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Paradoxical SR Ca^{2+} release in guinea-pig cardiac myocytes after β-adrenergic stimulation revealed by two-photon photolysis of caged Ca^{2+}

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In heart muscle the amplification and shaping of Ca^{2+} signals governing contraction are orchestrated by recruiting a variable number of Ca^{2+} sparks. Sparks reflect Ca^{2+} release from the sarcoplasmic reticulum (SR) via Ca^{2+} release channels (ryanodine receptors, RyRs). RyRs are activated by Ca^{2+} influx via L-type Ca^{2+} channels with a specific probability that may depend on regulatory mechanisms (e.g. β-adrenergic stimulation) or diseased states (e.g. heart failure). Changes of RyR phosphorylation may be critical for both regulation and impaired function in disease. Using UV flash photolysis of caged Ca^{2+} and short applications of caffeine in guinea-pig ventricular myocytes, we found that Ca^{2+} release signals on the cellular level were largely governed by global SR content. During β-adrenergic stimulation resting myocytes exhibited smaller SR Ca^{2+} release signals when activated by photolysis (62.3% of control), resulting from reduced SR Ca^{2+} content under these conditions (58.6% of control). In contrast, local signals triggered with diffraction limited two-photon photolysis displayed the opposite behaviour, exhibiting a larger Ca^{2+} release (164% of control) despite reduced global and local SR Ca^{2+} content. This apparent paradox implies changes of RyR open probabilities after β-adrenergic stimulation, enhancing local regenerativity and reliability of Ca^{2+} signalling. Thus, our results underscore the importance of phosphorylation of RyRs (or of a related protein), as a regulatory physiological mechanism that may also provide new therapeutic avenues to recover impaired Ca^{2+} signalling during cardiac disease.

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Depolarization of cardiac myocytes during an action potential leads to the opening of voltage-dependent L-type Ca^{2+} channels. The subsequent elevation of the Ca^{2+} concentration ($[Ca^{2+}]_i$) in the dyadic cleft activates Ca^{2+} release channels (referred to as ryanodine receptors or RyRs) forming macromolecular complexes located in the sarcoplasmic reticulum (SR) membrane and in the dyadic cleft. This Ca^{2+}-induced Ca^{2+} release (CICR) process amplifies and scales the Ca^{2+} signal via summation of discrete elementary Ca^{2+} signalling events termed Ca^{2+} sparks. In order for the Ca^{2+} signal to be transient the Ca^{2+} release from the SR has to terminate. Furthermore, cytosolic Ca^{2+} needs to be pumped back into the SR by the SR Ca^{2+} pump (SERCA) and transported out of the cell via the Na^{+}–Ca^{2+} exchange (for review see Bers, 2002).

The discovery of Ca^{2+} sparks as elementary Ca^{2+} signalling events has fundamentally changed the view of excitation–contraction coupling (EC coupling) on the cellular and subcellular level (Cheng *et al.* 1993; Niggli, 1999). Contrary to the notion of systems in which released Ca^{2+} has access to a common cytosolic pool (Stern, 1992), amplification and shaping of the Ca^{2+} signal is now understood to be fine-tuned by recruiting a variable number of functionally independent Ca^{2+} sparks, each of which is an all-or-none event with a high degree of positive feedback. After opening of an L-type Ca^{2+} channel each SR Ca^{2+} release site capable of generating a Ca^{2+} spark is activated with a specific probability, which may depend on a variety of variables, including regulatory mechanisms and pathophysiological or diseased states. Many of these variables can thus affect EC coupling by changing the Ca^{2+} spark trigger probability. Indeed, a reduced Ca^{2+} spark trigger probability has been identified as a cause underlying impaired EC coupling in myocytes from hypertrophied and failing rat hearts (Gomez *et al.* 1997).

Among possible mechanisms that may affect the Ca^{2+} sensitivity of the RyRs, and thus the Ca^{2+} spark trigger

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probability, variations of SR Ca^{2+} content and regulatory changes due to protein phosphorylation after β-adrenergic stimulation have received much attention recently. On the basis of receptor number, the β_1-adrenergic receptor (β_1AR) is the predominant β-receptor subtype in cardiac ventricular myocytes and couples to stimulatory G_s proteins, which leads to activation of adenylyl cyclase after receptor activation. Adenylyl cyclase synthesizes the second messenger cyclic adenosine monophosphate (cAMP), which increases the activity of protein kinase A (PKA). PKA phosphorylates many substrates, several of which play an essential role in Ca^{2+} signalling: (1) phosphorylation of L-type Ca^{2+} channels enhances the Ca^{2+} current, thus increasing both the trigger signal for CICR and the extent of SR loading with Ca^{2+} (Reuter, 1983); (2) phosphorylation of phospholamban (PLB) relieves its inhibitory effect on the SERCA, subsequently stimulating the Ca^{2+} pump, again increasing the Ca^{2+} load of the SR (Lindemann *et al.* 1983; James *et al.* 1989; Shannon *et al.* 2001; Bers, 2002); (3) it has recently been reported that PKA could also directly phosphorylate the RyRs, possibly inducing dissociation of calstabin-2 (formerly called FKBP12.6) and increasing RyR open probability (Lu *et al.* 1995; Valdivia *et al.* 1995; Marx *et al.* 2000). Dissociation of calstabin-2 may subsequently disrupt coupled gating of the RyRs (Marx *et al.* 2001). Generally, PKA-mediated phosphorylation of the RyRs is thought to increase the Ca^{2+} sensitivity of the release channels. Conversely, several research groups found that RyRs were not phosphorylated (Jiang *et al.* 2002), or, when phosphorylated, the RyR open probability appeared to remain unchanged (Li *et al.* 2002). In addition, others have found that RyR phosphorylation by Ca^{2+}-calmodulin-dependent kinase II (CaMKII) induced stimulatory or inhibitory effects in lipid bilayers and profound stimulatory effects in freshly isolated myocytes (Takasago *et al.* 1991; Hain *et al.* 1995; Lokuta *et al.* 1995; Li *et al.* 1997; Wang *et al.* 2004).

In intact cells changes of CICR after β-adrenergic stimulation could also result from increased SR Ca^{2+} load. Increased Ca^{2+} load leads to more Ca^{2+} release by the law of mass action (Fabiato, 1985; Bassani *et al.* 1995; Tripathy & Meissner, 1996; Santana *et al.* 1997; Satoh *et al.* 1997; Sitsapesan & Williams, 1997; Györke & Györke, 1998; Frank *et al.* 2000; Lukyanenko *et al.* 2001). In addition, the Ca^{2+} concentration inside the SR may have a regulatory effect on CICR by modulating the Ca^{2+} sensitivity of the RyRs, either via a Ca^{2+} receptor directly located on the luminal side of the RyRs (Györke & Györke, 1998; but see Tripathy & Meissner, 1996) or by involving a signalling pathway comprising calsequestrin as a Ca^{2+} sensor and one or more small accessory junctional SR proteins, such as triadin and junctin (Guo & Campbell, 1995; Zhang *et al.* 1997; Györke *et al.* 2004). In the failing heart, Ca^{2+} regulation is proposed to be one of several key players in the altered contractility (Houser *et al.* 2000; Hasenfuss & Pieske, 2002), but many other processes are affected too, such as structural changes, altered protein expression and decreased PKA-dependent phosphorylation of several proteins (Marks *et al.* 2002). Furthermore, heart failure may be associated with RyR hyperphosphorylation, possibly leading to dissociation of calstabin-2 from the RyRs and resulting in an increased SR Ca^{2+} leak, which may finally decrease SR Ca^{2+} content (Shou *et al.* 1998; Marx *et al.* 2000; Prestle *et al.* 2001). Ultimately, diminished SR Ca^{2+} content may then cause smaller Ca^{2+} transients and reduced cardiac force.

