J Physiol 588.1 (2010) pp 225–242

Increased Ca^{2+} leak and spatiotemporal coherence of Ca^{2+} release in cardiomyocytes during β-adrenergic stimulation

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β-Adrenergic receptor (β-AR) stimulation of cardiac muscle has been proposed to enhance Ca^{2+} release from the sarcoplasmic reticulum (SR) through ryanodine receptors (RyRs). However, the anticipated increase in RyR Ca^{2+} sensitivity has proven difficult to study in intact cardiomyocytes, due to accompanying alterations in SR Ca^{2+} content, inward Ca^{2+} current (I_{Ca}) and diastolic cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). Here, we studied whole-cell Ca^{2+} release and spontaneous Ca^{2+} leak (Ca^{2+} sparks) in guinea-pig ventricular myocytes with confocal Ca^{2+} imaging before and during β-AR stimulation by isoproterenol (Iso), but under otherwise nearly identical experimental conditions. The extent of SR Ca^{2+} loading was controlled under whole-cell voltage-clamp conditions. UV flash-induced uncaging of Ca^{2+} from DM-nitrophen was employed as an invariant trigger for whole-cell Ca^{2+} release. At matched SR Ca^{2+} content, we found that Iso enhanced the spatiotemporal coherence of whole-cell Ca^{2+} release, evident from spatially intercorrelated release and accelerated release kinetics that resulted in moderately (∼20%) increased release amplitude. This may arise from higher RyR Ca^{2+} sensitivity, and was also reflected in spontaneous SR Ca^{2+} leak. At comparable SR Ca^{2+} content and cytosolic $[Ca^{2+}]_i$, we observed a ∼4-fold increase in Ca^{2+} spark frequency in Iso that also appeared in quiescent cells within 2 min without increased SR Ca^{2+} content. This was likely to have been mediated by Ca^{2+}/calmodulin-dependent protein kinase (CaMKII), rather than cAMP dependent protein kinase (PKA). We conclude that Iso increases the propensity of RyRs to open, both in response to rapid elevations of $[Ca^{2+}]_i$ and at diastolic $[Ca^{2+}]_i$. While this could be beneficial in enhancing and synchronizing systolic whole-cell SR Ca^{2+} release, the same behaviour could also be proarrhythmogenic during diastole.

(Received 22 September 2009; accepted after revision 3 November 2009; first published online 9 November 2009)
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Abbreviations β-AR, β-adrenergic receptor; $[Ca^{2+}]_{SR}$, intra-SR Ca^{2+} concentration; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase; CICR, Ca^{2+}-induced Ca^{2+} release; EC, excitation–contraction; FDHM, full duration at half-maximum; I_{Ca}, L-type Ca^{2+} current; I_{NCX}, Na^+–Ca^{2+} exchange current; Iso, isoproterenol; P_o, open probability; PKA, cAMP-dependent protein kinase; PLB, phospholamban; NCX, Na^+–Ca^{2+} exchanger; RyR, ryanodine receptor; SERCA, sarcoplasmic reticulum Ca^{2+}-ATPase

Introduction

In cardiac muscle, the Ca^{2+} release channel, or ryanodine receptor (RyR), plays a central role in excitation–contraction (EC) coupling. Activated by the Ca^{2+} that enters the cardiac myocyte through voltage-gated L-type Ca^{2+} channels during the action potential, clusters of RyRs throughout the cell respond by releasing substantial amounts of Ca^{2+} from intracellular Ca^{2+} stores, the sarcoplasmic reticulum (SR). This amplification process, referred to as Ca^{2+}-induced Ca^{2+} release (CICR) (Fabiato, 1983), elevates cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) to the levels required to ensure cell contraction during systole.

Multiple targets involved in cardiac EC coupling are subject to regulation during exercise or stress, and respond synergistically to transient activation of the sympathetic nervous system by enhancing myocyte Ca^{2+} cycling and contraction (Hussain & Orchard, 1997; for review see Bers, 2002). Catecholamine-mediated activation of β-adrenergic receptors (β-AR) in the heart stimulates adenylate cyclase to produce cAMP, which increases the activation of cAMP-dependent protein kinase (PKA). PKA phosphorylation of L-type Ca^{2+} channels enhances Ca^{2+}

DOI: 10.1113/jphysiol.2009.181800

current (I_{Ca}) (Reuter & Scholz, 1977; Kameyama *et al.* 1985), providing a stronger trigger for SR Ca^{2+} release as well as the source for an increased pool of intracellular Ca^{2+}. Furthermore, Ca^{2+} uptake by the SR Ca^{2+}-ATPase (SERCA) is favoured by PKA-mediated phosphorylation of phospholamban (PLB), which relieves its inhibition of SERCA, thus accelerating relaxation and increasing SR Ca^{2+} content (Lindemann *et al.* 1983).

The RyR is known to serve as a substrate for phosphorylation early upon β-AR stimulation of cardiac myocytes (Yoshida *et al.* 1992). The physiological relevance of RyR phosphorylation, however, has been intensely debated during the past years. In particular, the possibly higher propensity of clusters of RyRs to spontaneously release (or leak) Ca^{2+} from the SR during diastole during β-AR stimulation has received extensive attention, for its potential implication in arrhythmogenesis and in the progression of contractile dysfunction under conditions of sustained β-AR stimulation, such as during heart failure (Marx *et al.* 2000; reviewed in Wehrens *et al.* 2005 and George, 2008). The fact that the contribution of the phosphorylated RyR to the normal, physiological response to sympathetic activation in the healthy myocyte still remains poorly understood has rendered the reconciliation of findings on its role in cardiac disease particularly difficult.

A significant number of studies carried out on isolated systems have provided invaluable information in revealing a multitude of potential changes in RyR behaviour upon phosphorylation (Takasago *et al.* 1991; Witcher *et al.* 1991; Hain *et al.* 1995; Lokuta *et al.* 1995; Valdivia *et al.* 1995; Uehara *et al.* 2002; Carter *et al.* 2006). However, the divergence of existing findings raises questions about the extrapolation to its role in modulating EC coupling and diastolic SR Ca^{2+} leak during β-AR stimulation of intact cells. Thus, the importance of studying RyR function during β-AR stimulation in its native environment is becoming increasingly evident, where accessory proteins and coupled gating between RyRs within a cluster may be essential in conferring physiological Ca^{2+} sensitivity to SR Ca^{2+} release (Marx *et al.* 2001; Gyorke *et al.* 2004). However, the altered Ca^{2+} sensitivity of the phosphorylated RyR as anticipated from observations in isolated systems has proven difficult to study in intact cardiomyocytes, mainly due to the aforementioned accompanying alterations in SR Ca^{2+} content and I_{Ca}, as well as in diastolic $[Ca^{2+}]_i$, with RyR open probability (P_o) being particularly sensitive to the Ca^{2+} concentration inside the SR ($[Ca^{2+}]_{SR}$) (Gyorke & Gyorke, 1998).

Previous studies in whole-cell voltage-clamped ventricular myocytes have shown that β-AR stimulation may synchronize triggered release of Ca^{2+} from the SR, increase SR Ca^{2+} release flux and thus the rate of whole-cell SR Ca^{2+} release (Song *et al.* 2001; Ginsburg & Bers, 2004). Furthermore, we have recently reported on stimulation of subcellular, local CICR in quiescent ventricular myocytes during β-AR stimulation in response to highly localized elevations of cytosolic $[Ca^{2+}]_i$ after two-photon excitation-induced Ca^{2+} liberation from caged Ca^{2+} (Lindegger & Niggli, 2005). Findings from studies on SR Ca^{2+} leak, manifested as local elevations of $[Ca^{2+}]_i$ resulting from the spontaneous release of Ca^{2+} from the SR through a single cluster of RyRs (Cheng *et al.* 1993), are conflicting, however. To date, observations on either increased amplitude or increased frequency of these elementary Ca^{2+} signalling events (Ca^{2+} sparks) during β-AR stimulation of intact ventricular myocytes or addition of cAMP to permeabilized cells, have been reported (Gomez *et al.* 1996; Tanaka *et al.* 1997; Li *et al.* 2002).

To our knowledge, no previous study performed on intact cells addressed the modulation of spontaneous SR Ca^{2+} release during β-AR stimulation under conditions of matched SR Ca^{2+} content and comparable diastolic $[Ca^{2+}]_i$, and how it translates to whole-cell CICR under similar experimental conditions. Therefore, we tested the hypothesis that if acute β-AR stimulation alters the Ca^{2+} sensitivity of the RyR, this should be reflected in the behaviour of whole-cell CICR and in spontaneous, Ca^{2+} spark-mediated SR Ca^{2+} leak. We studied SR Ca^{2+} release before and during β-AR stimulation by isoproterenol (Iso) under matched SR Ca^{2+} loading both in response to a rapid, artificial elevation of $[Ca^{2+}]_i$ that is invariant to β-AR stimulation, as well as at diastolic $[Ca^{2+}]_i$ under nearly identical experimental conditions. We found that clusters of RyRs exhibited a significantly higher propensity to open spontaneously in Iso, manifested as an elevation in SR Ca^{2+} leak that was likely to have been mediated by Ca^{2+}/calmodulin-dependent protein kinase, CaMKII, rather than PKA. This apparent increase in Ca^{2+} sensitivity of the RyR was also reflected in higher spatiotemporal synchronization of whole-cell CICR during β-AR stimulation, resulting in accelerated release kinetics and moderate increase in the amplitude of SR Ca^{2+} release. These results have been presented in preliminary form to the Biophysical Society (Ogrodnik & Niggli, 2009).

Methods

Isolation of guinea-pig ventricular myocytes

Single cardiac ventricular myocytes were isolated from adult male guinea-pigs according to established enzymatic procedures (Mitra & Morad, 1985). All animal handling procedures were performed with the permission of the State Veterinary Administration and according to Swiss Federal Animal protection law. All animal experimentation in the present study complies with

journal policies and regulations (Drummond, 2009). Guinea-pigs were killed by cervical dislocation. The hearts were rapidly excised and mounted on a Langendorff column by cannulation of the aorta for retrograde perfusion at 37°C for ∼5 min with a Ca^{2+}-free solution containing (in mM): 135 NaCl, 5.4 KCl, 1 $MgCl_2$, 0.33 NaH_2PO_4, 5 Hepes, 11 glucose, pH 7.3 (NaOH adjusted). Hearts were digested by subsequent addition of collagenase type II (0.5 mg ml^{-1}, Worthington, Switzerland) and protease type XIV (0.1 mg ml^{-1}, Sigma, Switzerland) to the perfusion solution for another 5–7 min. After digestion, the atria were removed and the ventricles transferred to a solution containing additionally 200 μM Ca^{2+}, where they were minced into small pieces. Single cardiac myocytes were liberated by gentle trituration of the digested ventricular tissue. Cells were washed and the Ca^{2+} concentration was progressively raised to 1 mM within ∼30 min. The cell suspension was placed on a gently rotating shaker, and stored at room temperature (21–22°C) until use, within 8 h after isolation.

