$ increase that either sensitize RyRs for CICR or lead to direct RyR activation. Functional cross-talk between $InsP_3R$ and RyR can operate in both directions. Hence, microdomain $[Ca^{2+}]_i$ elevations could sensitize $InsP_3R$ for $InsP_3$, which would favour $InsP_3R$ openings (Foskett *et al.* 2007).

Thus, the contribution of eventless SR-Ca^{2+} release via $InsP_3R$ could be more substantial for the regulation of Ca^{2+} signalling in cellular microdomains than previously thought. This is consistent with a number of not entirely explained experimental reports regarding functional interactions of $InsP_3R$ and RyR. It has been reported that $InsP_3R$ activity increases $[Ca^{2+}]_i$ in the vicinity of RyRs and thus facilitates CICR during ECC in adult cat atrial myocytes (Zima & Blatter, 2004), and that $InsP_3$-dependent Ca^{2+} release has a positive inotropic effect on ECC by facilitating Ca^{2+} release through RyR clusters in rabbit ventricle myocytes (Domeier *et al.* 2008). Furthermore, there is evidence that Ca^{2+} leak through $InsP_3Rs$ is present at sites where RyRs are located and that this Ca^{2+} leak can modulate RyR Ca^{2+} release events (Gordienko & Bolton, 2002).

This 'invisible' SR-Ca^{2+} leak, respectively, SR-Ca^{2+} flux via $InsP_3R$, was further examined in a series of SR-Ca^{2+} leak measurements. Here, $InsP_3R$ function was stimulated with ET-1. Compared with control, ET-1 may stimulate various Ca^{2+} influx pathways (e.g. via TRPC; Treves *et al.* 2004), which may have led to an increased SR-Ca^{2+} load before the nominally Ca^{2+}- and Na^{+}-free solution was added. This would lead *per se* to a transient increase in SR-Ca^{2+} leak until a new steady-state with matched leak/load relationship is reached. Nevertheless, increased $InsP_3R$ open probability will then concomitantly produce an 'excess' SR-Ca^{2+} leak in the Ca^{2+}- and Na^{+}-free solution compared with control as seen in Fig. 4*A*. This is largely independent of the total SR-Ca^{2+} content and results in an increased leak/load relationship.

Taken together, experiments using photorelease of $InsP_3$ and SR-Ca^{2+} leak measurements suggest that 'eventless' $InsP_3$-dependent SR-Ca^{2+} leak is the main mechanism of functional cross-talk between $InsP_3Rs$ and RyRs.

Pathophysiological implications

A change in the SR-Ca^{2+} leak/load relationship can effectively change $[Ca^{2+}]_{SR}$ transiently or even under steady-state conditions. It is well established that $[Ca^{2+}]_{SR}$ is involved in various forms of cellular instability and affected during cellular remodelling in response to pathophysiological stress (Bers, 2003; Shannon *et al.* 2003; Wehrens *et al.* 2005). Under chronic atrial fibrillation, $InsP_3Rs$ are targeted during cellular remodelling and were found to be upregulated, which may have positive inotropic effects on the global level (Zima & Blatter, 2004; Li *et al.* 2005; Bootman *et al.* 2007; Harzheim *et al.* 2009). Recently, subcellular mechanism(s) linking $InsP_3R$ activity to the development of atrial fibrillation and cardiac hypertrophy has been demonstrated (Higazi *et al.* 2009; Nakayama *et al.* 2010). Cardiac-specific blockage of the $InsP_3R$ pathway, therefore, could offer a new therapeutic strategy for treatment of atrial arrhythmogenicity.

References

Baddeley D, Jayasinghe ID, Lam L, Rossberger S, Cannell MB & Soeller C (2009). Optical single-channel resolution imaging of the ryanodine receptor distribution in rat cardiac myocytes. *Proc Natl Acad Sci U S A* **106**, 22275–22280.

Bers DM (2003). Sarcoplasmic reticulum Ca^{2+} and heart failure: roles of diastolic leak and Ca^{2+} transport. *Circ Res* **93**, 487–490.

Blatter LA, Kockskämper J, Sheehan KA, Zima AV, Hüser J & Lipsius SL (2003). Local calcium gradients during excitation-contraction coupling and alternans in atrial myocytes. *J Physiol* **546**, 19–31.

Bootman MD, Harzheim D, Smyrnias I, Conway SJ & Roderick HL (2007). Temporal changes in atrial EC-coupling during prolonged stimulation with endothelin-1. *Cell Calcium* **42**, 489–501.

