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Role of inositol 1, 4, 5-trisphosphate in the regulation of ventricular Ca^{2+} signaling in intact mouse heart

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Abstract

Inositol 1,4,5-trisphosphate (InsP_3) -mediated Ca^{2+} signaling is a major pathway regulating multiple cellular functions in excitable and non-excitable cells. Although InsP_3 -mediated Ca^{2+} signaling has been extensively described, its influence on ventricular myocardium activity has not been addressed in contracting hearts at the whole-organ level. In this work, InsP_3 -sensitive intracellular Ca^{2+} signals were studied in intact hearts using laser scanning confocal microscopy and pulsed local field fluorescence microscopy. Intracellular $[\text{InsP}_3]$ was rapidly increased by UV flash photolysis of membrane-permeant caged InsP_3 . Our results indicate that the basal $[\text{Ca}^{2+}]$ increased after the flash photolysis of caged InsP_3 without affecting the action potential (AP)-induced Ca^{2+} transients. The amplitude of the basal $[\text{Ca}^{2+}]$ elevation depended on the intracellular $[\text{InsP}_3]$ reached after the UV flash. Pretreatment with ryanodine failed to abolish the InsP_3 -induced Ca^{2+} release (IICR), indicating that this response was not mediated by ryanodine receptors (RyR). Thapsigargin prevented Ca^{2+} release from both RyR- and InsP_3 -containing Ca^{2+} stores, suggesting that these pools have similar Ca^{2+} reuptake mechanisms. These results were reproduced in acutely isolated cells where photorelease of InsP_3 was able to induce changes in endothelial cells but not in AP-induced transients from cardiomyocytes. Taken together, these results suggest that IICR does not directly regulate cardiac excitation-contraction coupling. To our knowledge, this is the first demonstration of IICR in intact hearts. Consequently, our work provides a reference framework of the spatiotemporal attributes of the IICR under physiological conditions.

Keywords

Inositol 1,4,5-trisphosphate; Caged InsP_3 ; Photolysis; Calcium; InsP_3 R; RyR

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1. INTRODUCTION

The electrical properties of ventricular sub-epicardial tissues (hereafter referred to as epicardium) have been widely described[1]. Alterations in the electrical behavior of the epicardium constitute an important arrhythmogenic mechanism and have been involved in numerous pathophysiological conditions [2, 3]. Historically, the regulation of both, systolic and basal myoplasmic $[Ca^{2+}]$ has been linked to the control of Ca^{2+} release from the sarcoplasmic reticulum (SR) through a Ca^{2+} release channel (ryanodine receptor; RyR)[4]. However, in myocytes from other cardiac tissues (i.e. atrium, purkinje fibers) another intracellular Ca^{2+} release channel, the inositol 1,4,5-trisphosphate receptor (InsP₃R)[5, 6], has also been suggested to be involved in the genesis of arrhythmias [7,8] (e.g. atrial fibrillation) and hypertrophy [9, 10]. In fact, the InsP₃R has been postulated to be a causal factor in the development of Ca^{2+} alternans as an arrhythmogenic mechanism[11,12]. One of the most intriguing problems in the field of intracellular Ca^{2+} homeostasis in heart is whether or not this InsP₃-sensitive pathway is also involved in Ca^{2+} signaling in ventricle.

InsP₃ is generated via phospholipase C by activation of Gq protein-coupled membrane receptors, as a result of activation by a variety of extracellular agonists (e.g. alpha adrenergic, muscarinic, purinergic, etc.). Intracellular InsP₃ can increase cytoplasmic $[Ca^{2+}]$ directly or indirectly. Direct activation of InsP₃R by InsP₃, results in Ca^{2+} release from the endoplasmic reticulum (ER) or from the SR, depending on the tissue. [16]. Indirectly, InsP₃R-mediated Ca^{2+} release causes capacitive Ca^{2+} entry [13–15] or Ca^{2+} -dependent K⁺ channel activation, [16].

The function of InsP₃R has been extensively characterized in cardiac endothelial and vascular smooth muscle cells. For example, in endothelium the InsP₃R is involved in the Ca^{2+} -dependent formation/release of the vasodilator, nitric oxide (NO)[17,18]. In vascular smooth muscle cells, InsP₃R controls a variety of cellular events, including cell contraction. In this tissue, a wide range of physiological stimuli can induce InsP₃R-mediated Ca^{2+} release to trigger/modulate contraction [19,20].

In isolated ventricular myocytes, type-2 InsP₃R (InsP₃R-2; the predominant isoform in these cells) [21] is mainly located in the perinuclear region [22]. The biophysical properties of this isoform have been defined at the single-channel level in planar lipid bilayers using both, recombinant and native channels[23–26]. The open probability of InsP₃R-2 exhibits different Ca^{2+} and InsP₃ dependencies compared to the type-1 isoform [23]. In contrast to the detailed knowledge of the biophysical properties of InsP₃R-2, there is a lack of evidence about the conditions under which InsP₃R can be physiologically activated at the organ level.

The central hypothesis of this study was that in epicardial ventricular myocytes, increases in myoplasmic [InsP₃] could modify the intracellular Ca^{2+} dynamics during the cardiac cycle, thereby altering electrical excitability. To test this hypothesis, InsP₃-induced intracellular Ca^{2+} release was activated by flash photolysis of the membrane-permeant form of caged-InsP₃ in the contracting ventricle of intact mouse hearts. Under these conditions, the changes in free $[Ca^{2+}]$ were monitored with laser scanning confocal microscopy (LSCM) and with pulsed-local field fluorescence microscopy (PLFFM) [27,28]. The kinetics of this InsP₃ induced Ca^{2+} release and its interaction with RyR-mediated Ca^{2+} release were also defined. This experimental imaging approach enabled us to identify the Ca^{2+} signaling arising from different cell types in the epicardium. Our results indicate that short-term increases in [InsP₃] did not modify the RyR-mediated Ca^{2+} -induced Ca^{2+} release (CICR) from the SR of the ventricular myocytes. However, the InsP₃ photoreleased from the caged compound was able to release Ca^{2+} from the endothelial cells lining the coronary vessels. Our results provide decisive evidence that in epicardium, the InsP₃ signaling is not a critical modulator

of intracellular Ca^{2+} dynamics associated to the action potential (AP) in ventricular cardiac myocytes. This suggests that under the experimental conditions used in this work, InsP_3 signaling is not involved in the regulation of excitation-contraction coupling (ECC) during the AP-induced Ca^{2+} transients.

