ONLINE SUPPLEMENTS

Material and Methods

Atrial myocytes from adult male C57/BL6 mice, obtained from the Central Animal Facility, University Hospital, University of Bern, were freshly isolated by Langendorff perfusion technique. All experiments were approved by the State Veterinary Office of Bern, Switzerland, according to Swiss Federal Animal protection law. The authors have also read, and the experiments comply with, the policies and regulations of *The Journal of Physiology* described by Drummond, 2009 (Drummond, 2009). Adult male C57/BL6 mice (3-5 months old) were heparinized (50 U ip) and subsequently killed after 5-15 minutes by cervical dislocation. Hearts were rapidly removed and mounted on a Langendorff column and perfused retrogradely for 8-16 minutes with collagenase type II (14 U/ml, Worthington type 2) and protease type IV (0.2 U/ml, Sigma, type XIV) in a Ca^{2+} free solution (in mmol/L: 140 NaCl, 5.4 KCl, 1.1 $MgCl_2$, 1 Na_2HPO_4, 5 HEPES and 10 glucose at pH 7.4, 37ºC). Atria (auricle) were removed and rinsed in 1% BSA solution before cells were dissociated mechanically from the tissue in Ca^{2+} free solution. The free $[Ca^{2+}]_o$ was increased progressively to 500-700 μM over 1-2 hours before cells were used for experiments within 4 hours after isolation. All experiments were performed at room temperature.

HeLa cells were a kind gift from N. Demaurex's lab, Geneva. HeLa cells were cultured on glass cover slips in DMEM, 10% FBS, 1% HEPES, 0.1% penicillin / streptomycin until 80% confluent. Once 80% confluency was reached, cells were used for experiments. Ca^{2+} responses were detected with K_5-fluo-3 after flash photolysis of 30 μM caged $InsP_3$ in the patch pipette.

Patch Clamp Recordings with caged $InsP_3$. Electrophysiological recordings were combined with simultaneous confocal Ca^{2+} imaging. Caged $InsP_3$ (Sichem) was dialyzed into the atrial myocytes via low resistance pipettes (1.5-3 MΩ) which were pulled from borosilicate glass micropipettes using a Zeitz DMZ puller (Zeitz instruments, Germany). The pipette solution contained (in mmol/L) 120 CsAsp, 10 HEPES, 20 TEA-Cl, 5 K-ATP,1 $MgCl_2$, 0.1 K_5-fluo-3 (Biotium), 2 GSH, (0.03, 0.06, 0.24) caged $InsP_3$ 6Na (Sichem), pH 7.2 with CsOH. Free Ca^{2+} was calculated using Patcher's Power Tools (IgorPro plug-in, MPI Göttingen, Germany) and was approximately 59 nM. During the experimental procedure, cells were perfused with external solution containing (in mmol/L) 140 NaCl, 5 HEPES, 1.1 $MgCl_2$, 5.4 KCl, 10 Glucose, 1.8 $CaCl_2$, 0.5 BaCl, 1 CsCl, pH 7.4 NaOH. Pharmacological experiments used 5 μM 2-aminoethoxydiphenyl borate (2APB, Fluka), 5 μM xestospongin C (Xesto, A.G Scientific Inc), 1 mM tetracaine (TET, Sigma) or 10 mM caffeine (Sigma) added to the external solution.

Cells were held at -80 mV and depolarized from -80 mV to 0mV ten times before line-scan images were taken; membrane voltages were controlled with an Axopatch 200 amplifier (Axon Instruments, Foster City, CA) driven by custom-written software developed under LabView software (National Instruments). Fluo-3 was excited with the 488 nm line of an argon laser alternatively with a solid-state laser (50 μW; Sapphire, Coherent) and fluorescence (> 515 nm) in line-scan mode (6 ms/line) was collected with a PMT on an MRC-100 or MicroRadiance confocal microscope (both BioRad, Glattbrugg, Switzerland). Line-scans of 1024 lines were collected in 2 equal, sequential segments. In line-scan recordings where $InsP_3$ was uncaged, UV light from a xenon short-arc flash lamp (300-350 nm, pulse duration ~ 400 μs, discharge energy up to 230 J) was delivered and applied at the beginning of line-scan recordings. For more details see Del Principe *et al*. (DelPrincipe *et al*., 1999). Line-scan images were processed using customized Image SXM software (Steve Barrett, University of Liverpool, UK). 3000 ms were used for analysis of each line-scan. Sparkmaster plug-in (Picht *et al*., 2007) for ImageJ (Wayne Rassband, NIH, USA) was used to identify and analyze Ca^{2+} release events. IgorPRO (WaveMatrics, Portland, OR, USA) software was used to analyze caffeine transients.