Experimental solutions

A sample of cells was transferred to an experimental chamber mounted on the stage of an inverted microscope (Diaphot TMD, Nikon, Küsnacht, Switzerland). Individual cells were continuously superfused with an extracellular solution containing (in mM): 140 NaCl, 5 KCl, 1.8 $CaCl_2$, 1 CsCl, 0.5 $BaCl_2$, 10 Hepes, 10 glucose, pH 7.4 (NaOH adjusted). In some experiments, a Na^+- and Ca^{2+}-free solution (0 Na^+, 0 Ca^{2+}) with similar composition was used, in which Li^+ was substituted for Na^+, and Ca^{2+} was omitted in exchange for 1 mM EGTA. In this solution, pH was adjusted with LiOH. A custom-made, gravity-driven superfusion system allowed for rapid switching (half-time of solution exchange, $t_{1/2} < 500$ ms) to or between superfusates containing additionally 1 μM isoproterenol ((–)-*N*-iso-propyl-L-noradrenaline hydrochloride, Iso) for β-AR stimulation, 10 mM caffeine for emptying of the SR, 5 μM H-89 for PKA inhibition or 5 μM KN-93 for CaMKII inhibition. Solutions containing Iso were prepared each experimental day from a fresh aliquot of a 10 mM stock in water, and solutions containing H-89 and KN-93 from 10 mM stocks in DMSO. Iso, caffeine, H-89 and KN-93 were from Sigma, Switzerland. Voltage-clamped cells were dialysed with a pipette solution containing (in mM): 120 caesium glutamate, 20 TEA-Cl, 20 Hepes, 5 K_2-ATP, 2 Na_4-DM-nitrophen (Calbiochem, La Jolla, CA, USA), 2 reduced glutathione (GSH), 0.5 $CaCl_2$, 0.1 K_5-fluo-3 (Biotium, Hayward, CA, USA), pH 7.20 (CsOH adjusted). All experiments were carried out at room temperature (21–22°C).

Electrophysiological recordings

Electrodes were pulled from filamented borosilicate glass capillaries (BF150-86-7.5, Sutter Instrument Company, Novato, CA, USA) on a horizontal puller (DMZ, Zeitz Instrumente, Augsburg, Germany) to a final inner tip diameter of 1–2 μm (series resistance 2–3 MΩ). Guinea-pig ventricular myocytes were voltage-clamped in the whole-cell configuration of the patch-clamp technique and held at a resting potential of −80 mV using an Axopatch 200 amplifier (Axon Instruments, Union City, CA, USA). The liquid junction potential for the described solution compositions was estimated to be around −15 mV (calculated using Patcher's Power Tools, Dr F. Mendez and F. Würriehausen, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany), and was not corrected for. After establishment of the whole-cell configuration, cells were allowed at least 5 min for dialysis to ensure equilibration with the intracellular solution. Following initial emptying of the SR by rapid application of 10 mM caffeine, the extent of SR Ca^{2+} reloading was controlled with a train of varying number of depolarizing steps of 200 ms duration to 0 mV at 0.5 Hz, to activate inward I_{Ca} carried by plasmalemmal voltage-gated L-type Ca^{2+} channels. SR Ca^{2+} content and changes therein were assessed from the integrated Na^+–Ca^{2+} exchange current (I_{NCX}), in addition to the Ca^{2+} transient amplitude accompanying rapid caffeine exposure. Currents were recorded with the use of custom-written data acquisition software developed in-house under Labview (National Instruments, Ennetbaden, Switzerland), running on an Apple Macintosh G3 computer. Recordings were digitized at a sampling frequency of 1.5–3 kHz using an A/D converter, and electrophysiological data were stored on hard disk for off-line analysis using IgorPro software (WaveMetrics, Lake Oswego, OR, USA).

Confocal Ca^{2+} imaging and whole-cell Ca^{2+} uncaging

Cells were loaded with the Ca^{2+}-sensitive fluorescent indicator fluo-3 by dialysis through the recording pipette and imaged with a 40× oil-immersion objective lens (Fluor, N.A. 1.3, Nikon). Fluo-3 was excited at $\lambda = 488$ nm with an optically pumped semiconductor laser (Sapphire 488-10, Coherent, Santa Clara, CA, USA) attenuated to 50 μW measured at the back plane of the objective. Fluorescence was detected at $\lambda > 515$ nm with a laser-scanning confocal microscope (MRC 1000, Bio-Rad, Glattbrugg, Switzerland) operating in the imaging (x,y) or the line-scan (x,t) mode (6 ms per line). Line-scans of 1024 lines were collected in two equal, sequential segments (each image 384 × 512 pixels). In Ca^{2+} uncaging experiments, UV flashes from a xenon short-arc flash lamp (total energy discharge up to 230 J in ∼400 μs) were delivered through the objective in an epi-illumination

arrangement. UV flashes were synchronized with the pixel clock of the line-scan image acquisition system, and applied during the laser-scan retrace to elicit rapid, artificial, and spatially homogeneous elevations in $[Ca^{2+}]_i$ by photolysis of DM-nitrophen to induce whole-cell Ca^{2+} release from the SR. UV flash energy was set to 100–110 J in order to reliably trigger moderate whole-cell SR Ca^{2+} release while at the same time minimize photoconsumption of DM-nitrophen. Acquired fluorescence line-scan images were processed and transformed into pseudo-ratiometric images off-line using a customized version of Image SXM software (Barrett, 2002), from which temporal Ca^{2+} transient profiles were extracted. Amplitudes and time courses of cytosolic Ca^{2+} transients were analysed after normalization to resting fluorescence levels (F/F_0) in IgorPro software. Time constants (τ) were extracted from monoexponential fits to UV flash-induced Ca^{2+} transient decays. Maximal rate of SR Ca^{2+} release ($d[Ca^{2+}]_{max}\, dt^{-1}$) and time to peak were calculated starting from the 3rd scan line of the upstroke of UV flash-induced Ca^{2+} transient profiles. In some Ca^{2+} uncaging experiments, the UV flash-induced Ca^{2+} transients were clearly characterized by a biphasic upstroke in $[Ca^{2+}]_i$ elevation, the initial (fast) phase corresponding to the very rapid uncaging of Ca^{2+} from DM-nitrophen (2 scan lines), and the subsequent (slow) phase to SR Ca^{2+} release. In these experiments, the amplitude of the initial (fast) phase was used to quantitatively verify the stability of the elevation in $[Ca^{2+}]_i$ induced by photolysis of DM-nitrophen (see Fig. 3*Fa* and *G*). In addition, in some Ca^{2+} uncaging experiments, the stability of the photolytic trigger was quantitatively assessed from the corresponding Ca^{2+} transients recorded in the constant presence of 10 mM caffeine, acquired in the beginning as well as at the end of the experimental protocol (see Fig. 3*Fb*). SR Ca^{2+} content and changes therein were assessed from peak Ca^{2+} transient amplitude accompanying rapid caffeine exposure, in addition to the integrated I_{NCX} as mentioned previously. Spontaneous SR Ca^{2+} release was analysed using ImageJ software (W. Rasband, National Institute of Mental Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij), in the SparkMaster plug-in (Picht *et al.* 2007). Under our experimental conditions, SR Ca^{2+} release events composed of multiple Ca^{2+} sparks (i.e. macrosparks or wavelets), as identified by their spatiotemporal characteristics, were frequent during β-AR stimulation with Iso. For simplicity, these SR Ca^{2+} release events were not separated into individual Ca^{2+} sparks, but were quantified as one single SR Ca^{2+} release event, as detected by the SparkMaster plug-in. Ca^{2+} spark amplitudes were not compared due to (1) the very rare occurrence of Ca^{2+} sparks in control, (2) frequent macrosparks in Iso and (3) minor, albeit existent differences in diastolic $[Ca^{2+}]_i$ under whole-cell voltage-clamp conditions after rest and after SR Ca^{2+} reloading protocols.

Expression of results and statistical analysis

Statistical analysis was performed with IgorPro software. All results, absolute values or relative to corresponding control values in percent, are expressed as means ± standard error of the mean (S.E.M.). Values were compared for significance using Student's *t* test. Statistical significance (*) was assumed for *P* values less than 0.01.

Results

β-AR stimulation increases Ca^{2+} transients by enhancing I_{Ca} and increasing SR Ca^{2+} content

It is well established that β-AR stimulation of ventricular myocytes acutely enhances I_{Ca} through voltage-gated L-type Ca^{2+} channels, thus increasing the main trigger for physiological SR Ca^{2+} release (Callewaert *et al.* 1988; Hussain & Orchard, 1997). Following a given (identical) preconditioning protocol, a depolarizing step of 200 ms duration (from −40 mV to 0 mV) will activate larger I_{Ca}, which reaches steady amplitude after 2 min 30 s of β-AR stimulation with Iso. This leads to an increase in CICR and, in consequence, a larger cytosolic Ca^{2+} transient (Fig. 1*A*). Stimulation of I_{Ca} also provides the source for an increased pool of intracellularly cycling Ca^{2+}. Together with the accompanying stimulation of SERCA activity, progressive accumulation of Ca^{2+} within the SR following each depolarization during β-AR stimulation is favoured, until steady-state is reached. Thus, SR Ca^{2+} release triggered by rapid, UV flash-induced Ca^{2+} uncaging from DM-nitrophen, a trigger that is invariant to β-AR stimulation, will also result in a larger cytosolic Ca^{2+} transient in Iso (Fig. 1*B*). The elevated SR Ca^{2+} content, as confirmed with rapid application of 10 mM caffeine, will increase CICR independently of any functional modification of the RyR resulting from β-AR stimulation. This is due to the direct dependence of RyR P_o on $[Ca^{2+}]_{SR}$ (Gyorke & Gyorke, 1998), as well as the larger driving force resulting from the higher gradient in $[Ca^{2+}]$ between the SR and the cytosol (Kettlun *et al.* 2003). A pronounced modulation of SR Ca^{2+} release during acute β-AR stimulation induced by other targets could therefore mask a subtle change resulting from altered RyR Ca^{2+} sensitivity.

SR Ca^{2+} content can be matched during β-AR stimulation by adaptation of the preconditioning protocol

In order to discern a difference in RyR Ca^{2+} sensitivity during β-AR stimulation, SR Ca^{2+} release would thus not only have to be studied in response to an invariant

cytosolic trigger or similar diastolic $[Ca^{2+}]_i$, but also under conditions of strictly matched SR Ca^{2+} content. Therefore, we investigated how SR Ca^{2+} content can be controlled under our experimental conditions. Rapid application of 10 mM caffeine was used to estimate SR Ca^{2+} content, but also to reset SR Ca^{2+} content from previous history. Subsequent activation of I_{Ca} was used to control the extent of SR Ca^{2+} reloading with a variable train of depolarizing steps. Initially, we estimated steady-state SR Ca^{2+} content in control conditions immediately after reloading with a train of 20 depolarizing steps of 200 ms duration to 0 mV at 0.5 Hz, following initial emptying. During β-AR stimulation with Iso, the SR was successively emptied and its content reassessed after reloading with one, two and four identical depolarizing steps (Fig. 2*A*). Figure 2*B* shows the acquired line-scan image of the caffeine-induced Ca^{2+} release (Fig. 2*Ba*), the Ca^{2+} transient profile (Fig. 2*Bb*), the accompanying I_{NCX} (Fig. 2*Bc*) and its integral, $\int I_{NCX}$ (Fig. 2*Bd*), respectively. From each cell, the Ca^{2+} transient amplitude ($\Delta F/F_0$) and the integral of the I_{NCX} accompanying rapid caffeine exposure were normalized to control steady-state SR Ca^{2+} content, and the relative loading conditions in Iso are shown in Fig. 2*C*. On average, SR reloading with 2 depolarizing steps in Iso best matched steady-state SR Ca^{2+} content in control (caffeine-induced $\Delta F/F_0$: 104.8 ± 11.0% and $\int I_{NCX}$: 106.0 ± 12.1% of control, respectively). In some cells, however, one or even four depolarizing steps resulted in a closer match. Therefore, in subsequent experiments, whole-cell CICR and spontaneous SR Ca^{2+} leak were studied following an adapted preloading protocol with 1–3 depolarizing steps in Iso to match steady-state SR Ca^{2+} content in control. Comparable SR Ca^{2+} content in Iso was confirmed in each experiment, and only the recording in Iso with the closest match was compared to control from each cell.

