SUPPLEMENTAL MATERIAL
for
Dynamic Calcium Movement in Cardiac Sarcoplasmic Reticulum during Release

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METHODS

Cell isolation

Single ventricular myocytes were isolated from New Zealand White rabbits using standard enzymatic techniques. All procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health and approved by the Institutional Animal Care and Use Committee. Animals were anaesthetized with sodium pentobarbital (50 mg/kg i.v.). Hearts were excised, rinsed in cold Ca-free solution, and mounted on a Langendorff perfusion system. Hearts were perfused at 37°C with Ca free solution (DMEM, Dulbecco's minimal essential medium, Gibco/Invitrogen, USA, gassed with 95% O_2-5% CO_2) for 5 min. Solution was then switched to a collagenase (0.3-0.7 mg/ml; Boehringer Mannheim, Germany) and Ca (10-20 μM) containing solution. After 15–25 min, perfusion was stopped and ventricles were minced into small pieces. Optionally the pieces were incubated for 5–20 min in fresh enzyme. Finally, enzyme activity was stopped with DMEM solution containing bovine serum albumin (BSA; 0.5-1%), and tissue was agitated or triturated to liberate single myocytes. Cells were washed and stored in DMEM solution adjusted to [Ca] = 150 μM. All experiments were performed at room temperature (21-24°C).

Fluo-5N loading and confocal imaging of $[Ca]_{SR}$

Isolated cardiomyocytes were loaded with the cell-permeable form of the low affinity Ca indicator Fluo-5N (K_d ~400 μM/L) as described previously.[1,2] In short, cells were incubated for 2 hours with 15 μM Fluo-5N/AM at 37°C, and 1.5 hours was allowed for deesterification and outward leak of cytosolic dye. Cells were superfused continuously with normal Tyrode's solution consisting of (in mmol/L): NaCl 140, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, glucose 10, and HEPES 5, pH adjusted to 7.4 with NaOH. Contraction was abolished by adding 30 μmol/L cyto-chalasin-D to the perfusion solution. Measurements of intra-SR free [Ca] ($[Ca]_{SR}$) were performed using laser scanning confocal microscopy (x-y resolution ~200 nm; Radiance 2100, Hertfordshire, BioRad, UK). Fluo-5N was excited with the 488 nm line of and argon-ion laser and fluorescence was measured at wavelengths > 500 nm. The linescan mode was used at a scanning speed of 166 lines per second with the scanning line parallel to either the longitudinal or transverse myocyte axis at a central focal plane (avoiding the nucleus). Cells were field-stimulated at 1 Hz using platinum electrodes until steady-state was achieved. Caffeine (10 mmol/L) was applied at the end of the experiment to determine the Ca insensitive fraction of the Fluo-5N fluorescence that was subsequently used to correct the raw fluorescence traces. $[Ca]_{SR}$ depletions were fitted with the product of a sigmoid decline and exponential recovery phase[2] and analyzed offline using custom-made software (Detector V2.3, E.P). With this approach, and care to only use myocytes with monophasic decreases in Fluo-5N fluorescence during excitation-contraction coupling[1-3] the Fluo-5N signals can be taken as a direct indicator of free $[Ca]_{SR}$.

Ca spark and blink measurements

For simultaneous recording of Ca sparks and corresponding $[Ca]_{SR}$ depletions (Ca blinks) we used the high affinity Ca indicator Rhod-2 and the low affinity Ca indicator Fluo-5N, respectively. Fluo-5N/AM loaded myocytes were permeabilized with saponin (0.005% for 30 s) as previously described.[4] The saponin free internal solution contained (in mmol/L unless indicated): K aspartate 100; KCl 15; KH_2PO_4 5; MgATP 5; EGTA 0.35; $CaCl_2$ 0.12; $MgCl_2$ 0.75; phosphocreatine 10; HEPES 10; Rhod-2 tripotassium salt 0.04; creatine phosphokinase 5 U/ml; dextran (MW: 40,000) 8%, and pH 7.2 (KOH). Free [Ca] and [Mg] of this solution were 150 nmol/L and 1 mmol/L, respectively.