2. MATERIALS AND METHODS

Hearts were obtained from adult Balb/c mouse using standard procedures described elsewhere[29]. All procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). All animal studies were approved by the *Institutional Animal Care and Use Committee*.

2.1 Whole Heart experiments

Hearts were cannulated through the aorta, mounted into a Langendorff system, and continuously perfused (at a rate of 1 ml/min) with Tyrode solution (in mM; 140 NaCl, 5.4 KCl, 2 CaCl_2 , 1 MgCl_2 , 0.33 Na_2HPO_4 , 10 HEPES, 10 glucose, pH = 7.4, equilibrated with 100% O_2). The hearts were loaded with rhod-2 AM or fluo-4 AM (30 μM ; Molecular Probes, Eugene, OR), and/or the membrane permeable and photolabile derivative of myo-inositol-1,4,5-trisphosphate (caged InsP_3 ; D-2,3-O-Isopropylidene-6-O-[2-nitro-4,5-dimethoxy]-benzyl-myo-inositol-1,4,5-trisphosphate-Hexakis [propionoxymethyl] ester (caged InsP_3); 40 μM ; SicheM, Bremen, Germany). Rhod-2 AM, fluo-4 AM and caged InsP_3 were dissolved in a 4% pluronic F-127 DMSO solution before being added to the perfusion solution. After 40 min, the hearts were perfused with Tyrode solution without Ca^{2+} -indicators or caged InsP_3 . Similar protocol was used to load cardiac cells with the potentiometric indicator di-8-ANEPPS. Di-8-ANEPPS 1 $\mu\text{g/ml}$ was dissolved in DMSO/Pluronic mixture and added to 1 ml normal Tyrode solution, to a final concentration of 2 μM . The loading time was ~30 minutes. Hearts were paced at 2 Hz. Fluorescence was measured from the epicardium of the left ventricle. Unless specified, all the Ca^{2+} transients are presented as normalized rhod-2 fluorescence records.

2.2 Ventricular myocytes isolation

Mouse ventricular myocytes were enzymatically dissociated using the Langendorff coronary retroperfusion technique[30]. Briefly, the aorta was cannulated and perfused with *Tyrode* solution followed by *Tyrode* Ca^{2+} -free solution at 31 °C. The heart was then perfused with *Tyrode* Ca^{2+} -free solution containing 2 mg/ml Collagenase 257 U/mg (Worthington Biochemical Corporation, NJ, USA) and 0.1 mg/ml of protease 4.4 U/mg (Pronase E, SIGMA, St. Louis, USA). The heart was perfused with *Tyrode* Ca^{2+} -free solutions, the ventricles cut, minced and mechanically filtered with a mesh to finish the dissociation procedure. After dissociation, cells were loaded (30 minutes, room temperature) with 5 μM fluo-4 AM and 2–10 μM caged InsP_3 in *Tyrode* containing 2 mM CaCl_2 and then rinsed continuously for 15–30 min before use.

2.3 Confocal Laser Scanning Microscopy

See supplemental material.

2.4 Pulsed Local Field Fluorescence Microscopy

See supplemental material.

2.5 Statistics

PLFFM data represent average of recordings from 8 different sites on the ventricular epicardium from different heart preparations. Each recording site contained between 25–100 ventricular cardiac myocytes. In LSCM, 10 to 24 individual regions of the epicardial surface were used from at least 6 different heart preparations. Results are shown as mean $\pm$ SE.

3. RESULTS

3.1 Cellular origin of InsP_3 -sensitive Ca^{2+} signals in whole hearts

The functional role of InsP_3 signaling in cardiac ventricle has been the focus of research for several years [8,31,32]. Most of the experimental approaches to study this fundamental signaling pathway had involved experiments in isolated myocytes. Usually these experiments are conducted by stimulating a Gq protein. Although in this experimental approach, a physiological downstream pathway is activated, it has been very difficult to determine if the cellular responses are due to a cytosolic increase of InsP_3 or if they result from the activation of some other parallel signaling mechanism.

To evaluate the specific role of InsP_3 on intracellular cardiac Ca^{2+} signals we performed InsP_3 photolysis experiments. Cellular Ca^{2+} fluorescence signals were recorded using a modified upright LSCM (see supplemental materials). Specifically, Ca^{2+} signals from the epicardial layer of an intact perfused heart were recorded by loading whole hearts with the Ca^{2+} indicator fluo-4 AM. The loaded hearts were perfused with zero Ca^{2+} Tyrode solution, only during the recording of images, to eliminate the moving artifacts induced by the heart contraction.

Figure 1A shows an XY confocal image obtained at a focal plane positioned on the epicardial surface of the heart. The image revealed a foremost fluorescence pattern that seems to correspond to cells surrounding the lumen of blood vessels, and a secondary pattern of lower intensity in the background formed by dim striations that most likely corresponded to myocytes. Similar images were obtained with other Ca^{2+} indicators (e.g. rhod-2; data not shown). The small diameter of these vessels (3–10 μm) suggests that these vascular structures most likely represent coronary capillaries. The vascular walls exhibited a peculiar fluorescent pattern characterized by thin fluorescence lines interrupted by larger regions of bright fluorescence (Fig. 1A, inset). This fluorescent profile was interpreted as endothelial cells lining the vessels interior. Similar distribution patterns of vascular walls were observed when the hearts were loaded with a membrane-labeling probe, the potentiometric indicator di-8-ANEPPS (Fig. 1B). Interestingly, cardiomyocytes were more visible using this membrane dye due to the distinct striated pattern that results from the T-tubules staining, and their limits corresponding to the surface sarcolemma are easily identified (see figure inset).

