Transient Ca^{2+} depletion of the sarcoplasmic reticulum at the onset of reperfusion.

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Supplementary Data

Methods

1. Heart preparation

Hearts were perfused with normal Tyrode solution (2 mM $CaCl_2$, 140 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 0.33 mM Na_2HPO_4, 10 mM HEPES, 10 mM glucose, pH=7.4) in a horizontal Langendorff apparatus. All reagents and chemicals were purchased from Sigma Chemical Company unless otherwise stated. Temperature of the bath solution, in which the heart was immersed, was controlled with a Peltier unit. All solutions were equilibrated with 100% oxygen.

After electrically ablating the pacemaker cells of the sinoatrial and atrioventricular nodes using an ophthalmic bipolar pencil (Mentor Ophthalmics, Santa Barbara, CA), the hearts were continuously paced at various rates by means of an electrical stimulator

ISOSTIM A320R (World Precision Instruments Inc, Sarasota, FL, USA) controlled by a PC.

2. ECG and Ventricular pressure measurements

Transmural ECG was obtained with the aid of two micro silver - silver chloride pellets were positioned inside the left ventricle, through the mitral valve, and outside the chamber. Both, the electrocardiographic signal and the ventricular pressure measurements were recorded as differential signals and acquired with a different A/D converter at a sampling frequency of 50 kHz. Both acquisition systems were controlled simultaneously by a Pentium III-based PC running a custom-designed, G-based software program (LabVIEW).

3. Fluorophores loading

All dyes were dissolved in 45 μL of DMSO with 2.5% pluronic and added to 1 ml of Tyrode. Perfusion with dye was started after the spontaneous heart rate became regular (within 10 min after cannulation). After 25-35 min of perfusion at room temperature, the solution was switched to normal Tyrode solution and the temperature was increased to 37^{o}C within 10 min. Increase in the temperature was supposed to induce the washing of Mag-fluo-4 from the cytosol, allowing the measurements of intra-SR Ca^{2+} signal. In most cases, a downward fluorescence signal reflecting depletion of SR was prevailing even before warming up, although some minor upward (cytosolic) component was still present. This last component completely disappeared within 10-20 minutes after the temperature reached 37^{o}C. After Mag-fluo-4 was removed from the cytosol, sufficient amount of the dye remained inside of SR to generate detectable signals for at least 2

hours. The other dyes were prepared and loaded in the same way as Mag-fluo-4 AM. The time of loading was 25-35 min.

4. Optical setup

Two solid-state YAG lasers (Enlight Technologies, Branchburg, NJ, USA) were used as illuminating source. Blue light (473 nm) and green light (532 nm) for excitation of fluorescein-based and rhodamine-based dyes were obtained from a MBL-10-3 CW Ng-YAG and a MGL-50B-1 CW Ng-YAG laser, respectively. Both lasers were time-multiplexed by two ferroelectric modulators 50075 Oriel optical shutters (Newport, Stratford, CT, USA) and optically mixed with ultra-fast dichroic mirrors. The excitation light pulses were focused by a standard microscope objective (40x, NA 0.45) into a small multimode optical fiber for the transmission of the exciting light to the epicardial layers. Emitted light was carried back through the same fiber, filtered to eliminate the reflected excitation component, and focused on an avalanche diode that was connected to an integrating current-to-voltage converter controlled by Digital Signal Processor (DSP 320, Texas instruments, Texas). Headstage units and high-voltage power supply for them were manufactured by IonOptix (Milton, MA, USA). Fluorescence signals were digitized at a sampling frequency of 5 MHz and filtered to a bandwidth of 500 kHz. The acquisition system was controlled by an Athlon-based PC running a custom-designed, G-based software program (LabVIEW).

One end of the optic fiber was gently placed on the tissue. This effectively allowed synchronous movement of the end of the fiber together with the heart surface. Such procedure considerably attenuated the motion artifacts generated by the beating hearts. However, to fully exclude that not significant artefactual fluorescence changes due to contraction could have altered our conclusions, additional control experiments were

performed in the presence and absence of the mechanical uncoupler blebbistatin, as described below.