Changes in cytosolic [Ca] and $[Ca]_{SR}$ were measured simultaneously with a laser scanning confocal microscope (Radiance 2000 MP, Bio-Rad, UK) equipped with a 40× oil-immersion objective lens (N.A.=1.3). Fluo-5N was excited with the 488 nm laser line of an argon ion laser and fluorescence was measured at 515±15 nm. Rhod-2 was excited with the 543 nm laser line of a HeNe laser and fluorescence was measured at wavelengths >600 nm. Images were acquired in linescan mode (333 lines per second; pixel size 0.12 μm). The scan line was positioned along either the longitudinal or transversal axis of the cell (see Results). Ca sparks were detected and analyzed using

SparkMaster.[5] For each detected Ca spark the corresponding Ca blink was analyzed as described previously.[6] The profiles of Ca blinks were fit as the product of a rising and decaying exponential function. Amplitudes of sparks and blinks are expressed as F/F_0, where F_0 is the initial fluorescence before release. All Ca blinks were corrected for the caffeine-insensitive component of Fluo-5N fluorescence after complete SR Ca depletion with 10 mmol/L caffeine. For $[Ca]_{SR}$ calibrations, F_{min} and F_{max} was assessed in each myocyte, and the apparent $K_d(Ca)$ was assumed to be 400 μM.[1] F_{min} was measured after depletion of the SR with 10 mmol/L caffeine in the presence of 5 mmol/L EGTA. F_{max} was measured following an increase of [Ca] to 10 mmol/L. Caffeine (10 mmol/L) keeps RyRs open allowing [Ca] equilibration across the SR membrane.[1] To prevent irreversible cell contraction during application of high [Ca] cells were pretreated for 5 min with the muscle contraction uncouplers 2,3-butanedione monoxime (10 mmol/L) and blebbistatin (10 μmol/L).

FRAP experiments

Cytosolic and intra-SR FRAP experiments were performed in line scan mode (488 nm excitation) at a scanning speed of 166 lines per second. Cytosolic FRAP experiments were performed on cells which were loaded with the cell permeable form of the Ca-independent fluorescent dye Calcein. Myocytes were incubated with 5 μM of Calcein/AM for 10 min followed by 30 min of superfusion with normal Tyrode's solution to allow deesterification. Intra-SR FRAP experiments were performed in Fluo-5N loaded cardiomyocytes after cell permeabilization with saponin as described before.[5] Permeabilization washes out cytosolic indicator; however, some Fluo-5N may be trapped in mitochondria, but because intra-mitochondrial [Ca] is low at the 150 nM [Ca] used here[7] mitochondrial Fluo-5N will only weakly fluoresce. Moreover, because the mitochondria are isolated, they cannot participate in the FRAP kinetics measured (i.e. they could only contribute to the lack of complete FRAP extent).

Confocal line-scan mode photobleach was performed,[8] restricting the area of photobleaching to the width of a sarcomere while allowing a high temporal resolution FRAP measurements. By bleaching either along the longitudinal or transverse direction of the myocyte, we selectively investigated intra-SR diffusion in the transverse or longitudinal direction of the SR (i.e. perpendicular to the direction of the bleach). By bleaching transversely along a z-line, FRAP occurs via diffusion perpendicular to the bleached line (i.e. due to diffusion in the longitudinal direction) because no concentration gradients exist along the direction of the bleach.

The FRAP protocol consisted of a 72 ms pre-bleach scan at 10% laserpower to determine the baseline fluorescence. This was followed by a photobleaching period lasting 450 ms at 75% laser power followed by 12 post-bleach scans of 72 ms duration immediately following the photobleach and distributed over 75 s. The laserpower of the short scans after the photobleaching during FRAP was the same as used for the pre-bleach scan. In between the recovery scans the laserpower was set to 0% to minimize photobleaching during the FRAP measurement. The recovery kinetics were fit with the sum of two exponentials.

All chemicals were from Sigma-Aldrich (St. Louis, MO). Fluo-5N/AM and Rhod-2 were purchased from Molecular Probes/Invitrogen (Carlsbad, CA).

Statistics

Data are presented as means±SEM of n measurements. Statistical comparisons between groups were performed with the Student *t* test. Differences were considered statistically significant a $P<0.05$.

Diffusion of Fluo-5N and Calcein during FRAP

For FRAP measurements we used Fluo-5N to assess intra-SR diffusion and calcein to assess cytosolic diffusion. These differ in molecular weight (MW) and so would be expected to differ in their apparent diffusion coefficient (see Table I). Correcting the relative D value for MW allows better comparison among the FRAP data. The MW-adjusted τ-fast for Fluo-5N FRAP suggests that diffusion within the SR is 3-4-fold slower (depending on direction) than in the cytosol. This would be consistent with greater tortuosity (or possibly viscosity) inside SR vs. cytosol.