The remarkable level of loading of the epicardial vascular bedding opened the possibility to record fluorescent signals not only from ventricular myocytes but from endothelial cells as well. To test this possibility, Ca^{2+} fluorescent signals were simultaneously measured with LSCM from myocytes and endothelial cells before and after the photolytic increase of InsP_3 . For this purpose, a UV photolytic stimulus was delivered through a 50 μm quartz multimode optical fiber coupled to a DPSS UV laser (*see material and methods*). The fiber was micro-positioned between a long working distance objective and the epicardial layer of the heart. Figure 2A shows experiments in which XY laser scan confocal images were obtained before and after the photolytic activation of caged InsP_3 . One region of interest (ROI, boxed regions) was defined for a myocyte and another one for an adjacent endothelial cell laying a capillary. The increase in fluorescence measured in both cell types revealed that the signal from ventricular myocytes was substantially smaller than that from endothelial cells. Pooled

data are presented in the bar graph of panel B, where different ROIs from both cell types were computed. The yellow bars correspond to the change in absolute fluorescence from 24 ventricular myocytes and from 22 endothelial cells ($n = 5$ hearts). To minimize the influence of dye loading and variable optical properties, the fluorescence signal was normalized by the basal fluorescence before the application of the photolytic stimulus (blue bars). These data indicate that the difference in the fluorescence change between the two cell types can be attributed to differences in IICR and not the result of non-specific factors. The time course of IICR in endothelial cells was evaluated by sequentially recording *XY* confocal images, at a rate of 0.635 frames/s, before and after InsP_3 photorelease. Figure 2C shows confocal images obtained at different times during the InsP_3 response. Immediately after the UV flash, an increase in fluorescence intensity in the endothelial cell (image b) was evident. This transient increase was followed by a slower relaxation to lower fluorescence levels (images c & d). The temporal attributes of the IICR are shown in Figure 2D, where the rising phase exhibited a time-to-peak value of less than 1.5 s and a decaying phase characterized by a time constant of ~ 20 s. Notice that after this transient response, a second component was also observed. This latter fluorescence phase slowly returned to its original (pre-pulse) value.

3.2 InsP_3 -sensitive Ca^{2+} signals during the cardiac cycle in whole hearts

As shown in Fig 1 and 2, LSCM is a very precise way to record fluorescent Ca^{2+} signals from different structures at the epicardial layer when the heart is not contracting. Unfortunately, the mechanical artifacts produced during contraction prevent the use of this technique on beating hearts under more physiological situations. PLFFM is an optical technique that allows attaching a very small multimode optical fiber to the surface of the heart allowing the fiber to follow the movement of the heart. We have previously shown that PLFFM is a very effective technique to eliminate the mechanical artifact introduced to the fluorescent signals by contracting murine hearts [27,28].

A modified PLFFM technique was used to evaluate the dynamics of intracellular Ca^{2+} , while intracellular $[\text{InsP}_3]$ was rapidly increased by the application of UV flash photolytic pulses delivered through the same recording fiber (Fig. 3). In addition, this approach allowed us the study of AP-induced intracellular Ca^{2+} transients under experimental conditions similar to those found *in situ*. Figure 3A shows AP-induced intracellular Ca^{2+} transients recorded from ventricular epicardium of an intact/beating heart, before, during, and after UV flashing in the absence of caged InsP_3 . Under these control conditions, the intracellular Ca^{2+} signaling did not exhibit any noticeable change attributable to the UV flash ($n = 10$ hearts). In contrast, when the cells were preloaded with an AM form of caged InsP_3 , the UV flash induced a substantial elevation of basal $[\text{Ca}^{2+}]$ of $\sim 30\%$ of the signal amplitude before the flash (Fig. 3B; $n = 6$ hearts). Although InsP_3 photolysis produced an increase of the resting fluorescence, the time course (time to peak) of this change was significantly slower (~ 1 s) than the one produced by the AP induced Ca^{2+} release (< 30 ms). The Ca^{2+} -dependent fluorescence signal induced by InsP_3 photolysis also exhibited a very slow relaxation time course (half duration of ~ 45 s; data not shown). Since InsP_3 photorelease was driven by short UV pulses (< 10 ms), it is likely that Ca^{2+} released through InsP_3 receptors must be regulated by a separate kinetic process from the one that regulates Ca^{2+} release through RyR.

3.3 Effects of InsP_3 photo-release on the kinetics of intracellular Ca^{2+} transients

Changes in the basal $[\text{Ca}^{2+}]$ usually have a large impact on the amplitude and kinetics of the Ca^{2+} transients induced by Ca^{2+} released from the SR. An increase in the steady-state fluorescence level after the photolysis of InsP_3 indicates that InsP_3 activates Ca^{2+} release from epicardial cells. A drawback of PLFFM recordings with a $200\ \mu\text{m}$ optical fiber is that

the recorded fluorescence signals might arise from a multi-cellular origin (Fig. 1). This implies that with PLFFM it is not possible to determine which fraction of the change in the fluorescence signal is coming from the ventricular myocytes. However, we have already shown (Fig. 2) that InsP_3 uncaging produced a substantially larger fluorescence change in endothelial cells than in ventricular myocytes. Thus, we cannot rule out the possibility that in epicardial myocytes the basal $[\text{Ca}^{2+}]$ was significantly increased by InsP_3 photorelease during the cardiac cycle.

To test this possibility, Ca^{2+} transients were recorded and compared before, during, and after inducing an elevation in basal $[\text{Ca}^{2+}]$ in ventricular myocytes (Fig 4A). Surprisingly, the main observation in these experiments was that the photolysis of caged InsP_3 consistently increased the basal fluorescence without obvious effects on the Ca^{2+} transient attributes. Figure 4A shows a representative experiment where basal $[\text{Ca}^{2+}]$ was increased by photolytic release of InsP_3 . No changes were observed in either the amplitude or the kinetics of the Ca^{2+} transients (time to peak 31.25 ± 3.15 ms vs. 30.95 ± 1.0 ms; decay time constant 102.1 ± 3.03 ms vs. 105.5 ± 2.6 ms; $n=16$ hearts). This lack of effect is more evident at an expanded time scale (Fig. 4A, upper panel), where the temporal course of representative Ca^{2+} transients before (black) and after (gray) the InsP_3 photorelease appeared essentially identical. This finding however, contradicted the widespread notion that any change in basal $[\text{Ca}^{2+}]$ always affects both, amplitude and kinetics of the Ca^{2+} transient.