Results

Since the magnitude of contraction and motion of the beating heart differ following ischemic episodes and reperfusion, we performed control experiments in intact mouse hearts perfused with Tyrode solution in the presence or in the absence of 10 μM blebbistatin in order to discard any significant artifactual fluorescence changes due to contraction. Blebbistatin, which effectively prevents contractions by specifically inhibiting the actin-myosin interaction[1], is helpful in eliminating possible motion artefacts.

Prior to application of blebbistatin in the experiments, it was necessary to make sure that the inhibitor does not affect other components of excitation contraction system. Dou et al. (2007) and Fedorov et al. (2007)[1,2] have shown that blebbistatin in a concentration of 10 μM has no effect on ECG, the morphology of AP as well as on the inward Ca^{2+} current. However, blebbistatin may potentially change Ca^{2+} dynamics by increasing the availability of ATP due to inhibition of contraction. Although the kinetic analysis of the Ca^{2+} transients did not reveal significant changes caused by treatment with blebbistatin[2,3] the time-dependent recovery of the amplitude of Ca^{2+} release (*i.e.* the restitution) was not previously analyzed. In control experiments we compared the restitution of Ca^{2+} release as well as the relaxation kinetics of Ca^{2+} transients in intact mouse hearts perfused with Tyrode solution in the presence or in the absence of 10 μM blebbistatin.

The restitution of Ca^{2+} release was examined by applying extrasystolic stimulation pulses at various times after a regular pacing pulse (Figure 1). As shown in Table 1, blebbistatin does not noticeably alter the kinetics of Ca^{2+} release as well as the time-course of the restitution. Elevation of $CaCl_2$ concentration in Tyrode solution up to 5 mM did not produce any signs of sensitivity of kinetic parameters to 10 μM blebbistatin (see Table 1). This suggests that blebbistatin does not significantly modulate Ca^{2+} handling and can indeed be used as a specific inhibitor of contraction. It should be noted that blebbistatin is not only sensitive to light[4] but also can interact with fluorescent dyes such as Fluo-3, Fluo-4[3], and Fluo-5F[2]. Our own experiments suggest that the blebbistatin can interfere with Mag-fluo-4 AM (data not shown). Nevertheless, the absence of reported side effects related to AP and Ca^{2+} handling makes blebbistatin more preferable than 2,3-butanedione monoxime (BDM) and cytochalasin-D, other popular uncouplers of excitation-contraction cycle[3,5].

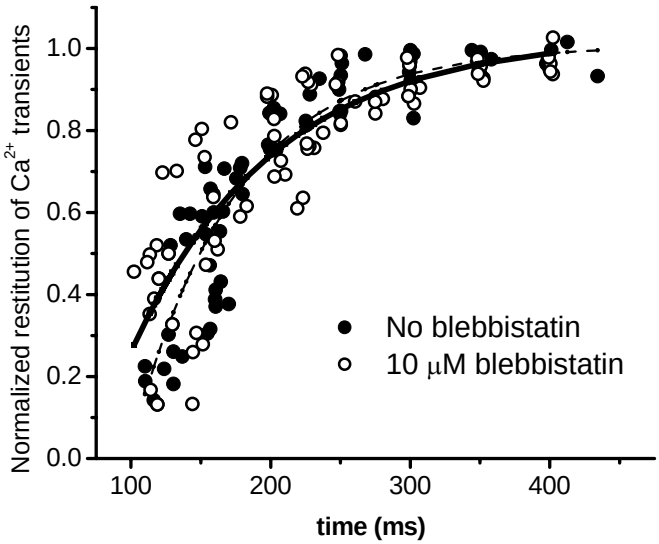

Figure 1. Effect of blebbistatin on Ca^{2+} transient restitution.

Table 1. Effect of blebbistatin (BBS) on Ca^{2+} dynamics in mouse hearts.

The experiments were conducted at 37°C and 2 Hz. Decay time constant (fall time) was determined as time between 10% and 90% of the relaxation. Data are means ± SD.

Parameter	**Solution**			
	2 mM $CaCl_2$		5 mM $CaCl_2$	
	No BBS	10 μM BBS	No BBS	10 μM BBS
Number of experiments	8	5	3	4
Time to peak (ms)	29±6	27±7	25±2	22±5
Fall time (ms)	68±17	76±12	60±27	54±17
Time to 50% peak recovery (ms)	147±21	133±28	107±42	125±48
Time constant of Peak recovery (ms)	168±20	154±32	125±48	115±12

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