Table I. Molecular weights and FRAP kinetics corrected for size

	MW(g/mol)	$(MW)^{0.5}$	Relative D*
Fluo-5N	958 g/mol	30.95	1
Calcein	622 g/mol	24.94	1.24

*D theoretically depends inversely on molecular weight $(MW)^{0.5}$

	τ slow (s)	τ-fast (s)	τ-fast (s)*
Intra-SR Fluo-5N			
longitudinal	36	1.56	1.56
Transverse	36	2.24	2.24
Cytosolic Calcein* (isotropic)	7	0.44	0.55

*τ Adjusted for molecular weight (MW)

Mathematical Model

General Information

All programming was done using Matlab (The Mathworks, Inc., Natick, MA, United States). The model is composed of a system of differential equations describing changes in [Ca] within SR and cytosolic compartments separated by the SR membrane. Ca was

conserved such that the sum of the Ca in the SR and the cytosol was held constant. The program was typically run for 5 seconds to assure steady-state had been achieved before simulating a release event.

Diffusion of all molecules (S) was described by a special case of Fick's Law:

$$d[S]/dt=D_S(d[S]/dx)$$

where D_S is the diffusion coefficient (Table II). All compartments were spaced at a distance, dx, of 50 nm. No attempt was made to distinguish the rate of diffusion of Ca bound and unbound ligands.

Table II: Diffusion Coefficients

Substance	Diffusion coefficient ($\mu m^2/s$)
SR Ca	As indicated
Fluo 5N	8
Cytosolic Ca	240
Rhod-2	42
Calmodulin	0.5
ATP	0.14
EGTA	0.036

Ca binding to buffering substances within the cell was described by the rate equation:

$$d[Ca\bullet L]/dt=k_{on}[Ca](B_{max}-[Ca\bullet L])-k_{off}[Ca\bullet L]$$

where L is the buffering ligand (Table III). For additional details concerning buffering and kinetics see Shannon *et al.*[9] and Bers.[10] The initial condition was set by calculating the amount of ligand bound using the Hill equation:

$$[Ca\bullet L]=B_{max}[Ca]/([Ca]+K_d)$$

Table III: Ca Buffering Parameters

Buffer	B_{max} μmol/l cyto)	k_{on} μM s^{-1}	k_{off} s^{-1}	K_d μM
Troponin C Ca	70	32.7	19.6	0.6
Ca-Mg (Ca)	140	2.37	0.033	0.0135
Ca-Mg (Mg)	140	0.003	3.33	1111
Calmodulin	24	34	238	7
Myosin	140	13.8	0.46	0.0333
Myosin (Mg)	140	0.0157	0.057	3.64
SR	19	100	60	0.6
SL	42	100	1300	13
SL (hi affinity)	15	100	30	0.3
Fluo-4/ Rhod-2	25	100	110	1.1
ATP*	5000	150	3000	200
ATP (Mg)*	5000	1.95	19.5	100
EGTA*	350	12.5	2	0.16
Calsequestrin	140	100	65000	650

*Only present in permeabilized myocytes

Ca fluxes were driven by Ca release from the SR which was represented by the waveform based on measurements in Shannon *et al.*[11,12] (see below). Released Ca was re-sequestered back into the SR by SR Ca pumps which operated as simple reversible enzymes:

$$J_{pump}=V_{max}(([Ca]_i/K_{mf})^H-([Ca]_i/K_{mf})^H)/(1+[Ca]_i/K_{mf})^H+([Ca]_i/K_{mf})^H$$

where the parameters are as in Table IV.

Table IV: SR Ca Pump Parameters

Parameter	Value
V_{max}	137 μM/s
K_{mf}	0.26 μM
K_{mr}	1.82 mM
H	2

Whole Cell Model

Whole cell Ca release was simulated in a half sarcomere with reflective boundaries (under the simplifying assumption that all junctions release during normal excitation-contraction coupling.[13,14] The half-sarcomere contains both SR and cytosolic compartments, each 50 nm in length along the long axis of the sarcomere. Longitudinally the half-sarcomere consisted of 20 SR compartments along with 20 corresponding cytosolic compartments. SR compartments one and two were considered to be junctional space containing calsequestrin. When displaying JSR signals that are averages of several compartments (to simulate optical measurements) the first 2 junctional compartments received five times greater weighting than the surrounding SR compartments to mimic the higher Fluo-5N concentration in these locations. Release was from compartment one into the corresponding cytosolic compartment, considered to be adjacent to the sarcolemma. Released Ca therefore diffused from this compartment through the adjacent compartments toward the center of the sarcomere. Ca was re-sequestered back into the SR through SR Ca pumps located in compartments 2-20 and diffused back towards compartment one through the SR.