This result can be interpreted in several ways. One possibility is that the Ca^{2+} indicator (rhod-2) was partially saturated with higher basal $[\text{Ca}^{2+}]$, so that the maximal changes in fluorescence during the Ca^{2+} transients were not detected due to the intrinsic non-linearity of the indicator. To test this possibility, basal $[\text{Ca}^{2+}]$ was elevated by other experimental maneuvers. Figure 4B shows the effects of increasing basal $[\text{Ca}^{2+}]$ by raising the extracellular $[\text{Ca}^{2+}]$ from 2 to 5 mM. This maneuver not only induced a $31 \pm 5\%$ elevation in the basal fluorescence ($n=5$, $p=0.004$) but also increased the amplitude of the Ca^{2+} transients by $34 \pm 4\%$ ($n=5$, $p<0.001$). In addition, the kinetics of the rising phase of the Ca^{2+} transients was modified (time to peak increased from 30 ± 2 ms to 38 ± 3 , $n=5$, $p=0.038$). However, an increase in the extracellular $[\text{Ca}^{2+}]$ would produce a larger sarcolemmal Ca^{2+} influx potentially affecting the Ca^{2+} transient kinetics. Consequently, we designed an experiment approach to exclusively increase basal $[\text{Ca}^{2+}]$ in the presence of physiological external $[\text{Ca}^{2+}]$ (i.e. 2 mM). To this end, an alternative pathway for cytoplasmic Ca^{2+} removal, the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX), was impaired by partially replacing extracellular Na^+ by Li^+ . Replacement of 50% of extracellular Na^+ by Li^+ (70 mM LiCl Tyrode) induced a 1.5-fold elevation in the basal fluorescence ($\pm 0.03\%$, $n=4$, $p<0.001$), as illustrated in Figure 4C. Likewise the experiments with increased extracellular $[\text{Ca}^{2+}]$, this elevation in basal fluorescence was accompanied by an increase in the amplitude of the AP-triggered Ca^{2+} transients ($55 \pm 9\%$ increase, $n=4$, $p=0.01$; pooled data not shown), and by a significant change in the kinetics of the Ca^{2+} transient raising phase (time to peak increased from 31 ± 1 ms to 44 ± 2 , $n=4$, $p=0.031$). This effect on the Ca^{2+} transient kinetics is better illustrated in the left lower panels of Figure 4C, where representative traces from the two conditions were normalized and superimposed. This figure also revealed that the effect on the transient kinetics was virtually identical with the two maneuvers used to increase basal $[\text{Ca}^{2+}]$: rise of extracellular Ca^{2+} and NCX impairment. Altogether, these observations suggested that saturation of the Ca^{2+} indicator loaded into the ventricular myocytes was not a contributing factor to our results. Consequently, the release of InsP_3 in ventricular myocardium did not induce significant changes of diastolic $[\text{Ca}^{2+}]$ to impact the Ca^{2+} release from intracellular Ca^{2+} stores.

3.4 Interaction between InsP₃R- and RyR- mediated Ca²⁺ signals

Since InsP₃R and RyR are usually present in the SR/ER, the resulting Ca²⁺ signals can interact with each other. Thus, it is possible that in presence of a large RyR mediated Ca²⁺ release, the amplitude of the InsP₃ sensitive signal will be functionally damped. To determine if IICR interacts with the RyRs-mediated CICR, we performed experiments in which Ca²⁺ release through the RyR was impaired by perfusing the hearts with a high concentration of the alkaloid ryanodine (40 μM).

As expected, ryanodine reduced the Ca²⁺ transient amplitude (to ~15% of the control; n=6) by removing the ECC signal (Figure 5A, upper trace, basal fluorescence subtracted). Therefore, the remaining Ca²⁺ transient was most likely resulting from the Ca²⁺ influx through the sarcolemma. As a consequence of the ryanodine treatment there was an increase in the resting basal fluorescence (Figure 5A, lower trace without basal fluorescence subtraction). This observation was consistent with an increase in cytoplasmic [Ca²⁺] due to an initial enhancement of SR Ca²⁺ leak induced by ryanodine. Under these conditions of ryanodine poisoning, the effects of photolytic release of InsP₃ were explored (Figure 5B; n=8). Likewise the effects under control conditions (Figs. 3 & 4), IICR did not alter the Ca²⁺ transients' properties, reflecting the sarcolemmal Ca²⁺ influx evoked during the AP. Taken together, these results demonstrated that in the ventricular epicardium, IICR does not affect the Ca²⁺ signals associated to ECC (sarcolemmal or RyR-mediated). Nevertheless, in presence of ryanodine the increase of IICR was smaller than in the control condition.

3.5 InsP₃ induced Ca²⁺ release in the absence of electrical excitability

In ventricular cardiac myocytes the cytosolic Ca²⁺ dynamics is tightly linked to the membrane potential, in particular to the time course and duration of APs. This relationship involves the activation of the RyR by the Ca²⁺ influx through the L-type Ca²⁺ channel. Furthermore, in the absence of APs this mechanism is impaired. The evaluation of IICR dependency on the membrane potential could also shed light on the conditions that are necessary for the IICR to occur. Thus, photolysis experiments were conducted in the presence of high concentrations (20 μM) of TTX in order to specifically assess the effects of IICR on the signaling pathways independent of APs. Figure 6A shows that TTX effectively eliminated AP-triggered Ca²⁺ transients, by preventing the sarcolemmal Ca²⁺ channels to activate SR RyRs (n=8). Under these conditions, photolysis of caged-InsP₃ was still able to elevate the basal Ca²⁺ (Figure 6A, left trace). Interestingly, the extent of this elevation was reduced (~40%) by ryanodine (Figure 6, middle trace), while it was completely abolished by the SR Ca²⁺ pump inhibitor thapsigargin (Figure 6, right trace). These results confirmed that the InsP₃ pathway is sensitive to the modification of the RyR and that a SERCA pump regulates the replenishment of InsP₃ sensitive Ca²⁺ stores. Finally, the InsP₃ induced Ca²⁺ release occurs even in absence of APs.