In the present study we found a strong correlation between global cellular Ca^{2+} release amplitude and SR Ca^{2+} load when the trigger signal was a spatially homogeneous photolytic Ca^{2+} transient induced by a UV flash. In contrast, highly localized Ca^{2+} release signals generated with diffraction-limited two-photon photolysis were less sensitive to SR Ca^{2+} load. In particular, larger localized Ca^{2+} release signals were recorded after β-adrenergic stimulation, even in conditions where the SR Ca^{2+} content was reduced. Based on this discrepancy between global and local Ca^{2+} signals we conclude that, in addition to the secondary effect via changes of luminal Ca^{2+}, β-adrenergic stimulation may change the gating properties of the RyRs themselves, either directly or indirectly. These findings have been presented in preliminary form to the Biophysical Society (Lindegger & Niggli, 2002).

Methods

Isolation of guinea-pig myocytes

Cardiac ventricular myocytes were isolated from adult male guinea-pigs using established enzymatic methods (DelPrincipe *et al.* 1999). All animal handling procedures were performed with the permission of the State Veterinary Administration and according to Swiss Federal Animal handling law. Guinea-pigs were killed by cervical dislocation, the hearts rapidly removed and mounted on a Langendorff system and retrogradely perfused with a Ca^{2+}-free solution at 37°C for about 5 min (Mitra & Morad, 1985). For enzymatic digestion, collagenase type 2 (0.12 mg ml^{-1}, Worthington, Switzerland) and protease type XIV (0.04 mg ml^{-1}, Sigma, Switzerland) were added to the perfusion solution for another 3–5 min. After digestion, the ventricles were cut into small pieces, placed on a gently rotating shaker in a solution containing 200 μM Ca^{2+} and kept at room temperature until use.

Solutions

For the experiments cells were transferred into a chamber mounted on the stage of an inverted microscope. The extracellular superfusion solution contained (mM): NaCl

140, KCl 5, $CaCl_2$ 1.8, CsCl 1, $BaCl_2$ 0.5, Hepes 10, glucose 10, pH 7.4 (adjusted with NaOH). Cells were continuously superfused using a custom-made rapid superfusion system ($t_{1/2} < 500$ ms). Where indicated, isoproterenol (isoprenaline, Iso) 1 μм ([−]-*N*-iso-propyl-L-noradrenaline hydrochloride; Sigma) was added from a frozen stock (1 mм in 10% ascorbic acid). Cyclopiazonic acid (CPA, 10 μм; Sigma) was added to block the SERCA and to avoid loading of the SR. Short applications of 20 mм caffeine (Sigma) were used to estimate SR Ca^{2+} content. In some control experiments the SR function was inhibited using 1 μм thapsigargin and 10 μм ryanodine (both from Alamone Laboratories, Jerusalem, Israel). The pipette-filling solution contained (mм): caesium aspartate 120, TEA-Cl 20, Na_4-DM-nitrophen 2 (Calbiochem, La Jolla, CA, USA), reduced glutathione (GSH) 1, $CaCl_2$ 0.5, K_2-ATP 5, Hepes 10 and K_5-fluo-3 0.05 (TefLabs, Austin, TX, USA), pH 7.2 (adjusted with CsOH). All experiments were carried out at room temperature (21°C).

Voltage clamp

Electrodes were pulled from filamented borosilicate glass capillaries (GC150F, Clark Electromedical Instruments, Pangbourne, UK) on a horizontal puller (DMZ, Zeitz Instrumente, Augsburg, Germany) to a series resistance of 1–2 MΩ. Cells were voltage-clamped in the whole-cell configuration of the patch-clamp technique and held at a resting potential of −70 mV or −40 mV using an Axopatch 200 amplifier (Axon Instruments, Foster City, CA, USA). Potentials were not corrected for the junction potential, which was calculated to be ∼12 mV for our pipette filling solution. The SR and cytosolic DM-nitrophen were loaded with Ca^{2+} by a variable number of L-type Ca^{2+} currents with voltage steps to 0 mV or +10 mV lasting 200 ms. Currents were digitized at 3 kHz using an A/D converter and custom-written data acquisition software developed by us under LabView (National Instruments, Ennetbaden, Switzerland) running on an Apple Macintosh G3 computer. Membrane current and voltage data were stored on a hard disk for later analysis using IgorPro software (WaveMetrics, Lake Oswego, OR, USA).

Confocal Ca^{2+} imaging

Cells were imaged with a 40 × oil-immersion objective lens (Fluor, N.A. = 1.3; Nikon) and loaded with fluo-3 by dialysis through the recording pipette. Fluo-3 was excited with the 488 nm line of an argon-ion laser (ILT-5000, Ion Laser Technology, Salt Lake City, UT, USA) at 50–150 μW on the cell. The fluorescence was detected at 540 ± 15 nm with a confocal laser-scanning microscope (MRC 1000, Bio-Rad, Glattbrugg, Switzerland). The amplitude and the time course of cytosolic Ca^{2+} transients were computed off-line using customized versions of either NIH Image or Image SXM and expressed as normalized fluorescence (F/F_0). Resting [Ca^{2+}] for experiments made in pipette filling solution (Fig. 1) was 20 nм and K_d was 400 nм (DelPrincipe *et al.* 1999). Mean Ca^{2+} concentration profiles were extracted from fluorescence images and calculated with IgorPro software using an established self-ratio calibration procedure (Cheng *et al.* 1993). Two-photon release signals were estimated as the maximal amplitude (mean of 5 points) minus the mean fluorescence during 100 ms prior to photolysis. Values were normalized to the maximal peak amplitude obtained in caffeine (Figs 5 and 6). Means of the normalized signals were plotted *versus* two-photon power and a sigmoidal function was fitted to the data. Under our conditions the fluo-3 fluorescence was not significantly quenched by the application of 20 mм caffeine (by 0.54 ± 0.16% of control, $n = 2$). Furthermore, the photolytic two-photon photolysis (TPP) Ca^{2+} signals remained unaffected by 20 mм caffeine.

Global UV flash and local two-photon photolysis of caged Ca^{2+} compounds

Photolytic Ca^{2+} concentration jumps were elicited with UV flashes from a xenon short-arc flash lamp (duration ∼400 μs, discharged energy up to 230 J) (Kaplan & Ellis-Davies, 1988). UV flashes were applied in an epi-illumination arrangement to trigger global and spatially homogenous Ca^{2+} releases from the entire SR (for details see DelPrincipe *et al.* 1999). In order to generate highly localized and diffraction limited photolytic Ca^{2+} sources, we used TPP of caged Ca^{2+} (DM-nitrophen) (Lipp & Niggli, 1998; DelPrincipe *et al.* 1999). The beam of a mode-locked titanium sapphire laser (Mira 900, Coherent) tuned to 710 nm, with < 100 fs pulse length at 80 MHz repetition rate, was guided through the camera port of the confocal microscope to produce a stationary diffraction-limited spot within the myocyte, parfocal with the plane of fluorescence detection. The excitation point spread function was determined to extend over ∼710 nm (full width at half-maximal amplitude; FWHM) in the *x*–*y*-direction and 1200 nm in the *z*-direction (Lipp & Niggli, 1998). Photolysis was elicited by the opening of a mechanical shutter (Uniblitz, Vincent Associates, Rochester, NY, USA). The shutter opening was set for a duration of 60 ms for all experiments, and the interval between subsequent shutter openings was 430 ms. Both the shutter and the UV flash were synchronized to the pixel-clock of the laser scanner to coerce synchronization with the image acquisition and the voltage-clamp recording system. The power of the two-photon laser was attenuated by means of a neutral density filter and an adjustable linear polarization filter, both placed in series. In order to rapidly estimate the Ca^{2+} dependence of the CICR process and to analyse signals of comparable amplitude before and after β-adrenergic stimulation, a filter wheel holding nine neutral density filters (Lambda 10-2, Sutter Instruments, Novato, CA, USA) was placed in the optical pathway. The filter wheel was controlled by a Motorola 68HC12 microcontroller, running software written by us under the LEMPS development system (GIBB, Bern, Switzerland). Using this system, a TPP power–response relationship could be measured within 6 s, as shown in an experiment where DM-nitrophen was photolysed in a droplet of pipette filling solution (Fig. 1). Ca^{2+} signals elicited by TPP in pipette solution did not saturate and displayed a power dependence with an exponent of 1.8, suggesting a two-photon process. In addition, this relationship implies that the amplitude of the photolytic Ca^{2+} release signals is proportional to the Ca^{2+} release flux. The width (FWHM) increased only slightly, consistent with the minimal power dependence of the volume excited by TPP, as determined by recording the fluorescent point spread function (PSF) in fluoresceine (not shown). Each power–response plot includes 10 different power levels (as a percentage of full power): 16, 22, 25, 31, 42, 53, 68, 84, 93 and 100, respectively.