Ca^{2+} leak measurements. Using the RyR2 blocker TET, Ca^{2+} leak was determined and using the SR-Ca^{2+} pump (SERCA) blocker cyclopiazonic acid (CPA), the increase in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) was determined with an adapted protocol from Shannon *et al*. (Shannon *et al*., 2002). Line-scans were recorded at 50 l/s on a laser scanning confocal microscope (MicroRadiance, BioRad, UK). Atrial myocytes were loaded with 5 μM fluo-3 AM (Biotium) for 20 min and left for de-estrification for 15 minutes before confocal images were collected. Fluo-3 (Biotium) was excited using an argon ion laser. Cells were field stimulated for at least 45 seconds at 1 Hz before line-scan images were recorded. After the pre-loading protocol, superfusion was rapidly switched from (in mmol/L) 140 NaCl, 5 KCl, 5 HEPES, 1 MgCl, 1.8 $CaCl_2$, 10 glucose, pH 7.4 with NaOH to a nominally Na^+ and Ca^{2+}-free solution (by substitution of Na^+ with Li^+ and addition of 0.5 mmol/L EGTA). A further solution switch was done after 10 s to a nominally Ca^{2+} and Na^+ free solution containing CPA for 30 s, before caffeine was applied to measure SR-Ca^{2+} load. The same protocol was repeated in the presence of 100 nM ET-1 in all solutions. CPA application was accompanied with an increase in $[Ca^{2+}]_i$.

Immunofluorescent staining. Atrial myocytes were enzymatically isolated as described earlier. Without increasing Ca^{2+}, 500 μl cell dispersion was left to settle for 2 hours at room temperature on previously prepared gelatin coated coverslips. Samples were washed twice before they were fixed in 4 % paraformaldehyde for 10 minutes. Prior to staining, samples were washed one more time with

PBS before cells were permeabilized and unspecific sites were blocked for 30 min in 0.1% Triton-X-100, 5 % BSA in PBS. Myocytes were stained against InsP3R2 and RyRs by incubating the samples with rabbit polyclonal $InsP_3R2$ (Abcam, 1:500, Cat.# ab77838, see also Wang *et al.,* 2012) and mouse monoclonal RyR (Abcam 1:200, Cat.# ab2827) antibodies overnight at 4ºC in 0.1% Triton-X-100, 5 % BSA in PBS. Cells were then again washed 3 times in 0.1% Triton-X-100, 5 % BSA in PBS for 10 minutes before incubating with the secondary antibodies (Alexa Fluo 488, 1:1000; Alexa Fluo 568, 1:1000) and DAPI , 1:20000 for 1.5 hours at room temperature. Cells were then washed 3 times with PBS before coverslips were mounted on slides with mounting medium (Fluoroshield). Cells were imaged using a fluorescent microscope (TMD300 microscope, Nikon) equipped with a high-sensitivity Visicam camera (Visitron Systems, Germany).

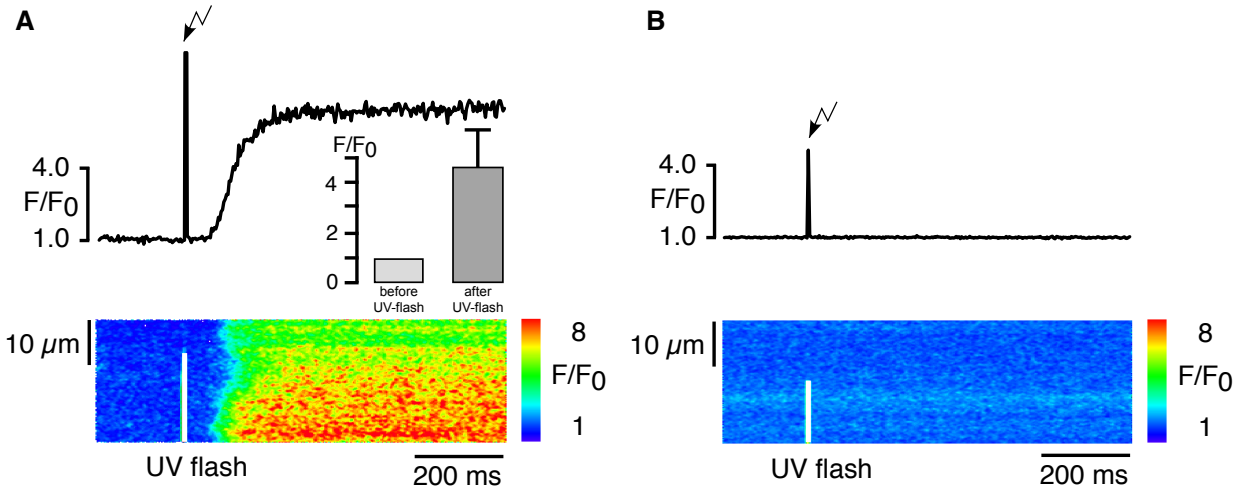
Verification of $InsP_3R$ Ca^{2+} release

