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Ca²⁺ homeostasis in sealed t-tubules of mouse ventricular myocytes

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Abstract

We have recently shown that in mouse ventricular myocytes, t-tubules can be quickly and tightly sealed during resolution of hyposmotic shock of physiologically relevant magnitude. Sealing of t-tubules is associated with trapping extracellular solution inside the myocytes but the ionic homeostasis of sealed t-tubules and the consequences of potential transtubular ion fluxes remain unknown. In this study we investigated dynamics of Ca²⁺ movements associated with sealing of t-tubules. The data show that under normal conditions sealed t-tubules contain Ca²⁺ at concentrations below 100 μM. However, blockade of voltage-dependent Ca²⁺ channels with 10 μM nifedipine, or increasing extracellular concentration of K⁺ from 5.4 mM to 20 mM led to several fold increase in concentration of t-tubular Ca²⁺. Alternatively, release of Ca²⁺ from sarcoplasmic reticulum using 10 mM caffeine led to restoration of t-tubular Ca²⁺ towards extracellular levels within few seconds. Sealing of t-tubules in the presence of extracellular 1.5 mM Ca²⁺ and 5.4 mM extracellular K⁺ led to occasional and sporadic intracellular Ca²⁺ transients. In contrast, sealing of t-tubules in the presence of 10 mM caffeine was characterized by significant long lasting increase in intracellular Ca²⁺. The effect was completely abolished in the absence of extracellular Ca²⁺ and significantly reduced in pre-detubulated myocytes but was essentially preserved in the presence of mitochondrial decoupler dinitrophenol. This study shows that sealed t-tubules are capable of highly regulated transport of Ca²⁺ and present a major route for Ca²⁺ influx into cytosol during sealing process.

Keywords

ventricular myocytes; t-tubules; hyposmotic stress; calcium; Ca²⁺

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1. Introduction

The work in the past 10-15 years highlighted significant and important roles of cardiac t-tubules in electrical and mechanical performance of cardiac ventricular myocytes ([1-3] for reviews). T-tubules are constant features of normal adult ventricular myocytes. However, it has been convincingly shown that t-tubules become significantly remodeled or lost during various cardiac pathologies including different types of heart failure and cardiomyopathies [4-12] as well as atrial fibrillation [13, 14] ([15] for review). The mechanisms underlying cardiac t-tubular remodeling are being actively studied. In particular, some of the associated or structural proteins such as caveolin-3 or junctophilin-2 have been shown to strongly affect the development and integrity of t-tubules [16-18]. Consistent with this, mice with cardiac-specific inducible knockdown of junctophilin-2 display loss of t-tubules and heart failure [19]. Similarly, reduced expression of caveolin-3 was shown to be associated with t-tubular remodeling and cardiac dysfunction [20]. Among other prominent players in t-tubular biogenesis and regulation in cardiac and skeletal muscle are Tcap [21-23] and amphiphysin 2 [24, 25]. Clearly, genetically induced disturbances in any t-tubular structural protein will likely contribute in various degrees to specific t-tubular remodeling. However, t-tubular remodeling is also observed in acquired diseases, including experimentally induced heart failure in otherwise normal animals. In those cases the exact driving force initiating and/or contributing to t-tubular remodeling and the mechanisms behind their disassembly (loss) are not clear.

It has been known for long time that a loss of t-tubules can be caused by osmotic shock with 1.5 M formamide [26]. This powerful experimental approach remains extremely useful for studies of cardiac t-tubules but, surprisingly, osmotic shock was not considered as one of the potential causes of t-tubular remodeling *in vivo*. In this regard, we have recently shown that in isolated mouse ventricular myocytes quick and significant loss of t-tubules can occur in response to resolution of hyposmotic shock of physiologically relevant magnitude [27]. In both types of osmotic shock (hyperosmotic or hyposmotic) the loss of t-tubules is due to their tight sealing [27, 28] rather than some kind of membrane disintegration. Similar “pinching off” of t-tubules has also been observed during culture of rat ventricular myocytes [29]. In our earlier work [30] we showed that the loss of t-tubular membrane can be observed in a number of experimental conditions which can collectively be identified as metabolic stress. The latter, in turn, can be easily linked to various osmotically related phenomena. Importantly, partial constrictions of t-tubules leading to failure of action potential propagation and spontaneous electrical activity were also observed in ventricular myocytes isolated from failing hearts [31]. Overall, it seems that membrane stretch, in particular caused by transient swelling of cells, may be one of the general driving forces that leads to constricting and sealing of cardiac t-tubules. Consistent with this, mechanical load on cardiac muscle is considered as one of the regulators of t-tubular remodeling (for review [32]).