InsP₃ can increase intracellular Ca²⁺ by two mechanisms. The first one by directly activating the InsP₃R in the intracellular store and second by indirectly activating the influx of Ca²⁺ through a store operated Ca²⁺ entry (SOCE) system. Thus, to distinguish between these two possibilities, InsP₃ photolysis experiments were conducted with the extracellular Ca²⁺ replaced by Mg²⁺. Figure 6B shows that the intracellular Ca²⁺ transients become progressively smaller immediately after external Ca²⁺ was replaced (n=7). Under these conditions, the photolytic release of InsP₃ still induced changes in the resting [Ca²⁺] (Figure 6C, left trace). The amplitude of this IICR was reduced (>50%) by the application of 40 μM ryanodine (Figure 6C, middle trace). Even more, the photolytic release of InsP₃ was unable to promote intracellular Ca²⁺ release after addition of thapsigargin (Figure 6C, right trace). These results favor the possibility that InsP₃ induces a Ca²⁺ signal by activating Ca²⁺ release from an intracellular organelle sensitive to both ryanodine and thapsigargin.

3.6 Dose dependency of InsP₃-sensitive Ca²⁺ response

Defining the kinetic characteristics of InsP₃-induced Ca²⁺ responses is an important step to understand the mechanism of the interaction between InsP₃ and its receptor. Two indicators of this specific interaction are: the existence of a saturating behavior of the agonist-induced responses and the acceleration of the reaction kinetics as the agonist concentration is increased. Hence, to define the properties of the agonist-receptor interaction in the InsP₃ signaling cascade, the amplitude and kinetics of IICR were evaluated as a function of the photolytic stimulus energy (Figure 7). This was achieved by varying the illumination time while the average laser power was held constant. Figure 7A shows that the amplitude of the IICR was profoundly dependent on the UV flash duration (5, 10, 20, 40, and 80 ms). This observation directly revealed the InsP₃-dependency of Ca²⁺ responses as function of magnitude of photolysis. Shorter UV flashes (i.e. 5, 10 and 20 ms) generated InsP₃ stimuli that triggered Ca²⁺ releases with rising velocities that increased with duration. Longer UV flashes (i.e. 40 & 80 ms) generated InsP₃ stimuli that induced maximal Ca²⁺ release responses with essentially identical time course. In these experiments, the removal of extracellular Ca²⁺ eliminated RyR-mediated intracellular Ca²⁺ transient (n=3).

In Figure 7B, the rise-time dependency of the InsP₃ responses was plotted as a function of the UV stimulus energy (calculated as the product of the mean beam power and the pulse duration). This relationship indicated that higher energy illumination flashes promoted faster activating Ca²⁺ responses. Similarly, the maximal IICR amplitude exhibited a strong dependence on the UV flash energy; higher energy pulses induced larger responses (Fig. 7C). However, the responsiveness of the system seemed to saturate at photolytic energies higher than 200 μJ. These results indicated that as illumination energy increased, the resulting Ca²⁺ transients displayed faster activation and larger amplitude. As a negative control, the inset of panel C shows fluorescence trace obtained while a 200 μJ UV flash was applied in the absence of caged InsP₃ and with the RyR-mediated Ca²⁺ transients absent. This result demonstrated that caged InsP₃ must be present in order for the UV flash to generate a basal [Ca²⁺] elevation.

3.7 Photo-release of InsP₃ in isolated cardiac cells

The intact organ preparation offers multiple physiological advantages with respect to the more reductionist approach of isolated cells. However, experiments in isolated cells usually provide several technical advantages to perform quantitative measurements using biophysical approaches. Although in this work we showed that we were able to instrument simultaneous photolysis and fluorescence recording experiments at the epicardial layer of intact beating hearts, the loading of AM dyes and caged compounds is often easier at the isolated cell level. Hence, to what extent the smaller response to photolytic stimuli in the epicardial myocytes was due to a lesser-caged InsP₃ compound loading? To answer this question we performed photolysis experiment on enzymatically isolated cardiac cells. Figure 8A shows the fluorescent Ca²⁺ signal from a ventricular cardiac myocyte that was loaded simultaneously with fluo-4 and caged InsP₃ AM compounds. Myocytes were electrically paced with extracellular field electrodes to elicit AP-induced Ca²⁺ transients. When a photolytic UV flash stimulus was applied (triggering a small and transient fluorescence artifact), InsP₃ failed to induce a significant change in ECC. A statistical analysis of the basal [Ca²⁺] and Ca²⁺ transients kinetic parameters from 9 cells is presented in Fig 8B. The photolytic stimulus did not induced any significant changes in the basal fluorescence, as well as in the amplitude, time to peak and decay time of the Ca²⁺ transients. These results are in accordance with those obtained from epicardial myocytes in the intact beating heart (Fig. 2).

To evaluate the difference in InsP_3 sensitivity between endothelial cells and myocytes, responses to photo-released InsP_3 were recorded simultaneously from both cell types. Mild enzymatic isolation allowed us to obtain few myocytes (less than 1%) that remained attached to endothelial cells. The sequence of images presented in Figure 8C corresponds to this type of cellular arrangement. These confocal images were obtained before, during, and after photolysis of intracellular caged InsP_3 from an un-paced and quiescent ventricular myocyte adhered to an endothelial cell. After the flash, the endothelial cell exhibited a transient Ca^{2+} elevation that reached its maximum at 4 s. At the same time however, the ventricular myocyte showed a much smaller and slower elevation in basal $[\text{Ca}^{2+}]$ (Fig. 8D). These results were identical to those found in the whole heart and indicate that these two cell types exhibit a considerably different InsP_3 -sensitive Ca^{2+} signals.

4. DISCUSSION

4.1 InsP_3 -mediated Ca^{2+} signaling in non-contracting hearts

Consistent with the remarkable flow-dependent myocardial energy supply characteristic of mouse hearts [33,34], our confocal images revealed extensive vascularization in this tissue (Fig. 1). This large degree of vascularization could reflect the remarkably high metabolic demand of the mouse heart imposed by an extraordinary elevated resting heart rate (~600 beats per minute)[35]. Two important consequences of this fact are that the vast net of capillaries increases the efficiency of the hearts' dye loading and that the endothelial cells lining the capillaries will also be highly loaded with fluorescence probes.