UV flash-induced SR Ca^{2+} release is increased during β-AR stimulation at matched SR Ca^{2+} content

Whole-cell SR Ca^{2+} release was induced by UV flash-photolysis of DM-nitrophen following loading of the SR to steady-state in control, and following an adapted preloading protocol during β-AR stimulation with Iso. Rapid application of 10 mM caffeine 2 s after the UV flash in both control and Iso was used to verify comparable SR Ca^{2+} content (Fig. 3*A*). SR Ca^{2+} release was small to moderate in all cells, as judged by the relative amplitude of the UV flash-induced to the caffeine-induced Ca^{2+} transient amplitude. Figure 3*B* shows that whole-cell SR Ca^{2+} release was higher in Iso compared to control, despite similar SR Ca^{2+} content, as confirmed by the amplitude of the caffeine-induced Ca^{2+} transient (Fig. 3*C*). Statistical analysis reveals a significant stimulation of whole-cell SR Ca^{2+} release in Iso ($\Delta F/F_0$: 123.4 ± 6.3% of control, Fig. 3*D*). Moreover, whole-cell release kinetics were also significantly accelerated in Iso ($d[Ca^{2+}]_{max}\, dt^{-1}$: 171.6 ± 17.0% of control, Fig. 3*E*). The amplitude of the initial (rapid) phase of UV flash-induced Ca^{2+} transients confirmed that the photolytical trigger for whole-cell SR Ca^{2+} release was comparable in control and Iso ($\Delta F/F_0$: 102.9 ± 2.9% of control, Fig. 3*Fa* and *G*), as did UV flash-induced Ca^{2+} transients recorded in the constant presence of 10 mM caffeine (Fig. 3*Fb*). Furthermore, Fig. 3*H* shows that SR Ca^{2+} content was reliably matched in these experiments (caffeine-induced $\Delta F/F_0$: 102.1 ± 3.6% and $\int I_{NCX}$: 96.4 ± 9.1% of control, respectively). These results support the notion of increased SR Ca^{2+} release flux and/or improved temporal

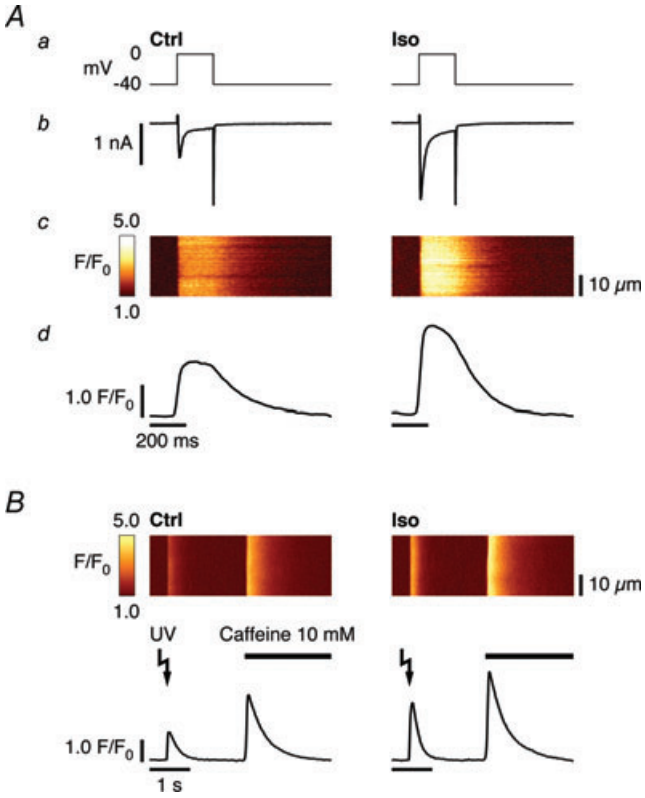

Figure 1. Iso increases Ca^{2+} transients by enhancing I_{Ca} and elevating SR Ca^{2+} content
A, depolarizing step (200 ms, from −40 mV to 0 mV, *a*), recorded current (*b*), acquired line-scan image (*c*) and average temporal Ca^{2+} transient profile (*d*). Compared to control (left), 2 min 30 s of β-AR stimulation with 1 μM Iso activates larger I_{Ca} and increases CICR, resulting in a larger cytosolic Ca^{2+} transient (right). *B*, whole-cell SR Ca^{2+} release in response to UV flash-induced Ca^{2+} uncaging from DM-nitrophen, following identical preconditioning in control (left) and Iso (right), recorded in a different cell. Stimulation of SERCA activity in Iso leads to more SR Ca^{2+} loading (confirmed with rapid application of 10 mM caffeine), and a larger cytosolic UV flash-induced Ca^{2+} transient, and may mask direct functional modulation of the RyR resulting from β-AR stimulation.

synchronization of triggered elementary SR Ca^{2+} release events, and also confirm the expected stimulation of SERCA activity, as whole-cell decay kinetics of the Ca^{2+} transient were significantly accelerated in Iso as well (τ: 202.4 ± 19.0 ms in control to 165.8 ± 12.4 ms in Iso, Fig. 3*I*, visible in Fig. 3*B*).

Increased spatial synchronization of whole-cell SR Ca^{2+} release during β-AR stimulation is revealed at near-threshold triggers

Enhanced whole-cell release kinetics suggest a higher RyR P_o, possibly as a result of increased Ca^{2+} sensitivity that

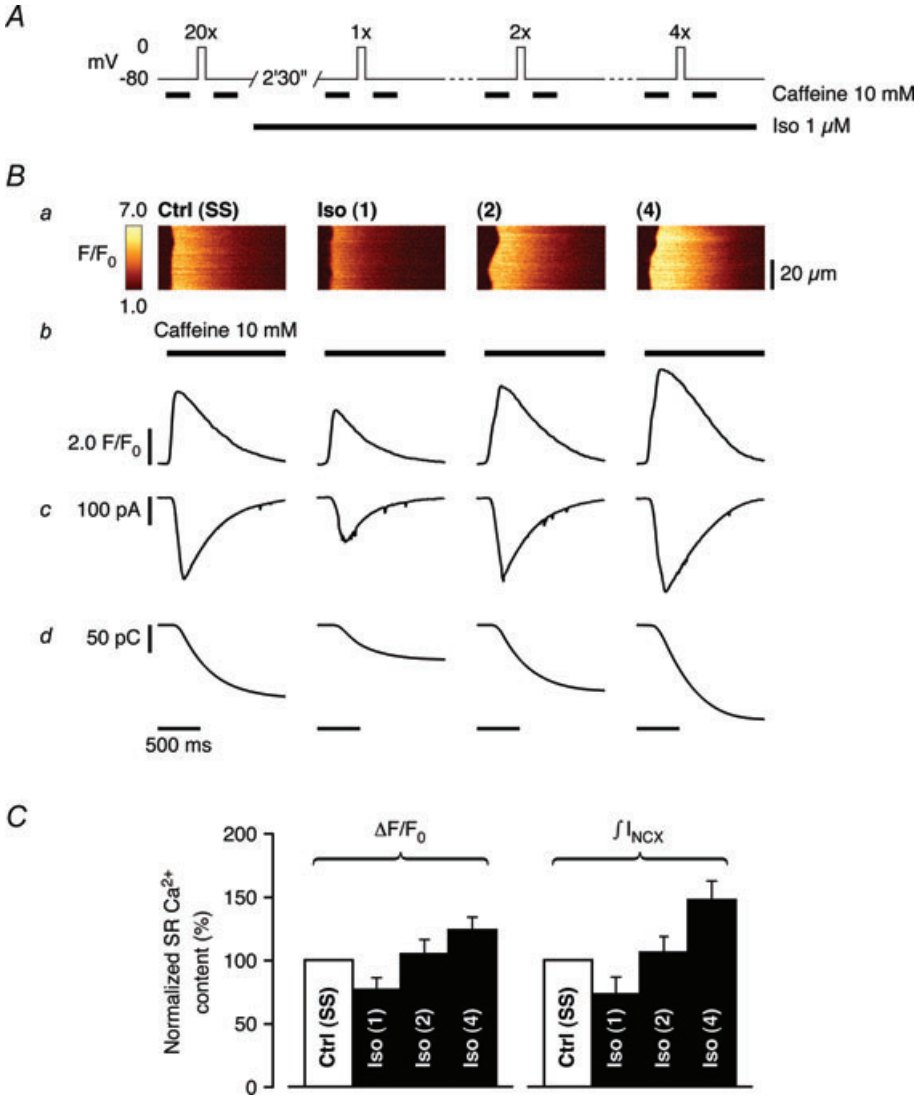

Figure 2. SR Ca^{2+} content can be matched in Iso by adaptation of the preconditioning protocol
A, adjusting SR Ca^{2+} loading under our experimental conditions. Following initial emptying with caffeine, SR Ca^{2+} content was assessed in control after reloading to steady-state (SS) with 20 depolarizing steps (200 ms, from −80 mV to 0 mV, 0.5 Hz). In Iso, the SR was successively emptied and its content reassessed with caffeine after reloading with 1, 2 and 4 depolarizing steps. *B*, acquired line-scan images of caffeine-induced Ca^{2+} release after the number of depolarizing steps indicated in *A* (*a*), corresponding Ca^{2+} transient profiles (*b*), accompanying I_{NCX} (*c*) and their integrals (*d*). As expected, SR Ca^{2+} content increased with the number of preloading steps in Iso. *C*, relative SR loading conditions normalized to control, as estimated from the amplitude of the Ca^{2+} transient ($\Delta F/F_0$) and integral of the I_{NCX} ($\int I_{NCX}$) accompanying rapid caffeine exposure after 1, 2 and 4 loading steps. On average, SR reloading with 2 depolarizing steps in Iso best matched SR Ca^{2+} content in steady-state in control ($\Delta F/F_0$: 104.8 ± 11.0% and $\int I_{NCX}$: 106.0 ± 12.1% of control, $n = 5$ cells), although in some cells 1 or even 4 depolarizing steps yielded a closer match.

could underlie synchronized opening of more RyRs or clusters of RyRs. Furthermore, increased single-channel conductance and/or gating properties of the RyR or clusters of RyRs would in principle also result in accelerated whole-cell release kinetics. In some Ca^{2+} uncaging experiments described in Fig. 3, the UV flash-induced Ca^{2+} transients were clearly characterized by a biphasic upstroke in $[Ca^{2+}]_i$ elevation, and spatial inhomogeneities in the Ca^{2+} transients were also evident in these cells. The initial (fast) phase corresponds to the very rapid, spatially homogeneous uncaging of Ca^{2+} from DM-nitrophen, and the subsequent (slower) phase to SR Ca^{2+} release, which in these cells is thought to occur near threshold of CICR (Lipp & Niggli, 1996). To further investigate the behaviour of this near-threshold CICR, we performed a detailed analysis of the release kinetics on the subcellular level in 1 of 4 cells where the Ca^{2+} transients in control and Iso were characterized by a clear biphasic upstroke and spatial inhomogeneities. Figure 4*A* shows that release kinetics markedly differ throughout the cell in control, as indicated by the temporal Ca^{2+} transient profiles from three different (subcellular) regions. SR Ca^{2+}