An important consequence of osmotically induced remodeling is a tight sealing of t-tubular lumen such that small molecules present in extracellular solution can be ‘efficiently’ trapped inside the myocytes [27, 28]. However, the fate of various components of trapped extracellular solution (e.g. ions) as well as the function and fate of sealed t-tubules

themselves remain essentially unknown. We are aware of only one work (Despa et al (2003) [33]) which provided indirect evidence that sealed t-tubules are likely to be 'alive' and capable of transporting Ca^{2+} . In this study we employ direct measurements of Ca^{2+} in sealed t-tubules using low affinity Ca^{2+} indicator Rhod-5N and show that under normal conditions sealed t-tubules contain Ca^{2+} at low concentration suggesting a massive, mostly hidden (due to uptake by SR), influx of trapped t-tubular Ca^{2+} into cytosol during the sealing process. The latter is unlikely due to 'leaky' (due to stress) t-tubular membrane since the data clearly show that Ca^{2+} in sealed t-tubules can be strongly regulated by various experimental manipulations including blockade of voltage-dependent Ca^{2+} channels, caffeine-induced release of Ca^{2+} from SR into cytosol, or even changes in extracellular K^+ . Overall, results of this study provide support for the general hypothesis that t-tubular remodeling, whether acute or chronic, is likely associated with sealing of t-tubular lumen leading to aberrant Ca^{2+} handling and consequent initiation and progression of a disease.

2. Materials and Methods

2.1. Animals

All experiments involving mice were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (8th Edition, Committee for the Update of the Guide for the Care and Use of Laboratory Animals, National Research Council; The National Academic Press, Washington, DC) and protocols approved by the veterinary staff of the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan.

2.2. Solutions (mM)

Solutions for isolation of ventricular myocytes (filtered using 0.22 μm filter):

- A. 122 NaCl, 5.4 KCl, 4 MgCl_2 , 0.16 NaH_2PO_4 , 3 NaHCO_3 , 15 HEPES, 10 Glucose, 0.1 μM EGTA, pH=7.35 with NaOH. 279.7 ± 6.4 mOsm/L.
- B. 25 ml A + 15 mg Collagenase.
- C. 180 ml A + 900 mg Bovine Serum Albumin + 250 mg Taurine. 288.7 ± 3.5 mOsm/L.

Modified Tyrode (Tyr): 137 NaCl, 5.4 KCl, 0.5 MgCl_2 , 0.3 CaCl_2 , 0.16 NaH_2PO_4 , 3 NaHCO_3 , 5 HEPES, 5.5 Glucose, pH=7.35 with NaOH. 278.7 ± 3.5 mOsm/L.

0.6 Na: Tyr containing 60% NaCl (82.2 mM). 204.7 ± 4.2 mOsm/L.

In experiments using elevated extracellular K^+ 20 mM KCl was added without adjusting osmolarity (e.g. by reducing the concentration of NaCl).

Osmolarity was measured using Vapro Osmometer 5520 (Wescor, ELITechGroup, France) and data are presented as mean $\pm$ SD.

2.3 Reagents

di-8-ANEPPS (Life Technologies, Carlsbad, CA, USA): 5 mg of voltage-sensitive dye di-8-ANEPPS was dissolved in 1 ml of DMSO and used as a stock (stored at +4 °C). Working solution was prepared as 50:50 v/v mix of di-8-ANEPPS stock and 20% Pluronic acid.

Avertin: 10 g of tribromoethanol alcohol (Sigma-Aldrich, USA) was mixed with 10 ml of *tert*-amyl alcohol (Sigma-Aldrich, USA) and used as a stock (stored at -20 °C). ~2.5% working solution was prepared in PBS with the addition of 1 mg/ml of heparin (Sigma-Aldrich, USA; 187 USP units/mg).

Caffeine (Sigma-Aldrich, USA); 200 mM stock solution was prepared in Tyr (stored at +4°C) and used at working concentration 10 mM.

Tetramethylrhodamine dextran ('3K dextran'; 3000 MW; Anionic form; Life Technologies, Carlsbad, CA, USA): 10 mg of dextran was dissolved in 1 ml PBS and used as a stock (stored at -20°C).

2,4-Dinitrophenol (DNP; Aldrich, USA): 200 mM stock solution was prepared in DMSO (stored at +4°C) and used at working concentration 200 µM.

Fluo-3 AM (Life Technologies, Carlsbad, CA, USA): 50 µg of Fluo-3 AM was dissolved in 50 µl DMSO and used as a stock (stored at -20°C). Working solution was prepared as 50:50 v/v mix of Fluo-3 AM stock and 20% Pluronic acid. Used at final concentration 10 µM.

Rhod-5N, tripotassium salt (Life Technologies, Carlsbad, CA, USA): 500 µg was dissolved in 1mL PBS and used as a stock (stored at -20°C).