The staining with fluo-4 AM seems to be less intense in the ventricular myocytes than in the endothelial cell (Fig. 1A). However, the fact that Di-8-ANEPPS labeling (Fig. 1B) showed a detailed structure of the epicardial myocytes at the cellular level suggests that the fluorescent dyes also reached the cardiac myocytes effectively.

The different responses to acute increases of InsP_3 exhibited by these two cell types (Fig. 2A) could have several explanations. First, the density and/or affinity of InsP_3R in endothelial cells could be higher than the one observed in cardiomyocytes. This difference would be consistent with the fact that in endothelial cells intracellular Ca^{2+} (i.e. NO release) is mediated by InsP_3R while in ventricular cardiomyocytes most of the beat-to-beat Ca^{2+} signaling (i.e. ECC) is conducted through RyR. Second, the loading of rhod-2, fluo-4, and caged InsP_3 could be lower in cardiomyocytes than in endothelial cells due to diffusional and steric constraints imposed by the capillary barrier. This second possibility seems unlikely as Korneyev et al. [36] demonstrated in rhod-2-loaded hearts, that coronary perfusion of EGTA-AM induced substantial changes in the amplitude and kinetics of epicardial Ca^{2+} transients recorded in intact beating mouse hearts. This evidence indicates that a second AM compound (in addition to the fluorophore) like the caged InsP_3 , can reach and efficiently load ventricular cardiomyocytes when is perfused through the coronary arteries. Third, as the endothelial cells seem to be more loaded, a photo-damaging effect could be more pronounced than in cardiomyocytes. This does not seem to be the case; in Fig 2D and 8D we showed that the InsP_3 responses in endothelial cells have kinetic features not compatible with photo-damage (e.g. slow activation and relaxation). Finally, it is possible that the periods of absence of extracellular Ca^{2+} could induce a reduction in the SR Ca^{2+} content in cardiomyocytes. This could eventually deplete the intracellular Ca^{2+} stores and consequently impair the IICR release in these cells. To test this possibility PLFFM experiments were conducted on intact beating hearts, where photolysis was performed during the cardiac cycle and in the presence of physiological (2 mM) $[\text{Ca}^{2+}]$.

4.2 InsP₃-mediated Ca²⁺ signaling during the cardiac cycle

LSCM has serious limitations to assess the intracellular Ca²⁺ dynamics in contracting hearts. In contrast, PLFFM is a superb technique to evaluate fluorescence signals in organs that move [27,28]. Fig. 3 illustrates that not only PLFFM can be used to record Ca²⁺ transients but also to photolyze through the same multimode fiber. To our knowledge, this is the first time that fluorescence was recorded while photolysis was simultaneously conducted through the same optical fiber. In addition, Fig. 3B shows, a combination of AP-induced Ca²⁺ transients and IICR. Nevertheless, PLFFM did not allow us to determine if the fluorescence recorded during diastole, after application of the laser flash, came from cardiomyocytes or from endothelial cells. This issue was investigated in Fig. 4A where the amplitude and kinetics of AP-induced Ca²⁺ transients was evaluated before and after the photorelease of InsP₃. The fact that there were no changes in these parameters, suggests that the cytosolic free [Ca²⁺] change was not enough to alter the Ca²⁺ transients properties upon the photolytic release of InsP₃ in the cardiomyocytes.

In cardiac myocytes, changes in diastolic [Ca²⁺] can modify the Ca²⁺ transients by different mechanisms [36,37]. For example, an increase in the free basal [Ca²⁺] will induce an increase in the turnover rate of the SERCA pump that reuptakes Ca²⁺ to the SR [38,39]. These changes in intra-SR [Ca²⁺] will produce an increase in the Ca²⁺ released from the SR. In addition, an increase in the free basal [Ca²⁺] will saturate a larger fraction of mobile and fixed cytosolic Ca²⁺ buffers, thereby reducing the active Ca²⁺ buffering capacity [36,40,41]. At a higher resting free [Ca²⁺] in the myoplasm, the cytosolic Ca²⁺ binding sites on the RyR will be mostly occupied [42,43], changing the rate of SR Ca²⁺ release and/or modifying the intra SR Ca²⁺ content as a result of an increased Ca²⁺ sparks frequency [44,45]. Consequently, if a photolytic release of InsP₃ increased the basal [Ca²⁺], a profound effect on the amplitude and kinetics of the RyR-mediated Ca²⁺ transients would be expected.

The lack of changes in the Ca²⁺ transient properties could result from sensitivity limitations of PLFFM to detect the changes mediated by an increase in the basal [Ca²⁺]. However, Fig. 4B–C showed that this was not the case as other maneuvers that increased basal [Ca²⁺] (i.e. increase in extracellular [Ca²⁺] and impairment of NCX) had a significant effect on the amplitude and kinetics of AP-induced Ca²⁺ transients. Thus, InsP₃ photolysis did not induce a large enough increase in the basal [Ca²⁺] to promote changes in the ECC. This observation is consistent with previous reports that have shown no effects of InsP₃ on ventricular myocytes [12].

4.3 Interactions between InsP₃R- and RyR- mediated Ca²⁺ release

Although the InsP₃-induced changes in basal fluorescence did not affect ECC, it is still possible that RyRs and InsP₃ Rs share a common Ca²⁺ pool. Fig. 5A confirms that Ca²⁺ changes during AP-induced Ca²⁺ transients are strongly determined by the RyR-mediated Ca²⁺ efflux from the SR. The properties of the ryanodine-resistant component of the Ca²⁺ transient (amplitude and kinetics) remained unchanged after caged InsP₃ photolysis. However, the amplitude of the basal fluorescence increase induced by InsP₃ photolysis was significantly reduced in the presence of ryanodine.