Brochet DXP, Xie W, Yang D, Cheng H & Lederer WJ (2011). Quarky calcium release in the heart. *Circ Res* **108**, 210–218.

Cheng H & Lederer WJ (2008). Calcium sparks. *Physiol Rev* **88**, 1491–1545.

Cheng H, Lederer WJ & Cannell MB (1993). Calcium sparks: elementary events underlying excitation-contraction coupling in heart muscle. *Science* **262**, 740–744.

Diambra L & Marchant JS (2011). Inositol (1,4,5)-trisphosphate receptor microarchitecture shapes Ca^{2+} puff kinetics. *Biophys J* **100**, 822–831.

Domeier TL, Zima AV, Maxwell JT, Huke S, Mignery GA & Blatter LA (2008). IP_3 receptor-dependent Ca^{2+} release modulates excitation-contraction coupling in rabbit ventricular myocytes. *Am J Physiol Heart Circ Physiol* **294**, H596–H604.

Eisner DA, Trafford AW, Diaz ME, Overend CL & O'Neill SC (1998). The control of Ca^{2+} release from the cardiac sarcoplasmic reticulum: regulation versus autoregulation. *Cardiovasc Res* **38**, 589–604.

Foskett JK, White C, Cheung K-H & Mak D-OD (2007). Inositol trisphosphate receptor Ca^{2+} release channels. *Physiol Rev* **87**, 593–658.

Gordienko D & Bolton T (2002). Crosstalk between ryanodine receptors and IP_3 receptors as a factor shaping spontaneous Ca^{2+}-release events in rabbit portal vein myocytes. *J Physiol* **542**, 743–762.

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Harzheim D, Movassagh M, Foo RS-Y, Ritter O, Tashfeen A, Conway SJ, Bootman MD & Roderick HL (2009). Increased InsP$_3$Rs in the junctional sarcoplasmic reticulum augment Ca^{2+} transients and arrhythmias associated with cardiac hypertrophy. *Proc Natl Acad Sci U S A* **106**, 11406–11411.

He JQ, Pi Y, Walker JW & Kamp TJ (2000). Endothelin-1 and photoreleased diacylglycerol increase L-type Ca^{2+} current by activation of protein kinase C in rat ventricular myocytes. *J Physiol* **3**, 807–820.

Higazi DR, Fearnley CJ, Drawnel FM, Talasila A, Corps EM, Ritter O, McDonald F, Mikoshiba K, Bootman MD & Roderick HL (2009). Endothelin-1-stimulated InsP$_3$-induced Ca^{2+} release is a nexus for hypertrophic signaling in cardiac myocytes. *Mol Cell* **33**, 472–482.

Janowski E, Cleemann L, Sasse P & Morad M (2006). Diversity of Ca^{2+} signaling in developing cardiac cells. *Ann NY Acad Sci* **1080**, 154–164.

Kockskämper J, Zima AV, Roderick HL, Pieske B, Blatter LA & Bootman MD (2008). Emerging roles of inositol 1,4,5-trisphosphate signaling in cardiac myocytes. *J Mol Cell Cardiol* **45**, 128–147.

Li X, Zima AV, Sheikh F, Blatter LA & Chen J (2005). Endothelin-1-induced arrhythmogenic Ca^{2+} signaling is abolished in atrial myocytes of inositol-1,4,5-trisphosphate (IP$_3$)-receptor type 2-deficient mice. *Circ Res* **96**, 1274–1281.

Lipp P, Laine M, Tovey SC, Burrell KM, Berridge MJ, Li W & Bootman MD (2000). Functional InsP$_3$ receptors that may modulate excitation-contraction coupling in the heart. *Curr Biol* **10**, 939–942.

Lipp P, Lüscher C & Niggli E (1996). Photolysis of caged compounds characterized by ratiometric confocal microscopy: a new approach to homogeneously control and measure the calcium concentration in cardiac myocytes. *Cell Calcium* **19**, 255–266.

Mackenzie L, Bootman M, Laine M, Berridge M, Thuring J, Holmes A, Li W & Lipp P (2002). The role of inositol 1,4,5-trisphosphate receptors in Ca^{2+} signalling and the generation of arrhythmias in rat atrial myocytes. *J Physiol* **541**, 395–409.