Nicardipine (Nic; Sigma-Aldrich, USA): 100 mM stock solution was prepared in DMSO (stored -20°C) and used at working concentration 10 µM.

Ouabain (Sigma-Aldrich, USA): 10 mM stock solution was prepared in both 0.6 Na and Tyr solutions and used at working concentration 1 mM.

2.4 Isolation of mouse ventricular cardiomyocytes

Ventricular myocytes were isolated from the hearts of adult (~2-6 month old) C57BL/6 mice of either sex anesthetized with Avertin (20 µl/g; intraperitoneal injection) using collagenase treatment. Briefly, hearts were perfused retrogradely (Langendorff perfusion) through aorta with appropriate solutions at a constant pressure of ~115 cm H₂O and T=~28 °C using a water-jacketed supply tube. Atria were removed and AV valve surgically destroyed. Solution A was applied for ~4 min, followed by solution B supplemented with 40 µM Ca²⁺, and the heart digested with solution recirculation for another 25 min. Approximately three-quarters of the lower part of the heart was cut off and the right ventricle outer wall removed. The left ventricle was opened, sliced into ~6-8 pieces with a blade, the tissue transferred to 15 mL tube containing 10 ml of solution B and manually triturated at ~28 °C for ~5 min using glass pipette with a fire-polished tip. Myocyte suspension was filtered and procedure repeated with the remaining tissue chunks up to 5 times (~5 min each trituration) with increasing proportion of solution C and using glass

pipettes with progressively smaller tip opening. The myocytes were usually optimally isolated at steps 4 to 5. Myocytes were stored in respective solutions at room temperature (RT) and used in experiments within 1 to 5 h post isolation.

2.5. Measurements of $[Ca^{2+}]$

Changes in intracellular calcium, $[Ca^{2+}]_{in}$, were monitored using Fluo-3 calcium indicator loaded into myocytes as follows. A small aliquot (a few hundred μ l) of enzymatically digested myocytes was diluted in 3 ml of solution C. Fluo-3 AM was then added to a final concentration of 10 μ M and myocytes left in this solution for 30 minutes at RT. Myocytes were then washed to remove extracellular dye and used for experiments up to one hour post loading using photomultiplier-based system described below.

Measurements of calcium in sealed t-tubules, $[Ca^{2+}]_{st}$, were performed using low affinity calcium indicator Rhod-5N trapped in t-tubules during resolution of hyposmotic shock [27]. A small aliquot (up to few hundred μ l) of enzymatically digested myocytes was diluted in a large volume (usually 10 ml) of 0.6 Na solution in order to induce swelling of myocytes and the timer started. Myocytes were then concentrated by centrifugation (2 minutes at 48 g), 10 μ l of cell suspension transferred into separate tube and 1 μ l of Rhod-5N stock added $\sim$ 2 min before removing hypo-osmotic solution to allow the dye to fill accessible t-tubules. Near isotonic solution consisting of 90 μ l of Tyr and 10 μ l Rhod-5N stock was added at 7 min in order to initiate myocytes shrinking and sealing of t-tubules while maintaining the presence of Rhod-5N in the extracellular solution. In order to remove most of extracellular Rhod-5N, myocytes were resuspended in $\sim$ 10 ml of Tyr 5 min after the hyposmotic shock, concentrated by centrifugation to $<\sim$ 100 μ l and resuspended again in $\sim$ 0.5-1 ml of solution C. As a positive control for the efficiency of Rhod-5N trapping and its stable retention in sealed t-tubules in separate experiments 1 mM Cd^{2+} was added during the detubulation process. Rhod-5N is significantly more sensitive to Cd^{2+} than to Ca^{2+} [34] (see also 'metal-ion response screening for Rhod-5N' at <http://products.invitrogen.com/ivgn/product/R14207>) such that even trace amounts of Cd^{2+} are sufficient to reveal the presence of Rhod-5N. Changes in Rhod-5N fluorescence were studied using either photomultiplier-based system or analysis of confocal images as described below.

In experiments employing blockade of Ca^{2+} channels 10 μ M nifedipine was applied $\sim$ 5 min before resolution of hyposmotic shock and kept throughout the rest of the procedure.

Since the relationship between the fluorescence of Ca^{2+} indicator and $[Ca^{2+}]$ is not linear, the transformation of fluorescence to concentration leads to asymmetrical error ranges (bars in graphs). Accordingly, relevant numerical data are presented as mean + top SE level/-bottom SE level, rather than mean $\pm$ SE.