In a series of control experiments we ruled out flaws in the applied experimental settings. HeLa cells are known to have a highly developed $InsP_3R$ pathway and were used as a bio-indicator for $InsP_3$ Ca^{2+} response. HeLa cells have several advantages, they express $InsP_3R$ at high density and $InsP_3R$ dependent elementary Ca^{2+} release events are well characterized. Although they express mainly type 1 and type 3 isoforms, $InsP_3Rs$ type 2 are also present (Tovey *et al*., 2001). This workaround was inevitable because there is still a deficit in the availability of $InsP_3$ indicators which could be used to monitor rapid intracellular $InsP_3$ -mediated signals. Indeed, by using UV-flash photolysis, robust $InsP_3$ Ca^{2+} transients were elicited (Fig. 1S). As shown in Fig. 1S, using identical settings and solutions containing 30 μM caged $InsP_3$, reliable global Ca^{2+} transients were photolytically triggered. This suggests that UV-flash triggered IP3ICR can be obtained similarly in cardiac cell preparations. The reliability of the experimental approach was further underscored by the dose dependency of $InsP_3$-induced increase in Ca^{2+} event frequency (Fig. 2C).

As a positive control for elementary Ca^{2+} release events we used neonatal rat cardiomyocytes that are known to exhibit both Ca^{2+} puffs and Ca^{2+} sparks (Luo *et al*., 2008). Fig. 2S A,B and C show individual photolytically triggered $InsP_3$ "Ca^{2+} puffs", "Ca^{2+} sparks" and local mini-waves identified by their spatio-temporal properties (Niggli & Shirokova, 2007).

For a variety of caged compounds it is known that only a small fraction of the total amount of the compound is being uncaged during a single UV-flash. This essentially depends on the physicochemical properties of the compound (e.g. quantum yield), the power of UV-light and the duration of illumination (Ellis-Davies, 2007), which in our hands is in the range of 200 μs. In addition, the pipette solution contains an infinite reservoir for caged $InsP_3$ compared to the cell volume. Finally, we would expect to end up in a biologically relevant concentration range of approximatively 10 nM - 1 μM $InsP_3$.

Figure 1S



Experimental approach to verify intracellular $InsP_3$ release.

Representative data showing averaged F/F_0 of fluo-3 and corresponding line-scan images of changes in $[Ca^{2+}]_i$ in HeLa cells under whole-cell conditions of the patch-clamp technique. $InsP_3$ was released by UV-flash photolysis. **A**. With 30 μM caged $InsP_3$ in the patch pipette there is an immediate global increase in Ca^{2+} after UV flash photolysis observed in 100% of all approached cells. The inset shows normalized fluorescence F/F_o before and after UV-flash (n=6); **B**. Control, with no $InsP_3$ in the patch pipette, shows no response to flash photolysis. n=5-12