Mackenzie L, Roderick HL, Berridge MJ, Conway SJ & Bootman MD (2004). The spatial pattern of atrial cardiomyocyte calcium signalling modulates contraction. *J Cell Sci* **117**, 6327–6337.

Nakayama H, Bodi I, Maillet M, DeSantiago J, Domeier T, Mikoshiba K, Lorenz J, Blatter L, Bers D & Molkentin J (2010). The IP$_3$ receptor regulates cardiac hypertrophy in response to select stimuli. *Circ Res* **107**, 659–666.

Niggli E & Shirokova N (2007). A guide to sparkology: the taxonomy of elementary cellular Ca^{2+} signaling events. *Cell Calcium* **42**, 379–387.

Pantazaka E & Taylor CW (2011). Differential distribution, clustering, and lateral diffusion of subtypes of the inositol 1,4,5-trisphosphate receptor. *J Biol Chem* **286**, 23378–23387.

Parker I, Choi J & Yao Y (1996). Elementary events of InsP$_3$-induced Ca^{2+} liberation in *Xenopus* oocytes: hot spots, puffs and blips. *Cell Calcium* **20**, 105–121.

Picht E, Zima AV, Blatter LA & Bers DM (2007). SparkMaster: automated calcium spark analysis with ImageJ. *Am J Physiol Cell Physiol* **293**, C1073–C1081.

Shannon TR, Ginsburg KS & Bers DM (2002). Quantitative assessment of the SR Ca^{2+} leak-load relationship. *Circ Res* **91**, 594–600.

Shannon TR, Pogwizd SM & Bers DM (2003). Elevated sarcoplasmic reticulum Ca^{2+} leak in intact ventricular myocytes from rabbits in heart failure. *Circ Res* **93**, 592–594.

Shuai J, Pearson JE, Foskett JK, Mak D-OD & Parker I (2007). A kinetic model of single and clustered IP$_3$ receptors in the absence of Ca^{2+} feedback. *Biophys J* **93**, 1151–1162.

Smith IF, Wiltgen SM, Shuai J & Parker I (2009). Ca^{2+} puffs originate from preestablished stable clusters of inositol trisphosphate receptors. *Sci Signaling* **2**, 1–7.

Sobie E, Dilly K, Cruz J, Lederer J & Jafri M (2002). Termination of cardiac Ca^{2+} sparks: an investigative mathematical model of calcium-induced calcium release. *Biophys J* **83**, 59–78.

Tovey SC, de Smet P, Lipp P, Thomas D, Young KW, Missiaen L, De Smedt H, Parys JB, Berridge MJ, Thuring J, Holmes A & Bootman MD (2001). Calcium puffs are generic InsP3-activated elementary calcium signals and are downregulated by prolonged hormonal stimulation to inhibit cellular calcium responses. *J Cell Sci* **114**, 3979–3989.

Treves S, Franzini-Armstrong C, Moccagatta L, Arnoult C, Grasso C, Schrum A, Ducreux S, Zhu MX, Mikoshiba K & Girard T (2004). Junctate is a key element in calcium entry induced by activation of InsP$_3$ receptors and/or calcium store depletion. *J Cell Biol* **166**, 537–548.

Wehrens XHT, Lehnart SE & Marks AR (2005). Intracellular calcium release and cardiac disease. *Annu Rev Physiol* **67**, 69–98.

Williams GSB, Chikando AC, Tuan H-TM, Sobie EA, Lederer WJ & Jafri MS (2011). Dynamics of calcium sparks and calcium leak in the heart. *Biophys J* **101**, 1287–1296.

Yamasaki Y, Furuya Y, Araki K, Matsuura K, Kobayashi M & Ogata T (1997). Ultra-high-resolution scanning electron microscopy of the sarcoplasmic reticulum of the rat atrial myocardial cells. *Anat Rec* **248**, 70–75.

Yao Y, Choi J & Parker I (1995). Quantal puffs of intracellular Ca^{2+} evoked by inositol trisphosphate in *Xenopus* oocytes. *J Physiol* **482**, 533–553.

Zima AV & Blatter LA (2004). Inositol-1,4,5-trisphosphate-dependent Ca^{2+} signalling in cat atrial excitation-contraction coupling and arrhythmias. *J Physiol* **555**, 607–615.

Author contributions

All experiments were performed at the Department of Physiology, University of Bern, Switzerland. M.E. conceived and designed experiments. T.H. collected and analysed the experimental data. M.E., T.H. and N.D.U. drafted and revised the article. All authors approved the final version for publication.

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