Statistics

Data are expressed as mean ± S.E.M, and n represents the number of analysed cells. Significance was tested with Student's t test and is denoted as $*$ ($P < 0.05$) or $**$ ($P < 0.02$). For estimates of CICR (Figs 5 and 6), recordings in which local Ca^{2+} signals at maximal photolytic power were not larger in control than in the presence of caffeine (i.e. contained only photolytic Ca^{2+} release and no CICR component) were excluded from the analysis. Furthermore, data where the photolytic signal amplitude at maximal laser power was less then 2 times the noise (S.E.M.) of the resting Ca^{2+} signal were not taken into account.

Results

In initial experiments we verified that the entire β-adrenergic signalling and second messenger cascade were present and functional under the conditions of our experiments. Successful β-adrenergic stimulation was also confirmed at the end of each subsequent experiment. After 2 min of 1 μM isoproterenol (Iso) superfusion, the time needed to get a robust β-adrenergic stimulation in most cells, L-type Ca^{2+} currents elicited by depolarizations

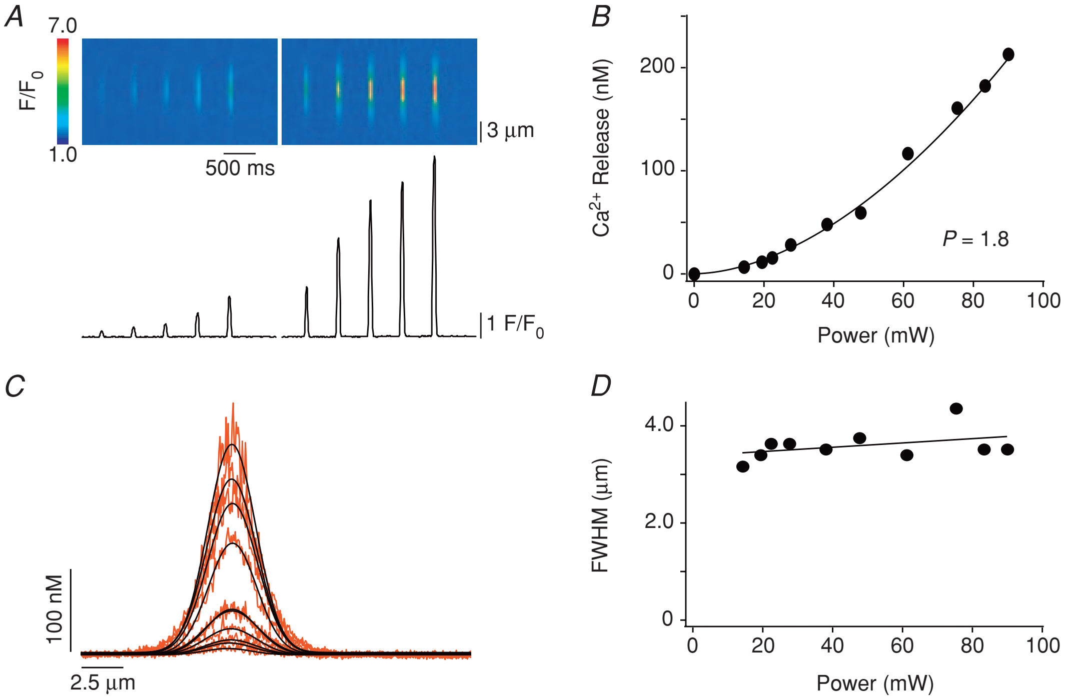

Figure 1. Two-photon photolysis in droplet

A, characteristic TPP power–response relationship obtained in pipette filling solution. By increasing the TPP power, more Ca^{2+} is released from the caged compound. *B*, when plotting the amplitude of the Ca^{2+} signal *versus* the photolytical power, data displayed a power dependence with an exponent of 1.8, suggesting a two-photon excitation process. *C*, horizontal spreading by diffusion of local Ca^{2+} signals of increasing amplitude. *D*, no strong dependence of the full width at half-maximal amplitude (FWHM) of the local Ca^{2+} signals *versus* power was found.

from −40 mV to +10 mV were considerably larger than in control (Fig. 2*A*). This resulted in an increase of the Ca^{2+} transient, as can be seen on the line-scan images and the traces averaged from the line-scans (Fig. 2*B* and *C*).

Activation of global cellular CICR with UV flash photolysis

In all subsequent experiments involving global cellular Ca^{2+} transients, we applied flash photolysis of caged Ca^{2+} to activate and examine CICR, since this is a trigger signal which itself is not affected by β-adrenergic stimulation, unlike the L-type Ca^{2+} current. To ensure a comparable and intermediate SR Ca^{2+} content, a specific loading protocol was carried out before each UV flash. It consisted of an initial SR emptying with a puff of caffeine, followed by a train of four L-type Ca^{2+} currents (depolarizing steps from −40 mV to 0 mV for 200 ms; Fig. 3*A*). To examine how CICR was affected by β-adrenergic stimulation while excluding the L-type Ca^{2+} current as the trigger signal, we applied UV flashes after 2 min at rest, either under control conditions or in the presence of Iso. Contrary to our expectations, the amplitude of the Ca^{2+} transient did not become larger after 2 min treatment with Iso. It even decreased to 62.3 ± 16.1% ($n = 7$, $P < 0.05$) of the control amplitude (Fig. 3*Cb* and *D*). This decline was even more surprising since loading protocols with trains of L-type Ca^{2+} currents were carefully kept identical for both, control and Iso-treated cells, to avoid any alterations of SR Ca^{2+} content (Fig. 3*Bi* and *ii*).