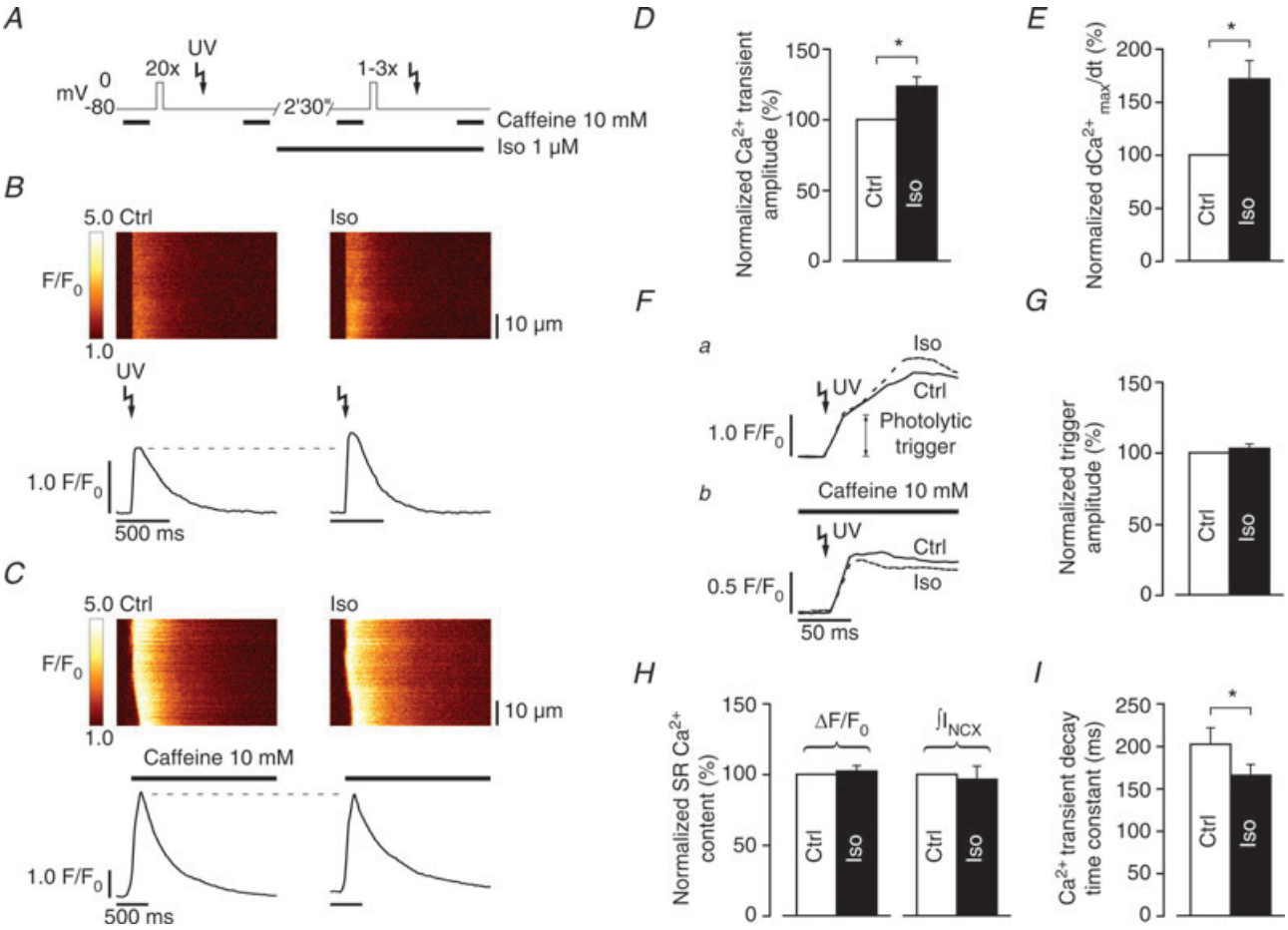

Figure 3. UV flash-induced SR Ca^{2+} release is increased in Iso at matched SR Ca^{2+} content
A, whole-cell SR Ca^{2+} release induced by UV flash-photolysis of DM-nitrophen in control (SR loaded to steady-state), and again following an adapted preloading protocol (1–3 preloading steps) in Iso. Rapid application of 10 mM caffeine 2 s after the UV flash in both control and Iso was used to verify comparable SR Ca^{2+} content. *B*, whole-cell SR Ca^{2+} release was higher in Iso (right) compared to control (left), despite similar SR Ca^{2+} content (*C*). On average, whole-cell SR Ca^{2+} release was significantly stimulated to $\Delta F/F_0$: 123.4 ± 6.3% of control ($n = 8$ cells, *D*). *E*, Iso accelerated whole-cell SR Ca^{2+} release kinetics ($d[Ca^{2+}]_{max}\ dt^{-1}$: 171.6 ± 17.0% of control, $n = 8$ cells). *F*, the amplitude of the initial (rapid) phase of UV flash-induced Ca^{2+} transients (*a*) confirmed that the photolytical trigger for whole-cell SR Ca^{2+} release was comparable in control and Iso, as did UV flash-induced Ca^{2+} transients recorded in the constant presence of 10 mM caffeine (*b*, data from another cell). *G*, average amplitude of the photolytical trigger extracted from the biphasic upstroke of UV flash-induced Ca^{2+} transients ($\Delta F/F_0$: 102.9 ± 2.9% of control, $n = 4$ cells). *H*, SR Ca^{2+} content was reliably matched in these experiments (caffeine-induced $\Delta F/F_0$: 102.1 ± 3.6% and $\int I_{NCX}$: 96.4 ± 9.1% of control, $n = 8$ cells). *I*, Iso also accelerated whole-cell Ca^{2+} transient decay kinetics (τ: 202.4 ± 19.0 ms in control to 165.8 ± 12.4 ms in Iso, $n = 8$ cells) (*$P < 0.01$).

release in Iso in the same cell, however, exhibited an obvious spatial synchronization, as reflected by the peaks of the corresponding three subcellular Ca^{2+} transient profiles. Therefore, we divided the line-scan images in control and Iso into 20 equal parts (each 1.8 μm wide), of which the temporal characteristics were analysed (Fig. 4*B*). Figure 4*C* shows the distributions of the time to peak of the Ca^{2+} transients from the 20 subcellular regions in control and Iso. Subcellular Ca^{2+} transients with time to peak in successive groups of two consecutive pixels were binned together. The distributions reveal that the average time to peak throughout the cell was significantly shorter in Iso (time to peak: 99.3 ± 5.4 ms in control to 78.9 ± 3.0 ms in Iso), which could explain the higher maximal rate of whole-cell SR Ca^{2+} release described above. The time to peak throughout the cell was less spread in time in Iso, suggesting increased spatial synchronization of SR Ca^{2+} release over larger distances. This is also highlighted by the relative amplitudes of the representative (integral normalized) Gaussians superimposing the distributions in Fig. 4*C*. Moreover, the distribution in Iso is notably better represented by its corresponding Gaussian in contrast to control, where the subcellular distribution of time to peak appears spatially uncorrelated, indicating a possibly increased coupling between SR Ca^{2+} release from neighbouring RyR clusters during β-AR stimulation. The accelerated release and decay kinetics in Iso also resulted in shorter duration of the Ca^{2+} transient, as reflected in the significantly shorter average full duration at half-maximum (FDHM) amplitude throughout the

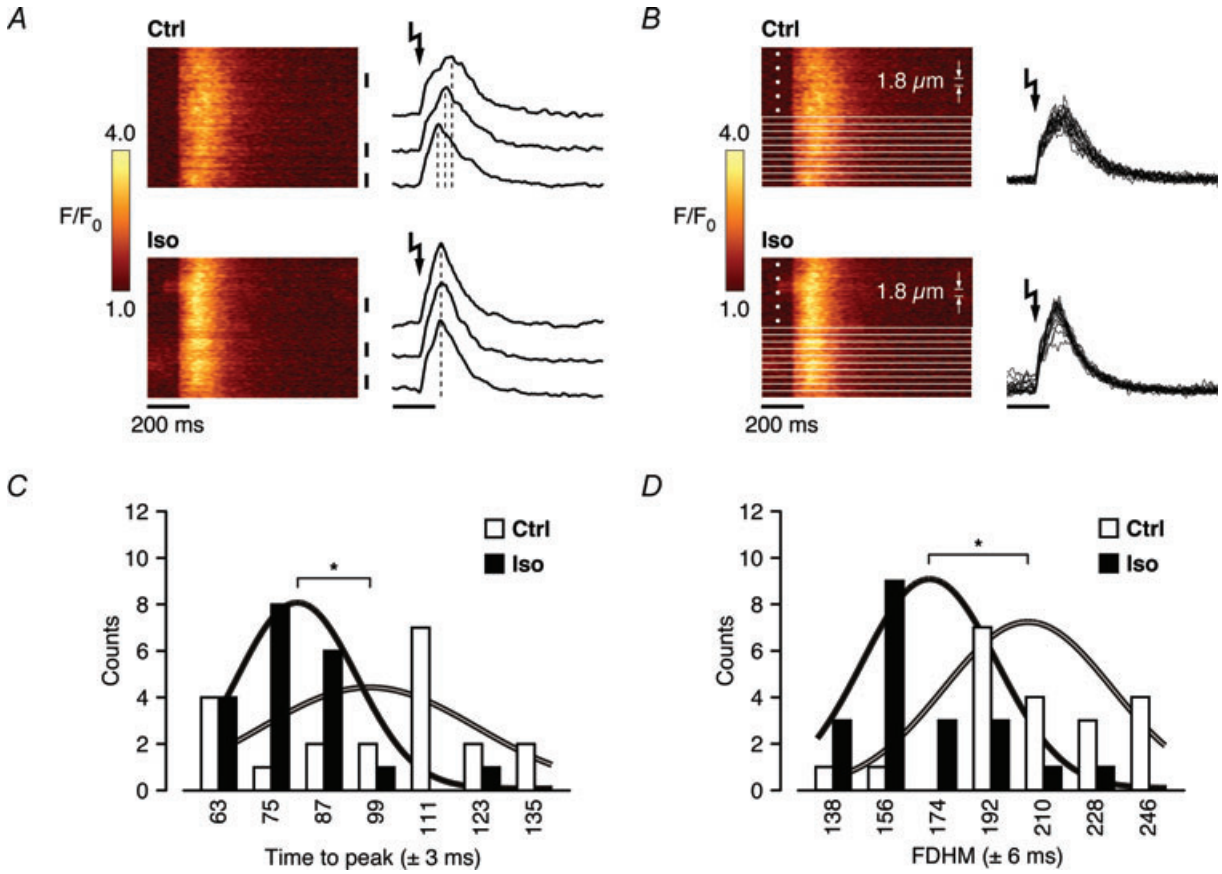

Figure 4. Increased spatial synchronization of whole-cell SR Ca^{2+} release in Iso is revealed at near-threshold triggers

A, near-threshold SR Ca^{2+} release kinetics exhibited clear spatial inhomogeneities in control, as indicated by the temporal Ca^{2+} transient profiles from 3 different (subcellular) regions. Iso synchronized SR Ca^{2+} release throughout the cell, as reflected by the coordinated peaks of the corresponding subcellular Ca^{2+} transient profiles. *B*, the line-scan images in control and Iso in *A* were divided into 20 equal parts (each 1.8 μm wide), on which a detailed analysis of the temporal characteristics was performed. *C*, the distribution of the time to peak in the subcellular regions revealed a significantly shorter average time to peak in Iso (99.3 ± 5.4 ms in control to 78.9 ± 3.0 ms in Iso), and was less spread in time throughout the cell (reflected by the relative widths and amplitudes of the representative, integral-normalized Gaussians). *D*, accelerated release and decay kinetics in Iso are also reflected in shorter average full duration at half-maximum (FDHM) amplitude throughout the cell (207.6 ± 6.3 ms in control to 168.0 ± 5.0 ms in Iso) (*$P < 0.01$).

cell (207.6 ± 6.3 ms in control to 168.0 ± 5.0 ms in Iso, Fig. 4*D*). The distributions of the FDHM of the Ca^{2+} transients from the 20 subcellular regions in control and Iso are shown in Fig. 4*D*, together with (integral normalized) Gaussians representing the distributions (subcellular Ca^{2+} transients with FDHM in successive groups of three consecutive pixels were binned together). These observations suggest that spatial synchronization throughout the cell contributes to an increased rate of whole-cell SR Ca^{2+} release and shorter time to peak of the cellular Ca^{2+} transient. β-AR stimulation thus reduces the duration of the Ca^{2+} transient as a consequence of both enhanced spatiotemporal summation of elementary SR Ca^{2+} release events and stimulation of SERCA activity.