Figure 2S

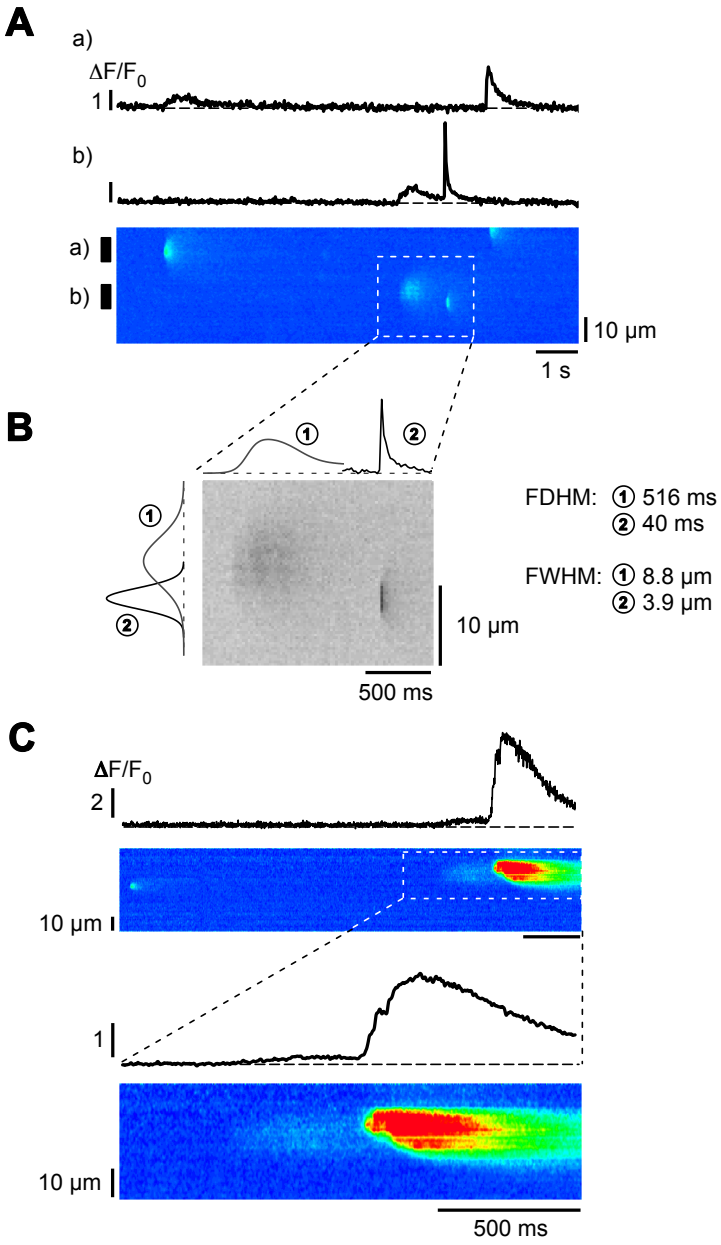


Figure 2S. Representative data showing averaged $\Delta F/F_0$ of fluo-3 fluorescence and corresponding line-scan images of changes in $[Ca^{2+}]_i$ in neonatal rat myocytes in response to $InsP_3$ photorelease. Cells were loaded with 5 μM $InsP_3$-PM (D-2,3-O-Isopropylidene-6-O-(2-Nitro-4,5-Dimethoxy) benzyl-myo-Inositol-1,4,5-Trisphosphate-Hexakis Propionoxymethyl Ester, SiChem) together with 4 μM fluo-3-AM (Biotium) for 120 and 30 min at room temperature, respectively. $InsP_3$ was liberated by UV-flash photolysis as described in the Material and Methods section. **A. a), b)** Individually triggered Ca^{2+} puff consecutively followed by a Ca^{2+} spark. **B.** Expanded part of the line-scan given above, now presented in unprocessed raw data format. From FDHM and FWHM analysis (1) can be classified as Ca^{2+} puff and (2) as Ca^{2+} spark (Picht *et al.*, 2007). **C.** Ca^{2+} mini-wave elicited in response of initial $InsP_3$ triggered local $[Ca^{2+}]_i$ increase. This local Ca^{2+} response shows that elementary Ca^{2+} release *via* $InsP_3R$ may be involved in the conditioning of CICR (e.g. Ca^{2+} mini-waves as given in this example) in cardiac cells.