Thus we wondered in what respect our flash photolytic experiments differed from the more physiological situation relying on L-type Ca^{2+} currents as triggers for CICR. First, our trigger signal was, on purpose, not increased by Iso. Second, we had chosen conditions to avoid exposing the SR to augmented Ca^{2+} currents during the loading protocol, in order to maintain the SR Ca^{2+} loads similar for both experiments. Could it be that the larger L-type Ca^{2+} currents are a prerequisite to obtain more release from the store, by either representing a more efficient trigger signal for CICR or by providing more Ca^{2+} influx for reloading the SR? To answer these questions and to distinguish between the two possibilities we slightly modified our loading protocol and started the application of Iso 2 min before performing the SR Ca^{2+} loading protocol. Or, in other words, we loaded the SR with larger Ca^{2+} currents (Fig. 3*Biii*) while keeping the trigger signal constant (i.e. the photolytic $[Ca^{2+}]$ jump). As it turned out, the amount of Ca^{2+} released was indeed larger than in control (160.4 ± 45.2%, $n = 7$; Fig. 3*Cc* and *D*). In addition to the increased L-type current after β-adrenergic stimulation used to reload the SR, SERCA stimulation could be seen during the decay of the signals. The maximal rate of decay increased to 137 ± 11.3% ($n = 7$) after 2 min rest in Iso and to 118 ± 12.6% ($n = 7$) after reloading in Iso. In guinea-pigs,

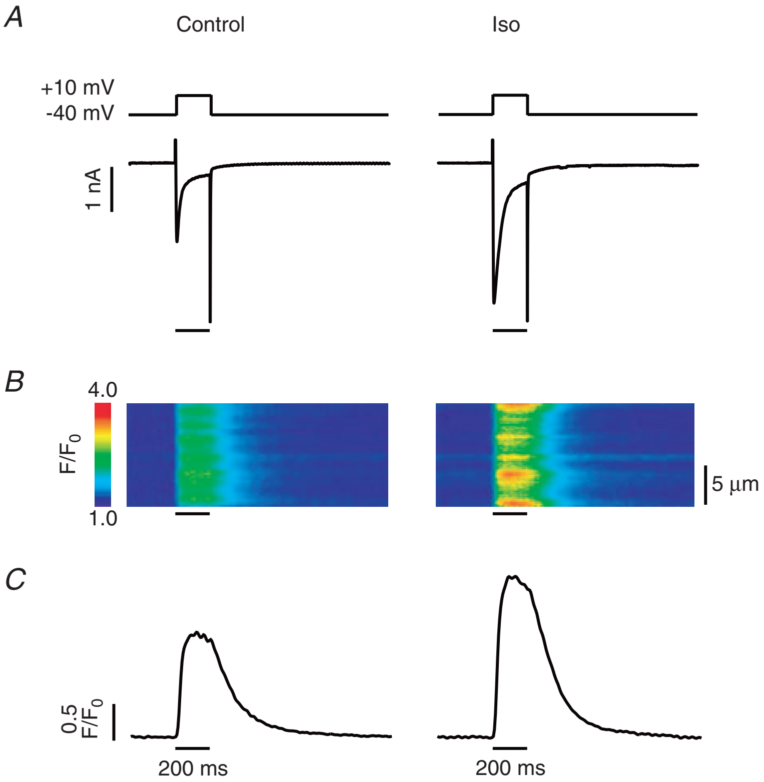

Figure 2. β-Adrenergic stimulation increases the amount of Ca^{2+} entry via L-type Ca^{2+} channels
A, voltage protocol and recorded current. Left: cells were held at −40 mV and depolarized to +10 mV for 200 ms to activate L-type Ca^{2+} current. Right: same protocol after 2 min Iso superfusion. Entry of Ca^{2+} was larger thereby enhancing the subsequent CICR. *B*, line-scan images recorded during the depolarizing step. *C*, averages of the line-scan images in *B*, expressed as normalized fluorescence.

cytosolic Ca^{2+} removal is more dependent on Na^{+}–Ca^{2+} exchange than, for example, in rat (Sham *et al.* 1995). Thus the effect of SERCA stimulation is less pronounced. Finally, as reported recently (Ginsburg & Bers, 2004), the maximal rate of Ca^{2+} release was indeed larger when cells were reloaded in Iso (147.8 ± 53.4%, $n = 7$). As expected, superfusion of 10 μM CPA prolonged the decay of the Ca^{2+} signal to 124 ± 12.5% and to 136 ± 23.7% in the presence of CPA and CPA plus Iso, respectively (Fig. 3*E*). But how can we explain the reduced Ca^{2+} signal amplitude after β-adrenergic stimulation, despite the identical SR Ca^{2+} loading protocol? First, run-down of L-type Ca^{2+} channels, occuring within a time window of 20–30 min, could not be held responsible for the decreased loading as shown in Fig. 3*Bi* and *ii*. Smaller Ca^{2+} transients could also result from a decrease in the Ca^{2+} sensitivity of the RyRs or from an acceleration of the SR Ca^{2+} loss occurring during the 2 min rest in Iso. The first possibility seemed less likely after β-adrenergic stimulation. To evaluate changes of SR Ca^{2+} content occurring during the resting period, we used the same loading protocol as above, but instead of triggering CICR with UV flashes, short puffs of caffeine were applied to completely empty the SR (Fig. 4). The resulting membrane currents reflecting electrogenic Ca^{2+} removal via the Na^{+}–Ca^{2+} exchanger (I_{NCX}) were integrated to estimate SR Ca^{2+} content (Trafford *et al.* 1998). Interestingly, the SR contained significantly less Ca^{2+} when resting for 2 min in Iso (58.6 ± 7.3%, $n = 6$, $P < 0.02$) than when resting in control solution (100%, $n = 10$; Fig. 4*C* and *D*). However, when the cells were Ca^{2+} loaded with L-type Ca^{2+} currents increased by