Ca^{2+} sparks in guinea-pig ventricular myocytes

Accelerated SR Ca^{2+} release kinetics during β-AR stimulation resulting from increased spatiotemporal synchronization would reflect a higher propensity of clusters of RyRs to open and contribute to the triggered opening of neighbouring clusters. The higher Ca^{2+} sensitivity of RyRs that would underlie this behaviour could thus also modulate the occurrence of elementary SR Ca^{2+} release events, Ca^{2+} sparks, during resting conditions. Although spontaneous Ca^{2+} sparks have been observed in myocytes from most mammalian hearts, few investigators have reported on Ca^{2+} sparks in guinea-pig ventricular myocytes, where their appearance is relatively rare. We studied the behaviour of Ca^{2+} sparks in guinea-pig ventricular myocytes, and how Ca^{2+} spark-mediated SR Ca^{2+} leak is altered upon β-AR stimulation with Iso, under conditions nearly identical to those in the experiments described in previous sections.

Ca^{2+} sparks are more frequent in quiescent guinea-pig ventricular myocytes during β-AR stimulation

Figure 5 shows the occurrence of Ca^{2+} sparks in a voltage-clamped ventricular myocyte. Line-scan images were acquired from a quiescent cell after loading of the SR with Ca^{2+} to steady-state (Fig. 5*A*). In our experiments, Ca^{2+} sparks were relatively rare under control conditions (Fig. 5*Ba*). However, superfusion of quiescent cells with Iso (1 μM for 2 min 30 s) markedly increased the number of readily visible Ca^{2+} sparks (Fig. 5*Bb*). The occurrence of macrosparks (or wavelets), with spatiotemporal characteristics typical of SR Ca^{2+} release events composed of multiple Ca^{2+} sparks, was also frequent during β-AR stimulation with Iso. Moreover, the more frequent appearance of Ca^{2+} sparks in Iso persisted when the extracellular solution was exchanged for a solution without Na^+ or Ca^{2+} (0 Na^+, 0 Ca^{2+}) (Fig. 5*Bc*), indicating that the SR Ca^{2+} leak is spontaneous in the sense that the Ca^{2+} sparks are not triggered by Ca^{2+} influx through L-type Ca^{2+} channels, the NCX or any other pathway dependent on extracellular Ca^{2+} (or Na^+).

Spontaneous SR Ca^{2+} release is increased at matched SR Ca^{2+} content and comparable diastolic $[Ca^{2+}]_i$ during β-AR stimulation

Intracellular Ca^{2+} retention is favoured upon β-AR stimulation of quiescent, intact ventricular myocytes, due to the aforementioned accompanying stimulation of SERCA activity. Therefore, the marked increase in spontaneous Ca^{2+} spark frequency shown in Fig. 5 could be a result of elevated SR Ca^{2+} content during rest. To compare spontaneous SR Ca^{2+} release in control and

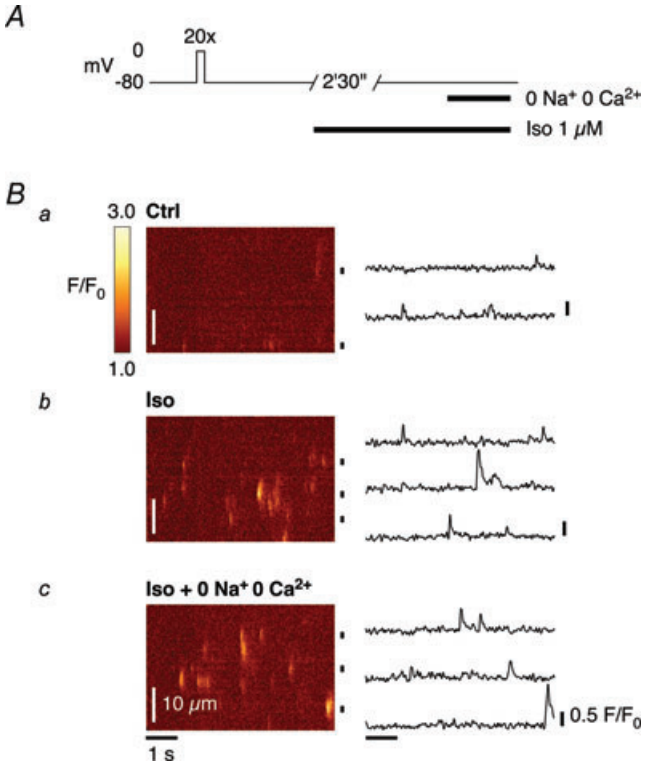

Figure 5. Ca^{2+} sparks are more frequent in quiescent guinea-pig ventricular myocytes in Iso
A, the occurrence of Ca^{2+} sparks in voltage-clamped ventricular myocytes was studied in quiescent cells, after loading of the SR with Ca^{2+} to steady-state in control conditions. *B*, Ca^{2+} sparks were relatively rare in control (*a*), but the number of readily visible Ca^{2+} sparks markedly increased during β-AR stimulation with 1 μM Iso for 2 min 30 s (*b*). Their appearance persisted when the extracellular solution was exchanged for a solution without Na^+ or Ca^{2+} (0 Na^+, 0 Ca^{2+}) (*c*), indicating that the SR Ca^{2+} leak is spontaneous and independent of extracellular Ca^{2+} (i.e. not triggered by any Ca^{2+} influx).

during β-AR stimulation at matched SR Ca^{2+} content, we recorded Ca^{2+} sparks in control cells immediately after reloading of the SR with Ca^{2+} to steady-state, and again in Iso following a preloading protocol identical to the one described in Fig. 3*A*. Analysis was performed on Ca^{2+} sparks during a 3 s long period starting 1.5 s after the final repolarization to −80 mV. Subsequent rapid application of 10 mM caffeine was used to verify comparable SR Ca^{2+} content (Fig. 6*A*). Figure 6*B* shows the occurrence of Ca^{2+} sparks after repolarization to the holding potential and complete decay of $[Ca^{2+}]_i$ to diastolic levels, and suggests that sparks were generally more frequent in Iso (a single spark in control to 4 in Iso in Fig. 6*B*). Statistical analysis revealed an ∼5-fold higher Ca^{2+} spark frequency in Iso ($0.92 \pm 0.34\ s^{-1}\ (100\ \mu m)^{-1}$ in control to $4.58 \pm 0.83\ s^{-1}\ (100\ \mu m)^{-1}$ in Iso, Fig. 6*C*), in recordings where the SR Ca^{2+} content was matched (caffeine-induced $\Delta F/F_0$: 100.8 ± 3.0% and $\int I_{NCX}$: 91.3 ± 3.8% of control, respectively, Fig. 6*D*). Furthermore, diastolic $[Ca^{2+}]_i$ was not significantly elevated in Iso in these experiments (110.0 ± 4.7% of control, not shown). The higher propensity for spontaneous SR Ca^{2+} release observed here could represent a significant SR Ca^{2+} leak during diastolic intervals.

Ca^{2+} spark frequency increases rapidly in quiescent cells during β-AR stimulation without significantly altering SR Ca^{2+} content

Increased SR Ca^{2+} leak during β-AR stimulation could also result in progressive net loss of Ca^{2+} from the cell during diastolic intervals through extrusion by the Na^+–Ca^{2+} exchanger (NCX). To address this issue, we designed an experimental protocol to investigate to what extent the observed increase in SR Ca^{2+} leak affects global SR Ca^{2+} content during rest, while simultaneously monitoring the time course of spontaneous SR Ca^{2+} release upon β-AR stimulation with Iso. The SR Ca^{2+} content at steady-state was initially assessed with caffeine under control conditions. Following reloading of the SR to steady-state, the cells were left to rest for 3 min, either for 30 s in control followed by 2 min 30 s in Iso, or 3 min in control (Fig. 7*A*). SR Ca^{2+} content was again assessed in both groups after the total resting period of 3 min, and compared to the one recorded initially. Line-scan images of quiescent cells were acquired every 15 s throughout the resting period. In Fig. 7*B*, a progressive, significant increase in Ca^{2+} spark frequency is clearly visible during superfusion with Iso (*a*, *b*′, *c*′), whereas the cells left to rest

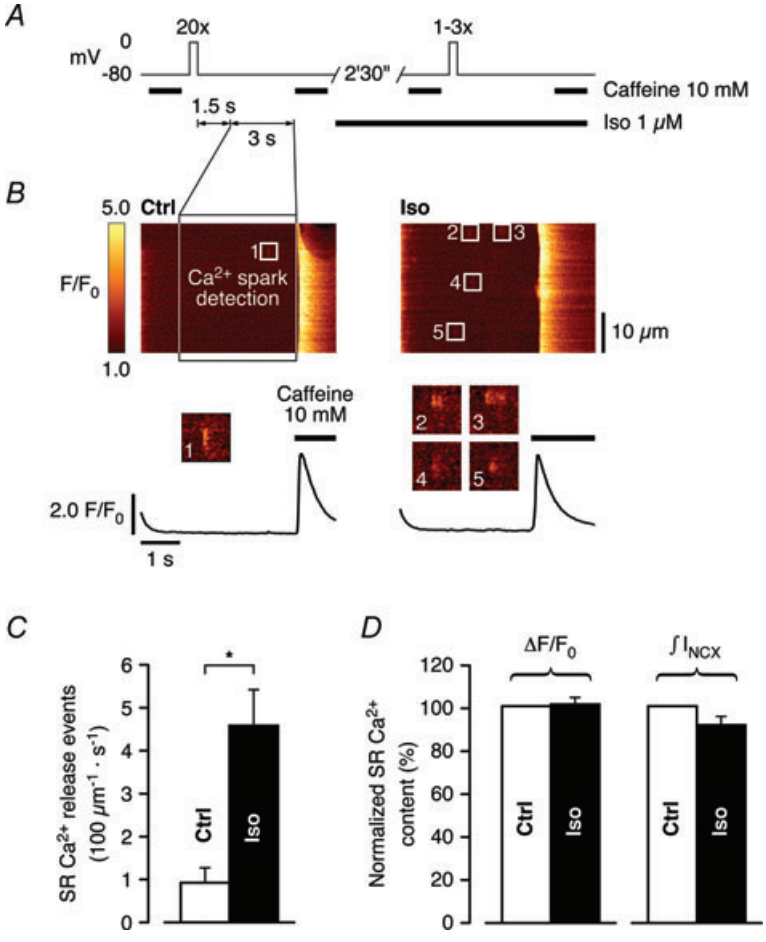

Figure 6. Spontaneous SR Ca^{2+} release is increased at matched SR Ca^{2+} content and comparable diastolic $[Ca^{2+}]_i$ in Iso
A, spontaneous SR Ca^{2+} release was compared in control and in Iso at matched SR Ca^{2+} content (cf. experimental protocol described in Fig. 3). Ca^{2+} sparks were recorded in control (SR loaded to steady-state), and again following an adapted preloading protocol (1–3 preloading steps) in Iso. Analysis was performed on Ca^{2+} sparks during a 3 s long period starting 1.5 s after the final repolarization to −80 mV. Subsequent rapid application of 10 mM caffeine was used to verify comparable SR Ca^{2+} content. *B*, Ca^{2+} sparks after repolarization and complete decay of $[Ca^{2+}]_i$ to diastolic levels were generally more frequent in Iso (a single spark in control, left, to 4 in Iso, right). *C*, on average, Ca^{2+} spark frequency increased ∼5-fold in Iso ($0.92 \pm 0.34\ s^{-1}\ (100\ \mu m)^{-1}$ in control to $4.58 \pm 0.83\ s^{-1}\ (100\ \mu m)^{-1}$ in Iso), in recordings where the SR Ca^{2+} content was matched to *D*, SR Ca^{2+} content was matched in these recordings (caffeine-induced $\Delta F/F_0$: 100.8 ± 3.0% and $\int I_{NCX}$: 91.3 ± 3.8% of control) ($n = 9$ cells, $^*P < 0.01$).