We frequently observed that with high concentrations of ryanodine the InsP₃-induced Ca²⁺ elevation seemed to be smaller than in the absence of ryanodine (compare Fig. 5B with 4A). This implies the existence of an intracellular Ca²⁺ pool shared by the two Ca²⁺ signaling systems. Although ryanodine-sensitive Ca²⁺ stores have been described in vascular endothelial cells [46,47], an intriguing question is how can ryanodine deplete the InsP₃-sensitive stores, while InsP₃ does not deplete the ryanodine-sensitive stores? The answer could imply either different relative density of InsP₃Rs vs. RyRs [48], and/or different

cellular localization of these channels. Specifically, the existence of two types of Ca^{2+} reservoirs, one containing only RyRs and another having a mixture of RyRs and InsP_3Rs is plausible [49]. If true, there may be simply not enough InsP_3Rs in the mixed pool to make a substantial difference in RyR-mediated Ca^{2+} release. Alternatively, the cells could contain separated Ca^{2+} stores with different sets of Ca^{2+} release channels. Our data support the former possibility. That is, that the dyadic SR involved in ECC would contain only functional RyR channels, while InsP_3R -containing stores present elsewhere could contain both types of channels. Yet, a third possibility would involve the intrinsic Ca^{2+} sensitivity of the InsP_3R channels. Since ryanodine raises the diastolic $[\text{Ca}^{2+}]$ [43] (Figure 5B) the degree of InsP_3R activation could actually be reduced. This peculiar response could result from an inhibitory effect induced by an elevated myoplasmic $[\text{Ca}^{2+}]$, as it has been suggested in experiments with immune cells [50]. Although this inhibitory effect has not been observed in lipid bilayers experiments [23,24], the variability of the inhibitory Ca^{2+} sensitivities among the InsP_3R isoforms [25,26] and the different experimental conditions (i.e. presence/absence of key regulatory proteins, subunit composition, etc.) could result in completely different behavior.

The fact that in absence of extracellular Ca^{2+} InsP_3 photolysis was still able to induce a change in the basal fluorescence favors an ER/SR Ca^{2+} release process. Specifically, the measured changes in basal Ca^{2+} have all the features of Ca^{2+} response mediated by InsP_3R and not by the permeation through a store operated capacitive pathway (SOCE) (Fig 6C). In addition, this intracellular receptor exhibits most of the kinetic features of a ligand-receptor activation mechanism [51–53]. For example, the photolysis of caged InsP_3 induced responses that were faster and larger as the energy of the photolytic stimulus was increased (Fig. 7).

4.4 InsP_3 photolysis in isolated cells

Is the absence of InsP_3 photorelease effect on the ECC a consequence of the compartmentalization of the caged compound between endothelial cells and cardiac myocytes? Is caged InsP_3 present in cardiac myocytes? These are central questions whose answers will further validate our conclusions.

The efficient myocyte internalization of compounds delivered by coronary perfusion was confirmed with experiments carried out in enzymatically-dissociated cells shown in Figure 8. After the flash-induced increase in intracellular $[\text{InsP}_3]$ the endothelial cell exhibited a transient Ca^{2+} elevation that reached its maximum at 4 s. At the same time however, the ventricular myocyte showed a much smaller and slower elevation in basal $[\text{Ca}^{2+}]$. These results were identical to those found in the whole heart indicating that these two cell types exhibit a considerably different InsP_3 -sensitive Ca^{2+} signals. Alternatively, the apparently different response to InsP_3 could result from a limited internalization of fluorophores and/or caged compounds. In these single-cell experiments however, heterogeneities in intracellular loading of either fluo-4 or caged InsP_3 are unlikely because both cells were incubated together for a long time (30–45 min) and at high concentrations of the fluorophore and caged InsP_3 (5 & 2 μM , respectively). Altogether, these results indicate that in the whole-heart preparation, membrane-diffusible compounds readily access intracellular compartments through coronary perfusion. Therefore, the assumption that in the whole-heart experiments the membrane-permeant caged- InsP_3 is effectively internalized into the ventricular myocytes is supported by our results; which on the other hand, validate our conclusion that in ventricular myocardium, a rapid rise in the intracellular $[\text{InsP}_3]$ does not significantly affect the ECC properties.

5. CONCLUSIONS

In this paper we showed for the first time, cellular responses at the whole-heart level induced by caged-InsP₃ photolysis. In the mouse epicardium, several cell types contribute to the resting fluorescence. InsP₃ photorelease induced Ca²⁺ changes in endothelial cells. The photolysis of InsP₃ and/or the resulting Ca²⁺ increase in the myocardium did not impact ECC. Finally, the cellular responses induced by InsP₃ activate mainly an intracellular Ca²⁺ store (not a SOCE Ca²⁺ influx) that shares InsP₃R and RyR. We believe that the methodology and hypothesis put forward in this work will help to understand InsP₃ signaling in cardiac function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Photolysis of caged-InsP₃ induced changes in intracellular [Ca²⁺] in beating, intact whole hearts from adult mice.
- In ventricles of contracting hearts photolysis of InsP₃ induced Ca²⁺ release in endothelial cells and epicardial myocytes.
- In ventricular myocytes the Ca²⁺ increase due to photolysis of InsP₃ did not impact the excitation contraction coupling process.
- The cellular responses induced by InsP₃ activate mainly intracellular Ca²⁺ stores that shares InsP₃R and RyR

Figure 1. Cellular origin of IP_3 -sensitive Ca^{2+} signals in whole hearts

A. XY Linescan confocal microscopy of AM fluo-4-loaded left ventricular wall, where regions of higher fluorescence are localized in cells lining the myocardial microvasculature. For these studies, cardiac contractility was eliminated by myocardial perfusion of 0 Ca^{2+} Tyrode solution. Since the membrane permeable form of Fluo-4 (AM) was used, the fluorescence signals originated from the cytoplasmic compartments. Details of a capillary bed are shown in the magnified region (notice the myocytes between two capillary vessels). Endothelial nuclei appear as the enlarged fluorescence spots within the capillaries. **B.** XY Linescan confocal microscopy of di-8-ANEPPS-loaded left ventricular wall. Since di-8-ANEPPS binds to surface membranes, fluorescence signals mainly labeled the capillary walls and the sarcolemma of cardiac myocytes. Details are shown in the magnified region, where surface sarcolemma and T-tube network of cardiomyocytes are clearly visible.