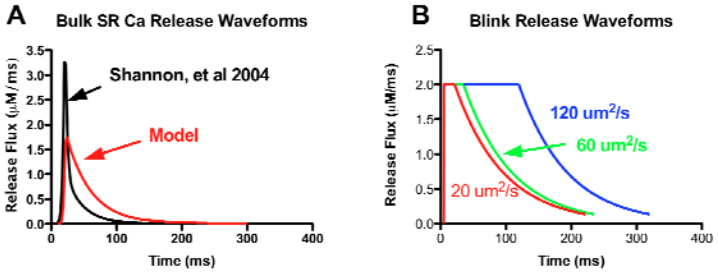

Figure 1. Kinetics of SR Ca release flux for A) the case of global E-C coupling and the first case for single junction release (in Fig 5D-F of the manuscript) and B) for single junction release where release shuts off when local $[Ca]_{SR}$ declines to 40% of the diastolic level (as in Fig 6 of the manuscript). Faster diffusion coefficients delay the time to shut off.

Transversely along the Z-line the half sarcomere consisted of two sets of 10 SR compartments, each meeting the longitudinal SR at compartment two. These compartments were considered to be passive conduits for Ca, without cytosolic compartments or SR Ca-ATPase associated with them.

The release waveform was determined from that described by Shannon *et al.*[11] (see Fig I). To simulate measured kinetics, we used an empirical product of three Boltzman equations:

$$J_{Release}=(1.43X10^{-5}+(8.5-1.43X10^{-5})/(1+10^{(20.54-t)*0.3976}))(12+(0.008749-12)/(1+10^{(-71.39-t)*0.012}))(75+(0.25-75)/(1+10^{(2.037-t)*0.1672}))$$

Where t is time in ms and $J_{Release}$ is in mmol/l cytosol/s.

Release from Single Junctions

For a single junction release (Ca spark/blink) we cannot use the same reflective boundaries, because Ca can diffuse from the SR in regions more than a half-sarcomere away (and of course cytosolic Ca can likewise diffuse away from the junction to neighboring regions). Here we used the geometry shown in Fig II, where the center (red) junction is the active junction that is diffusionally connected (intra-SR and cytosol) to two sarcomeres in each orthogonal direction (including one diagonal junction in each direction. The figure only shows two dimensions (*x-y*), but we included the same geometry in the vertical (*z*) direction (i.e. rotating Fig II 90° around the longitudinal axis). Thus we are including the 20 nearest junctions from one central release unit. We added another layer further out from this, but it did not appreciably alter the results.

Two Ca release waveforms were used. The first was identical to that for whole cell release. The second release form consisted of a rising exponential followed by a decaying exponential (see Fig IB). The rising exponential was defined as

$$J_{Release}=2(1-e^{(-30t)})$$

This release continued until a 40% decline in $[Ca]_{SR}$ in the releasing junction was reached at which point release terminated with an exponential decline as:

$$J_{Release}= J_0(e^{-0.0135(t-t0)})$$

where J_0 is the flux and t0 is the time (in ms) at which the threshold $[Ca]_{SR}$ is reached.

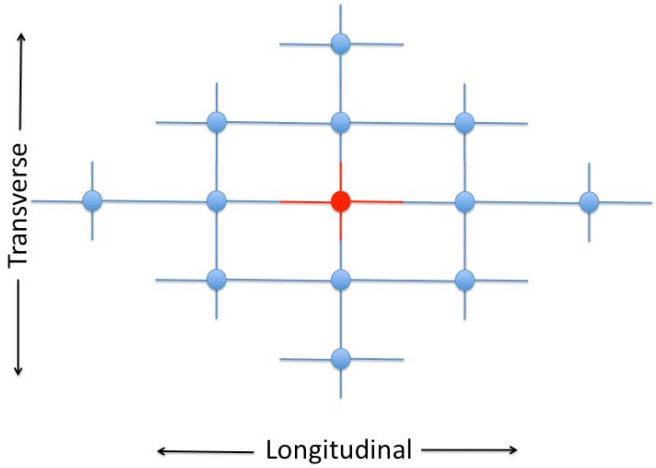

Figure II. Geometry of junctions considered for single junction release simulations. Longitudinal junctions spacing (center to center) are 2 μm, while transverse junctions are 1 μm.

References

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