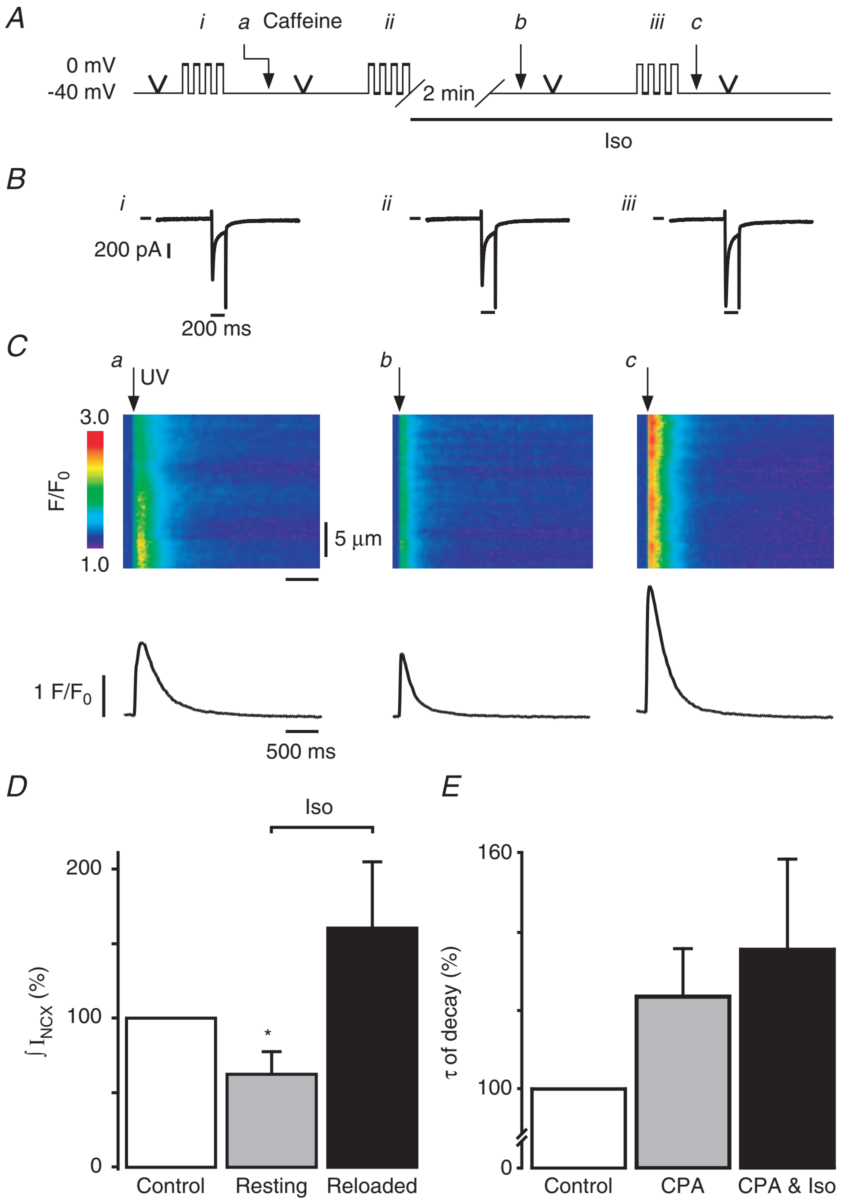

Figure 3. Whole-cell Ca^{2+} release signals are smaller after rest in Iso
A, voltage protocol. Caffeine (20 mM; arrowhead), briefly applied to empty the SR, was followed by 4 consecutive depolarizations from −40 mV to 0 mV for 200 ms to moderately load the SR (*Ai*). After 2 min a UV flash was triggered to induce a global and homogenous Ca^{2+} release from the SR (*Aa*) and immediately followed by another short application of caffeine. Four similar depolarizations were again applied (*Aii*) and followed by superfusion of Iso. After 2 min rest another UV flash (*Ab*) was triggered, followed by a short application of caffeine. Finally, 4 depolarizations were applied during β-adrenergic stimulation (*Aiii*) and followed by a UV flash (*Ac*). *B*, the third L-type Ca^{2+} current of a train of four consecutive depolarizations: *Bi*, in control: *Bii*, in control and before Iso application for 2 min: and *Biii*, during β-adrenergic stimulation. Line indicates 0 pA. *C*, line-scan images acquired during the UV flashes. *Ca*, control; *Cb*, resting in Iso; and *Cc*, stimulated in Iso. *D*, normalized amount of extruded Ca^{2+} ($\int I_{\mathrm{NCX}}$) in control (100%), after rest in Iso (62.3 ± 16.1%, $n = 7$, $P < 0.05$) and after reloading in Iso (160.4 ± 45.4%, $n = 7$). *E*, decays of global fluorescence signals were fitted with a monoexponential function and τ was normalized to control. As expected CPA prolonged the decay to 124 ± 12.5%, $n = 3$ and to 136 ± 23.7%, $n = 4$, in the continued presence of CPA and Iso.

β-adrenergic stimulation, the SR Ca^{2+} content was raised to 123.0 ± 11.7% ($n = 10$, $P < 0.05$). Thus, the amplitude of the global Ca^{2+} signals observed after triggering CICR with identical photolytic triggers mainly reflected the SR Ca^{2+} content prevailing under the various conditions. The reduced SR Ca^{2+} content after a resting period in Iso is an interesting finding by itself and could be the consequence of an increased SR Ca^{2+} leak, possibly resulting from an enhanced Ca^{2+} sensitivity of the RyRs after phosphorylation (Marx *et al.* 2000). This leak was presumably small since no significant change in resting $[Ca^{2+}]$ was measured between control and Iso (see below).

Local activation of CICR with two-photon photolysis

We have previously observed that Ca^{2+} refilling of the SR occurs much faster after local activation than after global activation of CICR, presumably because a rapid redistribution of Ca^{2+} within the SR network takes place after functional Ca^{2+} depletion of a highly confined Ca^{2+} release unit only (i.e. one junctional SR structure near a dyad) (DelPrincipe *et al.* 1999). Thus, localized Ca^{2+} release might be expected to behave differently than global cellular signals, possibly by being less dependent on the global SR Ca^{2+} content. Thus, local signals might be more sensitive to changes of RyR gating. Therefore, we next elicited highly localized photolytic Ca^{2+} signals by TPP of DM-nitrophen (Fig. 5), while following an SR loading protocol analogous to the one used for UV flash experiments (Fig. 3). TPP power–response relationships in six cells (Fig. 5*B*) were recorded in order to obtain local Ca^{2+} signals of varying amplitudes under each condition (i.e. in caffeine, in the presence or absence of Iso, at low and high SR Ca^{2+} load). Signals of

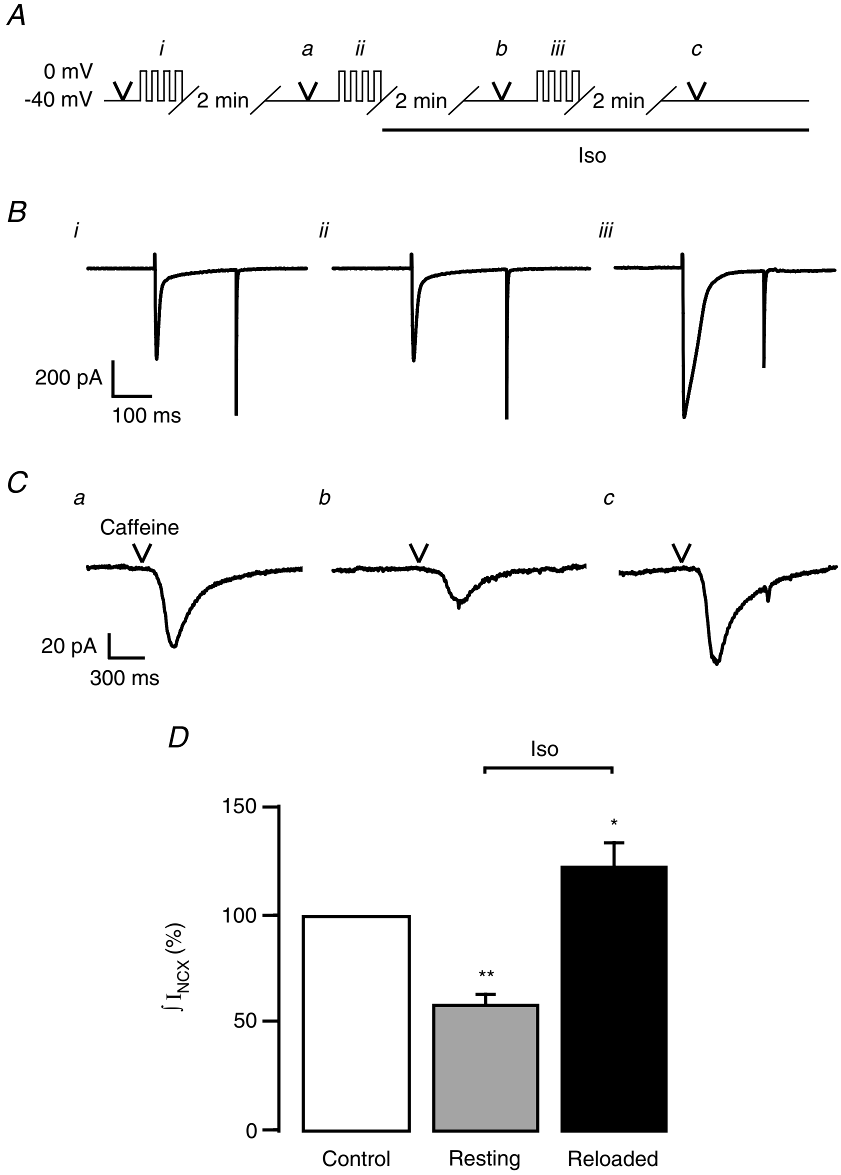

Figure 4. SR Ca^{2+} content governs the amplitude of whole-cell Ca^{2+} signals
A, protocol used to estimate total SR Ca^{2+} content. Short puffs of caffeine were applied in control (*Aa*), after a similar loading protocol and 2 min rest in Iso (*Ab*) and after reloading during β-adrenergic stimulation (*Ac*). *B*, third L-type Ca^{2+} current of a train of four consecutive depolarizations: *Bi*, in control; *Bii*, in control and before Iso application for 2 min; and *Biii*, during β-adrenergic stimulation. *C*, caffeine-induced NCX currents recorded in the 3 different conditions described above (*Ca*, *Cb* and *Cc*, respectively) were integrated to estimate the SR Ca^{2+} load. *D*, normalized $\int I_{NCX}$: control (100%, $n = 10$), resting in Iso (58.6 ± 7.3%, $n = 6$, $P < 0.02$) and stimulated in Iso (123 ± 11.7%, $n = 10$, $P < 0.05$).