in control did not exhibit any obvious change in Ca^{2+} spark frequency during rest (*a*, *b*, *c*). The time course in Fig. 7*C* shows that the increase in Ca^{2+} spark frequency started after ~30 s in Iso, and reached a ~4-fold higher level after 2 min. In contrast, Ca^{2+} spark frequency did not increase in control cells during 3 min rest, but rather exhibited a tendency to decrease over time. Statistical analysis was performed on data pooled from the initial three points in time (in Fig. 7*C*) after reloading of the SR to steady-state (0–30 s in control), as well as on data pooled from the final three points from both groups after rest (2 min 30 s–3 min in control and 2 min–2 min 30 s in Iso). Ca^{2+} spark frequency was not significantly lower after rest in control (from $0.93 \pm 0.21\ s^{-1}$ $(100\ \mu m)^{-1}$ to $0.66 \pm 0.19\ s^{-1}$ $(100\ \mu m)^{-1}$). However, after rest in Iso, Ca^{2+} spark frequency increased from $1.05 \pm 0.13\ s^{-1}$ $(100\ \mu m)^{-1}$ to $4.08 \pm 0.47\ s^{-1}$ $(100\ \mu m)^{-1}$ (Fig. 7*D*). Figure 7*E* shows that SR Ca^{2+} content decreased significantly after rest in control (caffeine-induced $\Delta F/F_0$: $82.0 \pm 3.3\%$ and $\int I_{NCX}$: $81.4 \pm 4.7\%$ of initial SR Ca^{2+} content, respectively), whereas this loss of Ca^{2+} was more limited after rest in Iso (caffeine-induced $\Delta F/F_0$: $89.5 \pm 3.5\%$ and $\int I_{NCX}$:

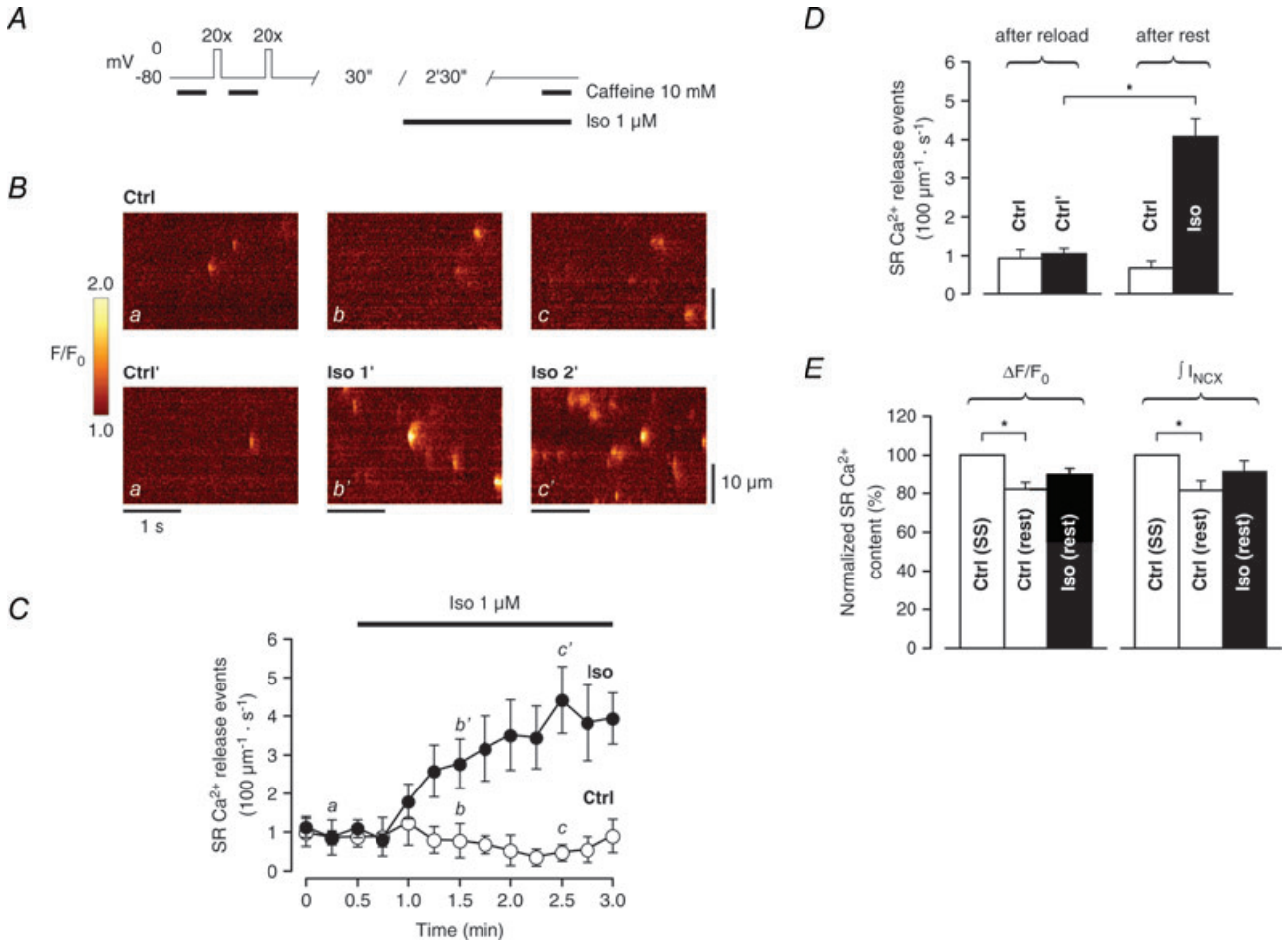

Figure 7. Ca^{2+} spark frequency increases rapidly in quiescent cells in Iso without significantly altering SR Ca^{2+} content

A, experimental protocol to study the time course of spontaneous SR Ca^{2+} release and how it affects SR Ca^{2+} content during rest. SR Ca^{2+} content was assessed with caffeine after loading of the SR with Ca^{2+} to steady-state in control. Following reloading of the SR to steady-state, cells were left to rest for 3 min (30 s in control + 2 min 30 s in Iso, or 3 min in control), after which SR Ca^{2+} content was again assessed with caffeine. *B* and *C*, a progressive increase in Ca^{2+} spark frequency appeared after ~30 s superfusion with Iso, reaching an ~4-fold higher level within 2 min (*a*, *b*′, *c*′). Cells in control did not exhibit any obvious change in Ca^{2+} spark frequency during rest (*a*, *b*, *c*). *D*, Ca^{2+} spark frequency was not significantly lower after rest in control ($0.93 \pm 0.21\ s^{-1}$ $(100\ \mu m)^{-1}$ to $0.66 \pm 0.19\ s^{-1}$ $(100\ \mu m)^{-1}$). After rest in Iso, Ca^{2+} spark frequency increased from $1.05 \pm 0.13\ s^{-1}$ $(100\ \mu m)^{-1}$ to $4.08 \pm 0.47\ s^{-1}$ $(100\ \mu m)^{-1}$; data were pooled from the initial 3 points and the final 3 points from both groups). *E*, SR Ca^{2+} content decreased significantly after rest in control (caffeine-induced $\Delta F/F_0$: $82.0 \pm 3.3\%$ and $\int I_{NCX}$: $81.4 \pm 4.7\%$ of initial SR Ca^{2+} content), whereas this loss of Ca^{2+} was more limited after rest in Iso (caffeine-induced $\Delta F/F_0$: $89.5 \pm 3.5\%$ and $\int I_{NCX}$: $91.4 \pm 5.5\%$ of initial SR Ca^{2+} content) (control: $n = 10$ cells, Iso: $n = 9$ cells, $^*P < 0.01$).

91.4 ± 5.5% of initial SR Ca^{2+} content, respectively). Moreover, diastolic $[Ca^{2+}]_i$ had a tendency to decrease in control after rest and slightly more in Iso, although the decrease was not significant in any group (94.0 ± 2.3% of initial diastolic $[Ca^{2+}]_i$ after rest in control to 91.5 ± 3.2% in Iso, not shown). Taken together, these results reveal that in control, guinea-pig ventricular myocytes behave as expected, exhibiting a rest decay of SR Ca^{2+} content with a tendency to parallel reduction of SR Ca^{2+} leak. However, despite the rapid, significant increase in Ca^{2+} spark frequency during rest in Iso, loss of Ca^{2+} from the cell through the NCX is limited, probably due to stimulation of SERCA activity. Higher SR Ca^{2+} content cannot explain the observed rapid increase in Ca^{2+} spark frequency in Iso, the time course of which is well in accordance with published data on the time course of RyR phosphorylation (Takasago *et al.* 1991; Yoshida *et al.* 1992), and could thus reflect a modulation of RyR P_o through phosphorylation.