Figure 2. Comparison of IP_3 -sensitive fluorescence signals from cardiomyocytes and endothelium

A. *XY* confocal images before (left) and after (right) UV flash photolysis of caged IP_3 in an intact whole heart. Regions of interest (red boxes) defined for an endothelial cell (Endo) and a ventricular myocyte (Myo) and collected with the same confocal settings (e.g. laser power, gain, iris size, etc.). **B.** Bar graph shows peak fluorescence values before (yellow bars) and after normalization ($\Delta F/F_0$; blue bars) of IP_3 -induced $[Ca^{2+}]_i$ increase in myocytes and endothelial cells. Myocytes fluorescence was 12% and 15.5% of the signal obtained from the endothelial cell before and after normalization, respectively. **C.** Time course of IP_3 -

sensitive Ca^{2+} signals from endothelial cells. $XY\text{Ca}^{2+}$ images taken from Fluo-4-loaded endothelial cells, at 5 s (a) before and 3 (b), 35 (c) & 52 (d) s after a UV flash. Red boxes represent the regions of interest defined in a single endothelial cell. **D.** Time course of spatially averaged fluorescence from the corresponding regions of interest, taken at a rate of 0.635 frames/s. Arrows indicate the times at which the above images were taken.

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Figure 3. IP₃-induced Ca²⁺ increase activated by UV flash illumination

A. AP-evoked $[Ca^{2+}]_i$ transients from ventricular epicardium showing a UV flash photolysis pulse delivered at the arrow mark in the absence of caged-IP₃. Under these conditions, the UV pulse did not produce changes in the basal fluorescence. **B.** AP-evoked $[Ca^{2+}]_i$ transients before and after a UV flash when caged-IP₃ was perfused into the heart. Photo-release of IP₃ (arrow mark) evoked an increase in the basal fluorescence. Ca^{2+} transients records are presented as normalized fluorescence ($\Delta F/F_0$). The measurements (from A and B) were conducted at 21°C and pacing frequency of 2Hz.

Figure 4. Effects of Ca^{2+} increase on the Ca^{2+} transient properties

A. AP-evoked $[\text{Ca}^{2+}]_i$ transients from ventricular epicardium before and after a UV flash photolysis pulse (arrow mark). For comparison, selected Ca^{2+} transients obtained before (black trace, left) and after (gray trace, right) photorelease of IP_3 are shown at expanded time scale in the upper panels. **B.** AP-evoked $[\text{Ca}^{2+}]_i$ transients before and after increasing extracellular $[\text{Ca}^{2+}]$ from 2 (black traces) to 5 (grey traces) mM (upper panel). Normalized Ca^{2+} transient records are overlapped to evaluate the effects of increasing extracellular $[\text{Ca}^{2+}]$ on the transient kinetics (lower left panel). Ca^{2+} transient parameters from 5 hearts before (black bars) and after (gray bars) increasing extracellular $[\text{Ca}^{2+}]$. **C.** Effect of the partial replacement of extracellular Na^+ with Li^+ on AP-evoked- Ca^{2+} transients (upper panel). Normalized Ca^{2+} transient Li^+ overlaps records to evaluate the effects of replacing extracellular Na^+ on the transient kinetics (lower left panel). Ca^{2+} transient parameters from 5 hearts before (black bars) and after (grey bars) replacing extracellular Na^+ by Li^+ . All the recordings shown in this figure were obtained at 2 Hz and 21°C using rhod-2 as Ca^{2+} indicator. All bar graph are shown as Mean $\pm$ SE.

Figure 5. Interaction between IP_3R -mediated and RyR -mediated Ca^{2+} release processes
A. The RyR -mediated Ca^{2+} transient component was eliminated by perfusion with $40\ \mu\text{M}$ ryanodine. Basal fluorescence was subtracted. Selected AP-evoked Ca^{2+} transients before and after the ryanodine actions (asterisk marks) shown at expanded time scale in the lower panel (fluorescence in these traces was not normalized and basal fluorescence was not subtracted). **B.** AP evoked Ca^{2+} transients before and after photorelease of IP_3 are shown in the presence of ryanodine.

Figure 6. Ca^{2+} source of the intracellular IP_3 -sensitive Ca^{2+} increase

A. IP_3 -induced Ca^{2+} release in a successive absence of action potentials (TTX 20 μM ; left trace), SR Ca^{2+} release (ryanodine 40 μM ; middle trace), and SR Ca^{2+} reuptake (thapsigargin 20 μM ; right trace). **B.** Time course of the amplitude of AP-induced Ca^{2+} transients during extracellular Ca^{2+} removal. **C.** IP_3 -induced Ca^{2+} release in a successive absence of extracellular Ca^{2+} (left trace), SR Ca^{2+} release (ryanodine, 40 μM ; middle trace), and SR Ca^{2+} reuptake (thapsigargin, 20 μM ; right trace). Arrows indicate the photorelease of caged- IP_3 .

Figure 7. Graded IP_3 -sensitive Ca^{2+} response

A. Prolonging UV flash duration increased the amount of photoreleased IP_3 . **B.** Rate of rise of the fluorescence signals shown in panel A as a function of the UV pulse energy (i.e. flash duration). **C.** Mean peak amplitude of fluorescence signals, expressed as $\Delta F/F$, normalized, and plotted as a function of the UV pulse energy (i.e. flash duration). Inset shows that when no caged- IP_3 was loaded the application of saturating (200 μJ) UV flash pulses (asterisk) did not change the levels of basal fluorescence (i.e. diastolic Ca^{2+}). These experiments were carried out in the absence of extracellular Ca^{2+} .

Figure 8. Origin of IP_3 -sensitive fluorescence signals at the single-cell level

A. AP-evoked Ca^{2+} transients after UV flash (small fluorescence artifact) photolytical release of caged IP_3 in paced (~ 0.6 Hz) isolated ventricular cardiomyocyte. **B.** Pooled data of Ca^{2+} signaling parameters from acutely isolated ventricular cardiomyocytes shown as percentage of pre-flash values ($n = 9$ cells). Pre-flash values of time to peak and decay time constant were 89 ± 30 ms and 157 ± 50 ms, respectively. **C.** Sequence of XY confocal images from a ventricular myocyte (ROI 1) attached to an endothelial cell (ROI 2) before, during, and after photorelease of caged IP_3 . **D.** Time course of the mean fluorescence of ROI 1 and ROI 2 selected in C. Images were taken at a frame rate of 40 Hz (25 ms per frame).