various amplitudes were required for later comparison of the signals and their properties, which may depend on the signal amplitude itself. As in our previous studies using two-photon photolytic activation of CICR (Lipp & Niggli, 1998; DelPrincipe *et al.* 1999), the first series of TPP power–response relationships was obtained in caffeine to estimate the photolytic component of the Ca^{2+} signals (normalized to the highest two-photon power applied, $n = 6$). Caffeine, in our hands, did not interfere with the TPP signals and the quench of fluo-3 fluorescence was negligible ($-0.5 \pm 0.2\%$, $n = 2$). After reloading the SR with four depolarizing steps another TPP power–response relationship was recorded in control solution ($120.4 \pm 4.7\%$, $n = 6$) and immediately followed by a short caffeine application to empty the SR. After SR reloading and a subsequent resting period of 2 min in Iso, another TPP power–response relationship was recorded. During these 2 min at rest, the SR Ca^{2+} content was known to decay more in Iso than in control solution, as we found above (see Fig. 4). Surprisingly however, despite reduced SR Ca^{2+} content and much unlike global Ca^{2+} signals, the local Ca^{2+} release signals were larger during β-adrenergic stimulation than in control ($144.5 \pm 7.4\%$, $n = 6$). When the SR was reloaded during β-adrenergic stimulation to an extent that was above control, only a small and not significant further increase of the signal amplitude was found, despite the higher SR Ca^{2+} load under these conditions (to $158.5 \pm 9.0\%$, $n = 6$). The weak effect of the reloaded SR on the local signals was expected since cells were kept at low SR Ca^{2+} load to avoid the appearance of regenerative Ca^{2+} signal spreading. A more prominent and significant increase in the signal amplitude has been observed with higher SR Ca^{2+} loads, such as after 12 prepulses (Lipp & Niggli, 1998). To estimate the Iso effect on SR Ca^{2+} release the photolytical component obtained in caffeine was subtracted from the total signals obtained for each trigger in control and in Iso at low and high SR Ca^{2+} load. After normalization

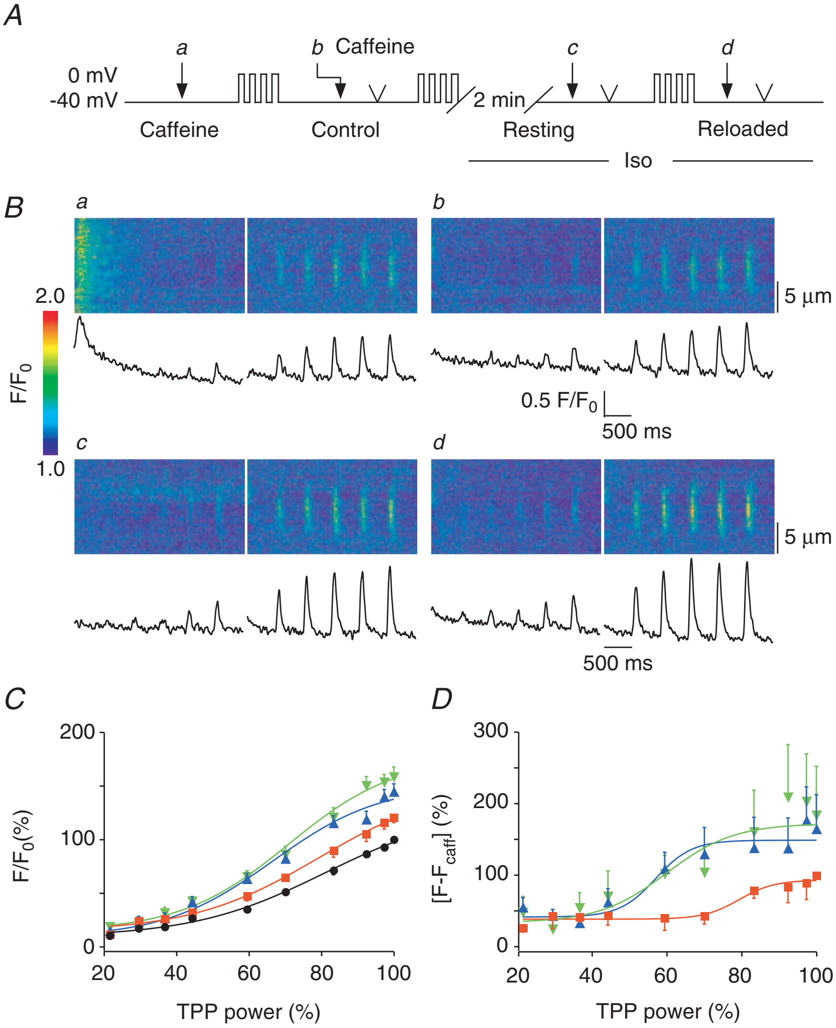

Figure 5. The amplitude of local Ca^{2+} signals is increased after β-adrenergic stimulation

A, protocol used to obtain the power–response relationships in conditions similar to those of Fig. 4. The cells were held at −40 mV and diffraction-limited Ca^{2+} releases of increasing power were triggered in caffeine (*Aa*), in control (*Ab*), after a similar loading protocol and 2 min rest in Iso (*Ac*) and after 4 consecutive depolarizations in Iso (*Ad*). *B*, line-scan images and averaged Ca^{2+} traces recorded during diffraction-limited releases. Ca^{2+} signals recorded in caffeine were used to estimate the amount of photolysis (*Ba*). Local Ca^{2+} signals were larger after 4 depolarizations in control (*Bb*) and further increased after Iso superfusion (*Bc* and *Bd*), independently of the global SR Ca^{2+} load (Fig. 4). *C*, normalized amplitude of Ca^{2+} signals for $n = 6$ cells in caffeine (●, 100% for the highest power), in control (■, $120.4 \pm 4.7\%$, $n = 6$), in Iso (▲, $144.5\% \pm 7.4\%$, $n = 6$) and after reloading in Iso (▼, $158.5\% \pm 9.0\%$, $n = 6$) are plotted *versus* power and fitted with sigmoidal functions. *D*, same data as in *C* but after subtraction of the photolytical component (signals in caffeine). CICR signals obtained in control (■, 100%, $n = 4$, for the largest release) were significantly larger after Iso superfusion, whether cells were resting (▲, $164.6 \pm 47.7\%$, $n = 5$) or being reloaded (▼, $184.2 \pm 67.6\%$, $n = 6$).

to the CICR amplitude obtained for the maximal trigger in control (100%, $n = 4$), data were plotted *versus* power (Fig. 5*D*) and fitted with a sigmoidal function. Interestingly, CICR started at lower power levels and was larger in Iso whether the SR was depleted (164.6 ± 47.7%, $n = 5$) or reloaded (184.2 ± 67.6%, $n = 6$). Taken together, these findings suggest that β-adrenergic stimulation with Iso enhanced local SR Ca^{2+} release irrespective of global SR Ca^{2+} content, much unlike the behaviour of global Ca^{2+} transients triggered with UV flashes.