Increased spontaneous SR Ca^{2+} release during β-AR stimulation is mediated by CaMKII rather than PKA

β-AR stimulation of cardiomyocytes activates dual signalling pathways, mediated by cAMP and Ca^{2+}/calmodulin-dependent protein kinases, PKA and CaMKII, respectively. While the cAMP/PKA pathway is known to undergo significant activation upon acute β-AR stimulation, it is unclear whether important activation of CaMKII occurs during the relatively short time frame of the experiments in the present study (Wang *et al.* 2004). Recent observations, however, suggest that CaMKII could potentially undergo significant activation during acute β-AR stimulation of quiescent cardiomyocytes, independently of the primary increase in Ca^{2+} cycling mediated by PKA (Curran *et al.* 2007). Therefore, we investigated whether the elevated Ca^{2+} spark frequency in Iso could be attributed to PKA or CaMKII. Using an experimental protocol similar to the one described in Fig. 7, we monitored the time course of spontaneous SR Ca^{2+} release during rest. Immediately after reloading of the SR to steady-state, the extracellular solution was exchanged for a solution containing additionally 5 μM of the widely used membrane-permeant inhibitors of PKA and CaMKII, H-89 or KN-93, respectively. The cells were left to rest for 3 min, initially for 30 s in H-89 or KN-93, followed by 2 min 30 s in H-89 or KN-93 together with 1 μM Iso. SR Ca^{2+} content was assessed with caffeine in both groups after the total resting period of 3 min, and compared to the steady-state SR Ca^{2+} content recorded initially. Interestingly, treatment with either H-89 or KN-93 almost completely suppressed the increase in Ca^{2+} spark frequency observed during rest in Iso (from 1.77 ± 0.36 s^{-1} (100 μm)$^{-1}$ in H-89 to 2.21 ± 0.57 s^{-1} (100 μm)$^{-1}$ in H-89 + Iso, n.s., and from 1.95 ± 0.44 s^{-1} (100 μm)$^{-1}$ in KN-93 to 2.44 ± 0.40 s^{-1} (100 μm)$^{-1}$ in KN-93 + Iso, n.s., Fig. 8*A*), supporting the hypothesis that downstream activation of these kinases could underlie the ∼4-fold increase again observed in Iso (from 1.32 ± 0.32 s^{-1} (100 μm)$^{-1}$ to 4.92 ± 0.56 s^{-1} (100 μm)$^{-1}$). However, during rest in H-89 treated cells, the sustained SR Ca^{2+} leak was accompanied by a dramatic loss of Ca^{2+} from the SR (caffeine-induced $\Delta F/F_0$: 65.4 ± 4.9% and $\int I_{NCX}$: 62.0 ± 6.5% of initial SR Ca^{2+} content). In contrast, in cells treated with KN-93, SR Ca^{2+} content rather exhibited a tendency to increase during rest, although this increase was not significant (caffeine-induced $\Delta F/F_0$: 108.8 ± 8.6% and $\int I_{NCX}$: 110.9 ± 11.9% of initial SR Ca^{2+} content). Similarly to the results presented in Fig. 7*E*, SR Ca^{2+} content was again unaffected after rest in Iso (caffeine-induced $\Delta F/F_0$: 99.6 ± 7.8% and $\int I_{NCX}$: 102.7 ± 7.4% of initial SR Ca^{2+} content, respectively). The comparable Ca^{2+} spark frequency despite much lower SR Ca^{2+} content in H-89 treated cells suggests a more likely implication of CaMKII, rather than PKA, in the stimulation of spontaneous SR Ca^{2+} release in Iso, which was normalized by KN-93 without significantly altering SR Ca^{2+} content.

Discussion

Cardiac hypertrophy and failure have been associated with abnormal cardiomyocyte Ca^{2+} handling (Gwathmey & Morgan, 1985; Beuckelmann *et al.* 1992; Gomez *et al.* 1997) that can be induced by chronic activation of the β-AR pathway (Engelhardt *et al.* 2004). Marx *et al.* provided a plausible mechanistic link between the hyperadrenergic state observed in the failing heart and spontaneously active RyRs, relating excessive PKA phosphorylation of the RyR to abnormal channel gating through altered interactions between the channel and accessory proteins (Marx *et al.* 2000). This postulate has recruited an ever increasing number of studies, many of which have severely challenged the proposed molecular and biochemical nature of RyR phosphorylation (target sites, kinase candidates, interactions with accessory proteins) and its functional impact in cardiac disease (for example Jiang *et al.* 2002; Ai *et al.* 2005; Xiao *et al.* 2005; Benkusky *et al.* 2007).

The intact cardiomyocyte: a complex Ca^{2+} signalling system

The understanding of the role played by RyR phosphorylation in pathogenesis has been considerably hindered by the fact that its physiological relevance during acute β-AR stimulation in the healthy myocyte still remains to be elucidated. Extensive attention has been devoted to resolving the physiological role of RyR phosphorylation in a multitude of studies carried out on isolated systems, cumulatively providing a

framework of potential changes that RyR behaviour could undergo upon phosphorylation. As previously mentioned, when addressed in intact ventricular myocytes, clear manifestation of alterations in the SR Ca^{2+} release mechanism of RyRs clustered in their native environment has proven difficult to discern from accompanying increases in SR Ca^{2+} content and I_{Ca}. Both exert a pronounced stimulation of SR Ca^{2+} release during β-AR stimulation that could mask a subtle change resulting from altered RyR Ca^{2+} sensitivity.

In the present study, we observed a significant stimulation of whole-cell SR Ca^{2+} release in Iso, induced by UV flash-photolysis of DM-nitrophen in voltage-clamped guinea-pig ventricular myocytes. Rapid Ca^{2+} uncaging from DM-nitrophen has the advantage of providing a stable trigger for CICR, while at the same time remaining absolutely invariant to β-AR stimulation. Thus, as SR Ca^{2+} content was strictly matched in these experiments, our observation suggests a modulation of the SR Ca^{2+} release mechanism triggered by rapid elevations in $[Ca^{2+}]_i$. Stimulatory regulation of single-channel or cluster gating properties, such as increased P_o (Ca^{2+} sensitivity), longer openings or higher single-channel conductance could all, in principle, underlie increased cellular SR Ca^{2+} release and higher rate of whole-cell SR Ca^{2+} release. Increased RyR P_o after PKA phosphorylation is supported by findings from several studies carried out in isolated systems, such as [^{3}H]ryanodine binding in cardiac microsomes (Takasago *et al.* 1989) and recordings of single channel activity of RyRs incorporated into planar lipid bilayers (Hain *et al.* 1995; Uehara *et al.* 2002; Carter *et al.* 2006). Interestingly, Valdivia *et al.* (1995) reported on more complex single-channel behaviour, where PKA phosphorylation induced a transiently higher RyR P_o with fast adaptation to lower steady-state P_o when activated by UV flash-photolysis of caged Ca^{2+}, implying a different gating mode of the phosphorylated RyR. In addition, there is evidence that the RyR can serve as a substrate for kinases other than PKA, particularly Ca^{2+}/calmodulin-dependent protein kinase (CaMKII). Sustained β-AR stimulation is believed to progressively increase CaMKII activity, and RyR phosphorylation by CaMKII has shown similar potentiation of RyR P_o (Takasago *et al.* 1991; Witcher *et al.* 1991; Wehrens *et al.* 2004). However, whether significant activation of this kinase occurs during acute β-AR stimulation is still unclear (Wang *et al.* 2004; but see Curran *et al.* 2007).

Macroscopic and microscopic changes of triggers for SR Ca^{2+} release

Similar to our findings, Ginsburg & Bers (2004) found that β-AR stimulation increased the maximal rate of whole-cell SR Ca^{2+} release after careful matching of SR Ca^{2+} content

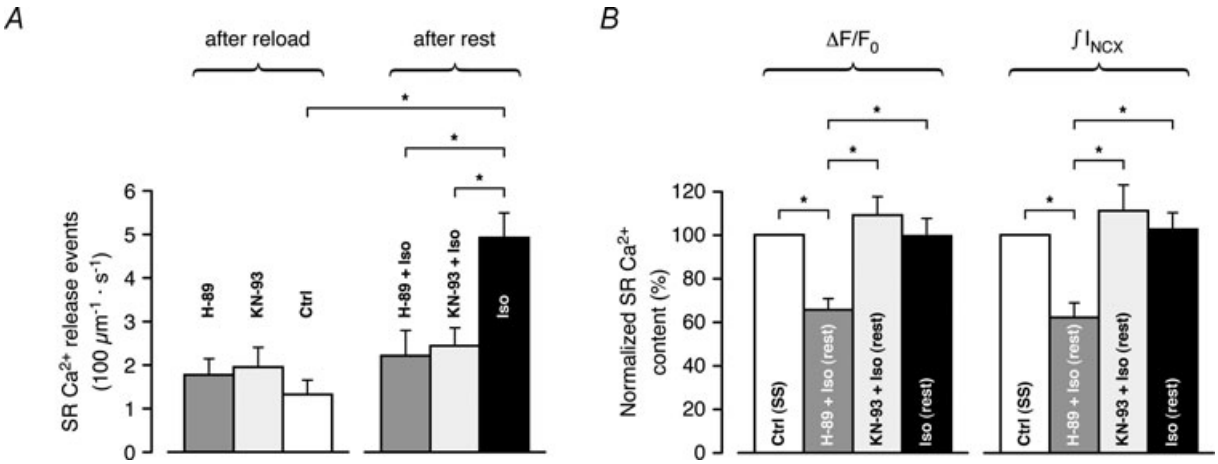

Figure 8. CaMKII rather than PKA mediates the increased spontaneous SR Ca^{2+} release in Iso
The time course of spontaneous SR Ca^{2+} release during rest was monitored following incubation with either the PKA inhibitor H-89 (5 μM) or the CaMKII inhibitor KN-93 (5 μM). H-89 or KN-93 was added to the extracellular solution immediately after reloading of the SR to steady-state. *A*, compared to Iso, which increased Ca^{2+} spark frequency from $1.32 \pm 0.32\ s^{-1}$ $(100\ \mu m)^{-1}$ to $4.92 \pm 0.56\ s^{-1}$ $(100\ \mu m)^{-1}$, treatment with either inhibitor almost completely suppressed the increase in Ca^{2+} spark frequency (from $1.77 \pm 0.36\ s^{-1}$ $(100\ \mu m)^{-1}$ in H-89 to $2.21 \pm 0.57\ s^{-1}$ $(100\ \mu m)^{-1}$ in H-89 + Iso, n.s., and from $1.95 \pm 0.44\ s^{-1}$ $(100\ \mu m)^{-1}$ in KN-93 to $2.44 \pm 0.40\ s^{-1}$ $(100\ \mu m)^{-1}$ in KN-93 + Iso, n.s.). *B*, during rest in KN-93 + Iso, SR Ca^{2+} content exhibited a tendency to increase in parallel, although this increase was not significant (caffeine-induced $\Delta F/F_0$: $108.8 \pm 8.6\%$ and $\int I_{NCX}$: $110.9 \pm 11.9\%$ of initial SR Ca^{2+} content). During rest in H-89 + Iso, however, the loss of Ca^{2+} from the SR was dramatic (caffeine-induced $\Delta F/F_0$: $65.4 \pm 4.9\%$ and $\int I_{NCX}$: $62.0 \pm 6.5\%$ of initial SR Ca^{2+} content). SR Ca^{2+} content was again unaffected after rest in Iso (caffeine-induced $\Delta F/F_0$: $99.6 \pm 7.8\%$ and $\int I_{NCX}$: $102.7 \pm 7.4\%$ of initial SR Ca^{2+} content) (H-89 ± Iso: $n = 8$ cells, KN-93 ± Iso: $n = 7$ cells, Iso: $n = 5$ cells, $^*P < 0.01$).

and triggering I_{Ca} in voltage-clamped rabbit and mouse ventricular myocytes. Interestingly, these experiments did not reveal an alteration of SR Ca^{2+} release amplitude itself. By reducing channel availability to compensate for increased P_o of L-type Ca^{2+} channels during β-AR stimulation, comparable macroscopic I_{Ca} was activated to trigger SR Ca^{2+} release. However, as the macroscopic I_{Ca} does not exhibit absolute microscopic invariance to β-AR stimulation, it is possible that this operation enhanced the coupling fidelity by reducing the number of L-type Ca^{2+} channels required to trigger the same amount of SR Ca^{2+} release during β-AR stimulation, hence increasing the number of redundant channel openings (Altamirano & Bers, 2007). In other words, changes of microscopic trigger features may alter coupling sufficiently to mask subtle changes of SR Ca^{2+} release itself.