Figure 3S

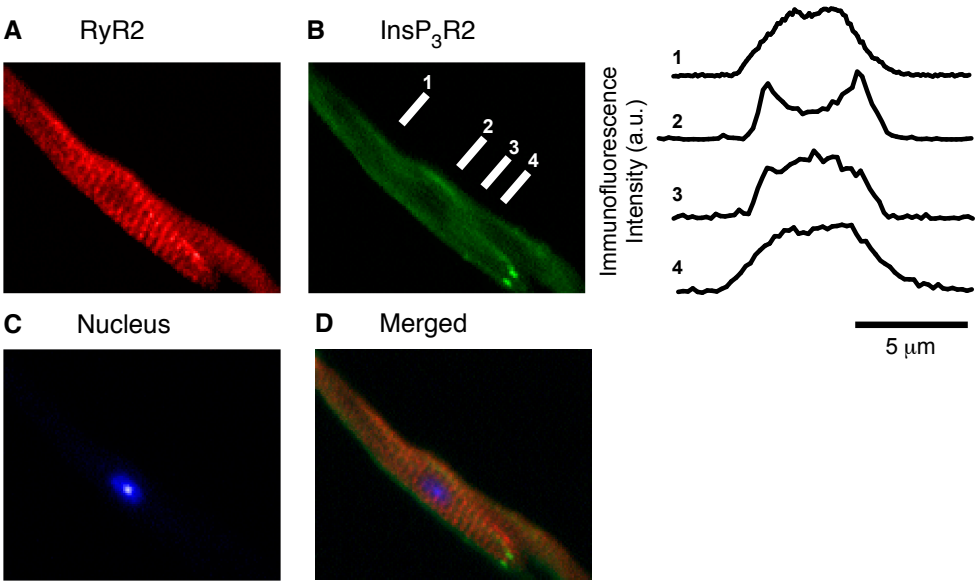


Figure 3S. Colocalization of RyR2 and $InsP_3R2$ in an atrial myocyte. **A**. Atrial myocyte was stained against RyR2 (primary antibody from Abcam 1:200, secondary Alexa Fluo 568 1:1000) **B**. Atrial myocyte was stained against $InsP_3R2$ (primary antibody from Abcam 1:500, secondary Alexa Fluo 488 1:1000), intensity profiles of immunofluorescence across the cell (right). These profiles were obtained by sampling the immunofluorescence intensity of the $InsP_3R2$ isoform across a line one pixel wide. **C**. The nucleus of atrial myocyte was stained with DAPI 1:20000 **D**. Merged image.

Ca^{2+} Spark characteristics.

Ca^{2+} spark characteristics were analyzed as mentioned in the Material and Methods section. Myocytes were divided into two groups.

Supplementary Table 1

events*100μm^{-1}s^{-1} <1.3 *10 myocytes, 4 animals*	**No $InsP_3$**	**$InsP_3$**	**p value**
Ampl ($\Delta F/F_0$)	0.79±0.05	0.87±0.06	0.33
FWHM (μm)	2.71±0.17	2.98±0.27	0.23
FDHM (ms)	51.6±2.6	50.03±3.18	0.67
fullwidth (μm)	**3.80±0.29**	**4.51±0.38**	**0.02**
fulldur (ms)	93.52±5.96	107.49±6.43	0.07
rise time (ms)	32.79±3.09	35.75±4.53	0.06
events*100μm^{-1}s^{-1} >1.3 *9 myocytes 5 animals*	**No $InsP_3$**	**$InsP_3$**	**p value**
Ampl ($\Delta F/F_0$)	1.11±0.05	0.83±0.06	0.19
FWHM (μm)	2.84±0.18	2.60±0.28	0.47
FDHM (ms)	47.87±2.75	47.62±3.35	0.97
fullwidth (μm	4.82±0.30	4.11±0.40	0.24
fulldur (ms)	120±6.28	99.74±6.78	0.31
rise time (ms)	50.60±3.25	36.88±4.77	0.18

REFERENCES

DelPrincipe F, Egger M & Niggli E (1999). Calcium signalling in cardiac muscle: refractoriness revealed by coherent activation. *Nat Cell Biol* **1,** 323–329.

Drummond GB (2009). Reporting ethical matters in the Journal of Physiology: standards and advice. *J Physiol* **587,** 713–719.

Ellis-Davies GCR (2007). Caged compounds: photorelease technology for control of cellular chemistry and physiology. *Nat Meth* **4,** 619–628.

Luo D, Yang D, Lan X, Li K, Li X, Chen J, Zhang Y, XIAO R-P, Han Q & Cheng H (2008). Nuclear Ca^{2+} sparks and waves mediated by inositol 1,4,5-trisphosphate receptors in neonatal rat cardiomyocytes. *Cell Calcium* **43,** 165–174.

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

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

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

Tovey SC, De Smet P, Lipp P, Thomas D, Young KW, Missiaen L, De Smedt H, Parys JB, Berridge MJ, Thuring J, Holmes A & Bootman MD (2001). Calcium puffs are generic $InsP_3$-activated elementary calcium signals and are downregulated by prolonged hormonal stimulation to inhibit cellular calcium responses. *J Cell Sci* **114,** 3979–3989.

Wang Y, Li G, Goode J, Paz JC, Ouyang K, Screaton R, Fischer WH, Chen J, Tabas I & Montminy M (2012). Inositol-1,4,5-trisphosphate receptor regulates hepatic gluconeogenesis in fasting and diabetes. *Nature* **485,** 128–132.