What could be responsible for this discrepancy between global and local Ca^{2+} signals? Could we derive conclusions regarding the underlying mechanism from this peculiar behaviour? First we excluded possible Ca^{2+} sources other than the SR by confirming the ryanodine sensitivity of the signal amplification by Iso (data not shown). Given that the source of the Ca^{2+} seemed to be the SR, only two basic possibilities remained: (1) either the SR Ca^{2+} content was elevated above the remainder of the SR network at the site where we performed photolysis or (2) local Ca^{2+} release from the SR was larger despite the reduced SR Ca^{2+} content and regardless of the smaller Ca^{2+} gradient across the SR membrane. The latter possibility would require a change of the gating or Ca^{2+} permeability of the RyRs. Because of the slow kinetics of the TPP (Ca^{2+} source lasting for 60 ms) one could imagine that some of the photoreleased Ca^{2+} could be locally transported into the SR. The extent of this local uptake would depend on the activity of the SERCA. When more Ca^{2+} is pumped into the SR, the local SR Ca^{2+} content could increase above the remainder of the SR network, which could explain our observations of enhanced local Ca^{2+} releases from the SR in the presence of Iso. To test for the possibility of local Ca^{2+} uptake we applied CPA to inhibit the SERCA. CPA would prevent the local amplification of the TPP Ca^{2+} signals if it were mediated by the SERCA. Thus, the following experiments were carried out (Fig. 6): a first TPP power–response relationship was obtained during superfusion of caffeine. After SR reloading another series of Ca^{2+} signals was recorded, and this was repeated after

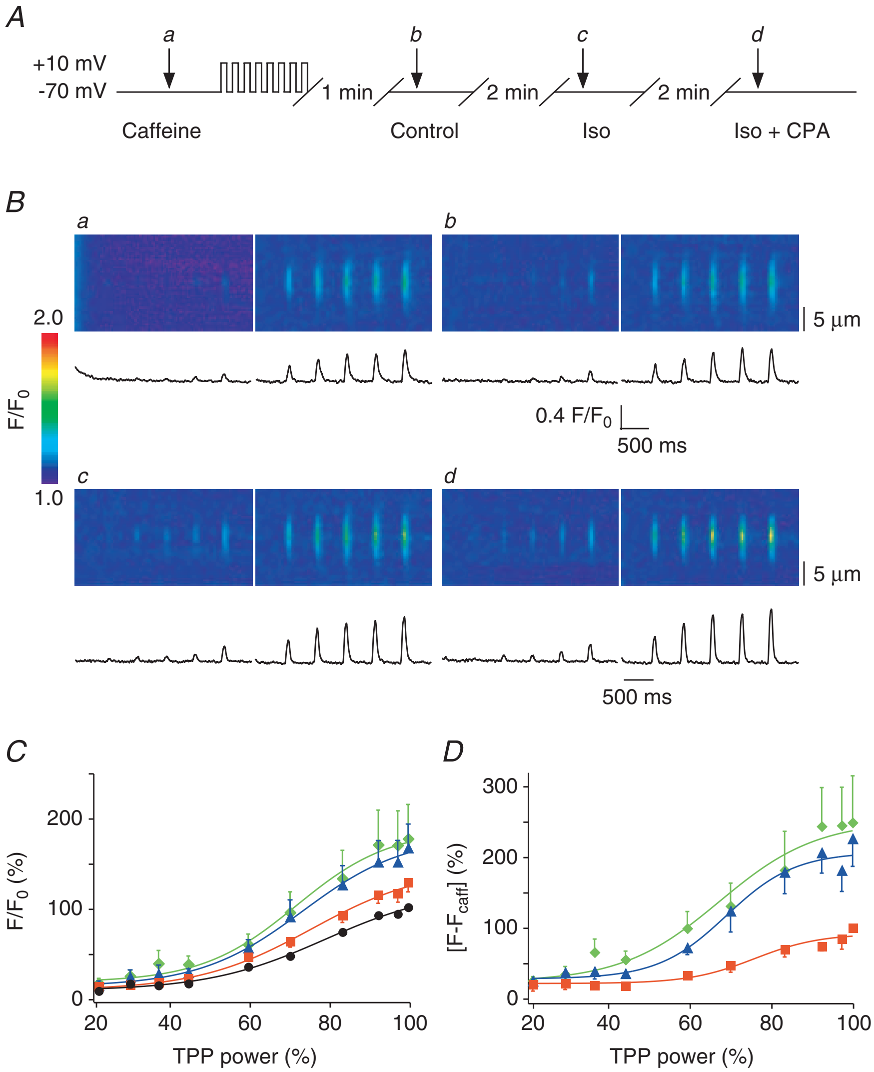

Figure 6. Enlargement of local Ca^{2+} signals is not prevented by superfusion of CPA

A, cells were held at −70 mV and a first TPP power–response relationship was triggered in caffeine to determine the amplitude of photoreleased Ca^{2+} signals (*Aa*). Cells were then reloaded by eight 200 ms depolarizing steps to +10 mV and after 1 min rest in control, a TPP power–response relationship was recorded (*Ab*). After 2 min superfusion in Iso another power–response relationship was measured (*Ac*), which was followed by 2 min of superfusion with Iso and CPA (10 μM), after which a last TPP power–response relationship was elicited (*Ad*). *B*, data obtained from one cell: *Ba*, line-scan images and traces obtained in caffeine; *Bb*, in control; *Bc*, in Iso; and *Bd*, in Iso and CPA. *C*, plot of the normalized amplitude of Ca^{2+} release *versus* TPP power in caffeine (●, 100% for the maximal trigger, $n = 14$), control (■, 126.8 ± 10.3%, $n = 14$), in Iso (▲, 163.7 ± 25.7%, $n = 14$) and in Iso with CPA (◆, 173.7 ± 36.8%, $n = 11$). *D*, same data as in *C* but after subtraction of the photolytical component (control: ■, 100%, $n = 11$; Iso: ▲, 226.4 ± 39.3%, $n = 10$; and Iso + CPA: ◆, 249.2 ± 66.1%, $n = 9$).

treatment with Iso. Again, local Ca^{2+} signals were larger during β-adrenergic stimulation than in control and the signals obtained in the presence of functioning CICR were significantly larger than in caffeine, as expected. Finally, the SERCA was blocked by 2 min superfusion of 10 μM CPA in the presence of Iso. As it turned out, CPA could not prevent the amplification of CICR by β-adrenergic stimulation (Fig. 6*B*). Figure 6*C* presents data obtained from 14 cells and normalized to the local release signal recorded in caffeine for the maximal TPP power (100%). The Ca^{2+} signal recorded in control was $126.8 \pm 10.3\%$, demonstrating the presence of the biological signal amplification by CICR. The signal obtained during Iso superfusion had an amplitude of $163.7 \pm 25.7\%$ ($n = 14$), and in the presence of both CPA and Iso, the amplitude was $173.7 \pm 36.8\%$ ($n = 11$). After subtraction of the photolytical Ca^{2+} release component, it became evident that CICR was increased to $226.4 \pm 39.3\%$ ($n = 10$) by Iso and remained unaffected at $249.2 \pm 66.1\%$ ($n = 9$) when the SERCA was inhibited (Fig. 6*D*). This set of data suggests that CPA could not suppress the amplification of local CICR by β-adrenergic stimulation. To confirm successful inhibition of the SERCA by CPA in these experiments, decay rates after UV flash-triggered Ca^{2+} transients were analysed (Fig. 3*E*) confirming successful inhibition of the SERCA by CPA.

Finally changes in resting Ca^{2+} were not significant between control and Iso ($2.5 \pm 5.3\%$, $n = 14$) or between control and Iso + CPA ($7.7 \pm 10.5\%$, $n = 11$), suggesting that cytosolic Ca^{2+} did not interfere with the increased local signals. In summary, these findings indicate that local loading of the SR with Ca^{2+} did not play an important role in the amplification of the TPP release signals by β-adrenergic stimulation. Thus, the increased TPP Ca^{2+} fluorescent signals were resulting from more Ca^{2+} being released from the SR despite reduced Ca^{2+} content.