Physiological role of RyR sensitization

Nonetheless, I_{Ca} remains the physiological trigger for SR Ca^{2+} release. Redundant Ca^{2+} supply from influx through L-type Ca^{2+} channels together with the tight ultrastructural organization of these channels with respect to clusters of RyRs in the SR membrane most likely serve to ensure EC coupling fidelity. The stimulation of whole-cell SR Ca^{2+} release in Iso observed in the present study may thus be minor in comparison to the more pronounced stimulation exerted by increases in SR Ca^{2+} content and I_{Ca} during physiological β-AR stimulation. However, under conditions where the tight coupling between L-type Ca^{2+} channels and clusters of RyRs is disrupted, the significance of our observations could be more relevant. For example, metabolic inhibition was found to fracture the pattern of Ca^{2+} transients as a result of reduced ability of I_{Ca} to recruit Ca^{2+} release locally (Fukumoto *et al.* 2005). Moreover, Louch *et al.* (2004) related dispersion of T-tubules, a network of plasmalemmal invaginations that allow the coupling of L-type Ca^{2+} channels to clusters of RyRs throughout the cell interior, to reduced synchrony of whole-cell SR Ca^{2+} release. Similarly, T-tubule disorganization is thought to contribute to dyssynchronous SR Ca^{2+} release in the failing heart (Song *et al.* 2006). Such loss of ultrastructural organization could compromise the tight coupling between L-type Ca^{2+} channels and clusters of RyRs by rendering the physiological trigger for SR Ca^{2+} release, I_{Ca}, more spatially homogeneous and by consequence less efficient, but at the same time more similar to the trigger used in the present study.

Consistent with this hypothesis, we found that SR Ca^{2+} release appeared spatially uncorrelated throughout the cell in control near the threshold for CICR when triggered by rapid, spatially homogeneous uncaging of Ca^{2+} from DM-nitrophen, and that this was particularly reflected in the subcellular distribution of time to peak of the Ca^{2+} transient. Moreover, we found that Iso synchronized whole-cell SR Ca^{2+} release over larger distances at strictly matched SR Ca^{2+} content. This observation further supports the notion of increased Ca^{2+} sensitivity of the RyR in Iso, as it is presumably not only reflected in the (primary) opening of clusters in response to the rapid elevation in $[Ca^{2+}]_i$, but also in the (secondary) coupling between neighbouring clusters, as suggested by the narrow, correlated distribution of time to peak of the Ca^{2+} transient throughout the cell. Song *et al.* (2001) first proposed that β-AR stimulation may synchronize triggered release of Ca^{2+} from the SR and increase SR Ca^{2+} release flux at comparable SR Ca^{2+} content in whole-cell voltage-clamped ventricular myocytes, although they did not compensate for the stimulation of I_{Ca}. Iso has also been shown to reduce the dyssynchrony of Ca^{2+} transients in myocytes isolated from infarcted hearts (Litwin *et al.* 2000). On a cellular level, increased RyR Ca^{2+} sensitivity during β-AR stimulation may thus play an important compensatory role in the shaping of Ca^{2+} transients under certain pathological conditions, where the coupling between L-type Ca^{2+} channels and clusters of RyRs is compromised and SR Ca^{2+} content is reduced (e.g. during heart failure).

SR Ca^{2+} leak and arrhythmias

Spontaneous release of Ca^{2+} through clusters of RyRs (or single RyRs) accounts for the leak of Ca^{2+} from the SR during diastole. Ca^{2+} spark-mediated SR Ca^{2+} leak can initiate propagating Ca^{2+} waves under certain conditions (Cheng *et al.* 1993) that give rise to arrhythmogenic, transient inward currents (Berlin *et al.* 1989). Recently, transient inward currents carried by the NCX were shown to directly evoke triggered arrhythmias in the heart during β-AR stimulation (Fujiwara *et al.* 2008). Thus, thorough understanding of Ca^{2+} spark-mediated SR Ca^{2+} leak and its regulation during β-AR stimulation is essential, as it may play an important role not only in SR Ca^{2+} load but also in arrhythmogenesis. Tanaka *et al.* (1997) studied Ca^{2+} sparks in intact rat ventricular myocytes, and found an increased Ca^{2+} spark amplitude during β-AR stimulation, but did not take possible alterations in SR Ca^{2+} content into account. Conversely, Gomez *et al.* (1996) did not observe an increased Ca^{2+} spark amplitude during β-AR stimulation at comparable SR Ca^{2+} content, but noted a 3-fold increase in Ca^{2+} spark frequency under these conditions in whole-cell voltage clamped rat ventricular myocytes, suggesting significantly higher RyR Ca^{2+} sensitivity at diastolic $[Ca^{2+}]_i$.

In agreement with the findings by Gomez *et al.* (1996) we found an ~4-fold increase in Ca^{2+} spark frequency at comparable SR Ca^{2+} content in Iso, and that this

significantly higher diastolic SR Ca^{2+} leak appeared within 2 min of β-AR stimulation with Iso, consistent with reports on the time course of RyR phosphorylation (Takasago *et al.* 1991; Yoshida *et al.* 1992). An increase in Ca^{2+} sensitivity of the RyR is also supported by the frequent occurrence of SR Ca^{2+} release events composed of multiple Ca^{2+} sparks (macrosparks) in Iso, and reflects an increased propensity of Ca^{2+} sparks from one cluster to trigger release from neighbouring clusters, similar to the coupling between neighbouring clusters that contributes to synchronization of whole-cell SR Ca^{2+} release revealed at near threshold triggers. The significant increase in SR Ca^{2+} leak observed after repolarization and complete decay of $[Ca^{2+}]_i$ to diastolic levels together with the apparent loss of local control could, in principle, when enhanced by parallel increase in SR Ca^{2+} content during β-AR stimulation, produce the intense, synchronous Ca^{2+} waves required to trigger delayed afterdepolarizations (Fujiwara *et al.* 2008).

Li *et al.* (2002) attributed increased Ca^{2+} spark frequency after addition of cAMP to permeabilized cells entirely to higher SR Ca^{2+} content. However, the strong rest potentiation of SR Ca^{2+} content they observed in mouse myocytes could have masked a more subtle stimulation of RyR P_o, which in the present study could be revealed in guinea-pig ventricular myocytes exhibiting rest decay of SR Ca^{2+} content. Similarly, the maximally Ca^{2+} loaded SR in mice lacking PLB used in the study by Li *et al.* could have overridden any functional modulation of RyR Ca^{2+} sensitivity resulting from phosphorylation. Carter *et al.* (2006) found that the functional manifestation of PKA phosphorylation of the RyR may critically depend on differential, possibly species-dependent basal phosphorylation levels, as well as the extent of phosphorylation, underscoring the sensitivity of the RyR to the reigning balance between kinase and phosphatase activity. Li *et al.* also addressed this issue, and noted that addition of cAMP only resulted in less than 50% of maximal phosphorylation. It is thus possible that RyR phosphorylation by PKA did not reach the threshold required for functional manifestation in these experiments on myocytes from wild-type mice and from mice lacking phosphorylatable PLB.

The dramatic increase in Ca^{2+} spark frequency in our experiments (from very rare Ca^{2+} sparks in control to 4- to 5-fold higher in Iso) during acute β-AR stimulation of guinea-pig ventricular myocytes could result from RyR phosphorylation by CaMKII. In fact, Curran *et al.* (2007) recently reported on increased diastolic SR Ca^{2+} leak in intact, quiescent rabbit ventricular myocytes in Iso that could be attributed to CaMKII. Interestingly, activation of CaMKII in the experiments by these investigators appeared to be independent of elevated Ca^{2+} cycling mediated by PKA. Rather, it required nitric oxide synthase activation (Curran *et al.* 2009), and other oxidation-dependent mechanisms have also been reported to activate CaMKII (Erickson *et al.* 2008). In good agreement with the results by Curran *et al.* we found that the dramatic increase in Ca^{2+} spark frequency in Iso observed in the present study could be normalized with the widely used CaMKII inhibitor KN-93, without altering SR Ca^{2+} content. Conversely, although increased Ca^{2+} spark frequency in Iso could not be observed in cells treated with the PKA inhibitor H-89, SR Ca^{2+} leak was accompanied by a strong depression of SR Ca^{2+} content. Increased SR Ca^{2+} leak resulting from sensitization of RyRs by CaMKII phosphorylation could underlie the progressive depletion of the SR, in the absence of a counterbalancing stimulation of SERCA activity. Inhibition of PKA phosphorylation of PLB by H-89 would thus explain the inability of the SR to sustain the elevated Ca^{2+} spark frequency observed in Iso. In line with these arguments, Curran *et al.* also reported that the SR Ca^{2+} content required to maintain comparable Ca^{2+} spark frequency in Iso in cells treated with H-89 was significantly lower, similarly indicating a failure of H-89 to prevent the increased SR Ca^{2+} leak in Iso. Although we cannot exclude a contribution of PKA to the increased SR Ca^{2+} leak observed in the present study, the results presented here suggest a predominant role of CaMKII, rather than PKA, in the stimulation of spontaneous SR Ca^{2+} release in Iso.

In addition to being arrhythmogenic, increased diastolic SR Ca^{2+} leak is thought to contribute to progressive impairment of cardiac function by reducing SR Ca^{2+} content during chronic activation of the β-AR pathway (Wehrens *et al.* 2006). We found that despite the significant increase in SR Ca^{2+} leak, loss of Ca^{2+} from the cell through the NCX is limited, probably due to stimulation of SERCA activity, which rather tended to sustain the elevated spontaneous SR Ca^{2+} release. This is also supported by the finding that inhibition of SERCA stimulation results in a dramatic reduction of SR Ca^{2+} content. Thus, the higher Ca^{2+} sensitivity of the RyR observed here does not appear sufficient to alone depress SR Ca^{2+} content during acute β-AR stimulation of resting guinea-pig ventricular myocytes. However, it could potentially exacerbate the reduction of SR Ca^{2+} content in heart failure where SERCA activity is reduced and extrusion of Ca^{2+} from the cell by the NCX is increased (Schmidt *et al.* 1998; Pieske *et al.* 1999; Gomez *et al.* 2002). Finally, we showed that SR Ca^{2+} leak can be significantly higher during β-AR stimulation at levels where SR Ca^{2+} content is comparable to control. This finding could provide a potential explanation of the occurence of exercise- or stress-induced arrhythmias in conditions such as heart failure where SR Ca^{2+} content is reduced and stimulation of SERCA activity during β-AR stimulation is limited, particularly if oxidative stress further elevates SR Ca^{2+} leak (Yano *et al.* 2005), or in the presence of mutations in the RyR or closely associated proteins (Liu & Priori, 2008).

Conclusions

Taken together, our results suggest that β-AR stimulation increases the Ca^{2+} sensitivity of the RyR, resulting in a higher probability of clusters to open and trigger release from neighbouring clusters. Modulation of the SR Ca^{2+} release mechanism is reflected in slightly larger Ca^{2+} transients as a consequence of enhanced spatiotemporal synchronization of whole-cell SR Ca^{2+} release. Although the stimulation may be modest when EC coupling fidelity is normal, it could play a compensatory role when coupling is compromised and SR Ca^{2+} content is reduced. Higher Ca^{2+} sensitivity of the RyR during β-AR stimulation is also manifested in an elevated diastolic SR Ca^{2+} leak that is predominantly mediated by CaMKII rather than PKA, and an increased propensity of Ca^{2+} sparks to trigger neighbours. The higher spontaneous SR Ca^{2+} leak is sustained by stimulation of SERCA activity, limiting the loss of Ca^{2+} from the SR. Thus, the same mechanism that could be beneficial in enhancing and synchronizing whole-cell SR Ca^{2+} release could also be responsible for the generation of delayed afterdepolarizations.

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Author contributions

All authors contributed to the conception and design of the study, analysis and interpretation of data, drafting the article and revising it critically for important intellectual content, and final approval of the version to be published.

Acknowledgements

We would like to thank the Swiss National Science Foundation (grant 31-109693 to E.N.), the Swiss Cardiovascular Research and Training Network (SCRTN) and the Swiss Foundation for Research on Muscle Diseases for support. We thank Drs Nicolas Lindegger and Nina Ullrich for helpful comments on the manuscript, and Daniel Lüthi for technical assistance.