Discussion

The notion of a cardiac Ca^{2+} signalling system relying on the recruitment of functionally independent elementary signalling events (Ca^{2+} sparks) has opened the door for a new concept of EC coupling incorporating a probabilistic paradigm, whereby each Ca^{2+} spark event is triggered under local control by an L-type Ca^{2+} channel with a specific non-zero probability (Cheng *et al.* 1993; Lipp & Niggli, 1998; Niggli, 1999). Such an arrangement not only allows for a new understanding of modulatory changes of EC coupling, but also for a novel mechanism of EC coupling failure under pathological conditions, whereby the Ca^{2+} spark trigger probability could be altered by modulatory, metabolic and pathophysiological mechanisms (Gomez *et al.* 1997; Marx *et al.* 2000; Pacher *et al.* 2002; Isaeva & Shirokova, 2003). Two mechanisms are thought to be very important for this type of regulation: the Ca^{2+} content of the SR (Terentyev *et al.* 2003) and the phosphorylation of various Ca^{2+} signalling proteins (Takasago *et al.* 1991; Simmerman & Jones, 1998; Marx *et al.* 2000). These two regulatory pathways are expected to exhibit a high degree of cross-talk because of at least two reasons: (1) functional changes of Ca^{2+} signalling proteins after phosphorylation will affect SR Ca^{2+} content (Hussain & Orchard, 1997); (2) changes of Ca^{2+} concentrations may alter the extent of phosphorylation, for example through the CaMKII pathway.

In the present study we used a combination of biophysical techniques in an attempt to dissect these two regulatory pathways, for example by using photolysis of caged compounds as a trigger for CICR to remove some variables (e.g. changes of I_{Ca} after phosphorylation of L-type Ca^{2+} channels). With this combination of approaches we were able to simplify the system, eliminate some variables while controlling and measuring others. By comparing features of global (i.e. whole-cell) Ca^{2+} fluorescent signals with those of subcellularly localized signals (i.e. diffraction-limited two-photon photorelease signals) we could derive conclusions about the relative significance of either mechanism for CICR (i.e. the SR Ca^{2+} load or the phosphorylation of Ca^{2+} signalling proteins) under the two conditions.

Whole-cell signals are dominated by SR Ca^{2+} load

It is known that an increase in luminal Ca^{2+} can enhance the Ca^{2+} sensitivity on the cytosolic side of RyRs reconstituted in lipid bilayers (Sitsapesan & Williams, 1997). This finding may also be related to the generation of spontaneous Ca^{2+} sparks and Ca^{2+} waves in isolated cardiac myocytes under conditions of SR Ca^{2+} overload (Wier *et al.* 1987; Lukyanenko *et al.* 1999; Terentyev *et al.* 2003). In contrast, a decrease of SR Ca^{2+} content is thought to control termination and refractory behaviour of CICR in cardiac myocytes (DelPrincipe *et al.* 1999; Sobie *et al.* 2002; Terentyev *et al.* 2002; Szentesi *et al.* 2004). Our data show that whole-cell Ca^{2+} signals are largely governed by the SR Ca^{2+} load, a feature which possibly masks subtle effects due to changes of RyR Ca^{2+} sensitivity arising from phosphorylation. Indeed, irrespective of how many RyRs open before or after their phosphorylation, only a specific releasable fraction of SR Ca^{2+} will be liberated into the cytosol (previously determined to be typically 57% under our conditions (DelPrincipe *et al.* 1999)). Thus, the number of opening RyRs will only affect the kinetics of SR Ca^{2+} release, but not increase the amount of Ca^{2+} that can be released (unless CICR would be more exhaustive after RyR phosphorylation, for which there is little evidence). Thus, with or without β-adrenergic stimulation, the extent of whole-cell SR Ca^{2+} release is dominated (i.e. limited) by SR Ca^{2+} content and by processes which terminate CICR by functional SR Ca^{2+} depletion.

Local signals reveal effects of β-adrenergic stimulation

Interestingly, the present study revealed that subcellularly localized SR Ca^{2+} signals exhibited a strikingly different behaviour than whole-cell signals. Even though local Ca^{2+} signals also depend somewhat on the SR Ca^{2+} load, particularly at more elevated SR Ca^{2+} loads (Lipp & Niggli, 1998; DelPrincipe *et al.* 1999), the localized Ca^{2+} releases are not expected to significantly lower the global SR Ca^{2+} content. Since SR Ca^{2+} release only occurs from one or a few functional units of the SR, we think that local depletion of intra-SR Ca^{2+} will be less pronounced, since neighbouring SR sites do not release and Ca^{2+} can, in fact, rapidly diffuse to the active sites within the SR network. Thus, under these conditions more open channels allow more Ca^{2+} to be released (Sobie *et al.* 2002), even at reduced SR Ca^{2+} content. Thus, the declining global SR Ca^{2+} load no longer terminates the release flux, contrary to the whole-cell activation. Thus, TPP signals might be a suitable technique to allow observation of localized SR Ca^{2+} release process modulation.

Estimates of CICR

Subtraction of fluorescent signals obtained in caffeine from signals in control, Iso or Iso and CPA was used to estimate the amount of CICR. As seen in Figs 5*D* and 6*D*, a threshold seems to appear at about 70–80% of the maximal trigger power in control, while it is shifted toward smaller triggers in Iso or in Iso and CPA. For the maximal trigger, Iso approximately doubled the caffeine-insensitive component (165 to 249%); as for the lowest trigger power range no clear separation could be made. This suggests that very low photolytic power may not detectably increase the open probability of the RyRs at the low SR Ca^{2+} load used in this study, thereby triggering no CICR. Finally, the sigmoidal function fit to the data point tends to become more shallow and saturate. In the droplet, saturation occurs at very high powers and is thought to result from depletion of DM-nitrophen within the diffraction limited excited volume. In a droplet this is expected to occur at higher photolytic power levels than in cells, because DM-nitrophen diffusion may not be entirely free in the cytosol. In addition, inside living cells many other factors may also contribute to saturation and shallow release functions, including mobile and stationary Ca^{2+} buffers and saturation of CICR.

Conclusion

In conclusion, we found that when CICR was activated in a synchronized way throughout the cell, the Ca^{2+} signals were predominantly governed by SR Ca^{2+} content, thus defining the amplitude of the resulting Ca^{2+} transient. Although β-adrenergic stimulation may lead to synchronization of SR Ca^{2+} release channels (Song *et al.* 2001; Viatchenko-Karpinski & Györke, 2001; Ginsburg & Bers, 2004), this will not necessarily affect the amount of global Ca^{2+} release from the SR. This observation on the global level is therefore consistent with the notion that whole-cell SR Ca^{2+} release is terminated by emptying of the Ca^{2+} store, leading to deactivation of the RyRs by reducing their Ca^{2+} sensitivity (DelPrincipe *et al.* 1999; Terentyev *et al.* 2002; Szentesi *et al.* 2004). However, when CICR was activated on a local level (i.e. with TPP), the system behaved in a completely different and apparently opposite way, that cannot be explained by global changes in SR Ca^{2+} content. Insensitivity of these unexpected signals to inhibitors of the SERCA also suggested that these effects were not mediated by increased local SR Ca^{2+} loading via the SERCA that could, in principle, occur before and during the photorelease process. Increased local Ca^{2+} releases were thus observed regardless of the decreased SR content, suggesting a direct modulatory effect of the β-adrenergic signalling cascade on the SR Ca^{2+} release pathway. Increased Ca^{2+} release from the SR despite reduced Ca^{2+} content can only occur when the open probability of the RyRs is somehow increased, either by activation of more channels or by changing the gating properties of the channels (or both). Based on these observations, we conclude that increased local Ca^{2+} release signals are the result of the PKA-dependent phosphorylation of a Ca^{2+} signalling protein other than the L-type Ca^{2+} channels, phospholamban and the SR Ca^{2+} pump, and possibly the ryanodine receptors themselves (or other SR proteins).

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