2.6. Confocal imaging

Confocal imaging was performed using Olympus FV-500 microscope at the Microscopy and Image Analysis Laboratory (University of Michigan, Ann Arbor). Labeling of sarcolemmal membrane was achieved using di-8-ANEPPS by adding $\sim$ 0.5 μ l stock solution to 300 μ l of cell suspension in Tyr for $\sim$ 10-30 min at RT. Confocal images were visualized and analyzed using ImageJ software (<http://imagej.nih.gov>) followed by further analysis in Microsoft

Excel. In experiments with fluorescent 3K dextran and Rhod 5N trapped in sealed t-tubules, myocytes were manually outlined and mean intracellular fluorescence per unit area calculated. The data were corrected for background fluorescence observed in the presence of dextran or Rhod 5N but without application of osmotic shock [27].

In vitro calibration of Ca^{2+} -induced Rhod-5N fluorescence was performed as follows. Rhod-5N solution (10 mg/ml in Tyr) containing 1 mM Ca^{2+} or 1 mM Cd^{2+} was placed in imaging dish and Z stacks of images taken at 1 μm step to cover the distance from the bottom of the glass up to $\sim 20 \mu\text{m}$ (about the height of the myocyte) using optical settings employed for imaging myocytes. Averaged (over the stack) intensity of Ca^{2+} -induced fluorescence was normalized to that in the presence of Cd^{2+} and used as calibration value (Fig. 1).

2.7. Microscope based photomultiplier system

A photomultiplier system (PMS) based on a Nikon Eclipse TE300 microscope equipped with appropriate optical elements, including 60x Oil CFI APO Lambda S Objective (Nikon Instruments Inc, Japan), was used to record fluorescence from sealed t-tubules (Rhod-5N) and the cytosol (Fluo-3). Luxeon 700 mA Rebel Light Emitting Diodes (LED; Quadica Developments Inc., Brantford, Ontario, Canada) were used as a light source. The fluorescent signal was recorded using Model 814 PMT (Photomultiplier) Housing (Photon Technology International, Inc, Birmingham, NJ, USA) and R928P Photomultiplier tube (Hamamatsu Photonics K.K., Japan).

530 nm green LED, D540/25 excitation filter, Q565LP2 dichroic mirror and HQ575/30 emission filter were used for recordings Rhod-5N fluorescence.

505 nm cyan LED HQ500/20 excitation filter, 515 nm dichroic mirror and HQ530/30 emission filter were used for recordings of Fluo-3 fluorescence.

All filters and mirrors were from Chroma Technology Corp (Bellows Falls, VT, USA).

In vitro calibration of Ca^{2+} -induced Rhod-5N fluorescence for PMS system was performed similar to that for confocal imaging studies and produced indistinguishable results.

2.8. Statistics

Data are presented as a mean $\pm$ Standard Error (with the exception of the data on absolute $[\text{Ca}^{2+}]$ above). Statistical significance was determined using a two sample t-test assuming equal variances and considered significant if $p < 0.05$. In figures *, **, and *** correspond to p values of 0.05, 0.01 and 0.001, respectively.

3. Results

3.1. Concentration of Ca^{2+} in sealed t-tubules

In ventricular myocytes t-tubules can be sealed by two similar approaches. One of them employs hyper-osmotic shock with 1.5 M formamide leading to nearly complete removal of t-tubular network [26, 28]. We have recently shown that similar level of detubulation can

also be achieved by hyposmotic shock with solution having only 27% less osmolarity than normal Tyrode [27]. Therefore, most of the experiments in this study employed the latter approach.

Sealed t-tubules can retain low-molecular weight dextrans for significant time [27, 28]. In particular, with hyposmotic approach the fluorescence of trapped 3K dextran declined less than 10% per hour (data not shown). This finding suggested the use of trapped ion indicators for direct measurements of ionic homeostasis in sealed t-tubules. In this study we aimed to directly assess $[Ca^{2+}]_{st}$ using trapping of a low affinity Ca^{2+} indicator Rhod-5N since it was expected that $[Ca^{2+}]_{st}$ may likely be close to that in the extracellular solution (1 mM or 1.5 mM in this study).

Rather unexpectedly, in the presence of 1 mM extracellular $[Ca^{2+}]$ the fluorescence from myocytes with likely trapped Rhod-5N was very close to the background level (Fig. 1A, top). This could certainly be due to a number of reasons. For example, lower concentration and potentially lower intrinsic intensity of Rhod-5N fluorescence (even in the presence of high $[Ca^{2+}]_{st}$) relative to that of 3K dextran used in previous studies can account for the low values. Alternatively, the smaller Rhod-5N (~0.9K MW) could simply leak out from partially constricted t-tubules before imaging the cells. In order to address the above issues we took advantage of a unique property of Rhod-5N – its high sensitivity to Cd^{2+} . Specifically, it has been shown that Rhod-5N is more sensitive to Cd^{2+} than to Ca^{2+} (by more than 4 orders of magnitude; [34]). Therefore, inclusion of 1 mM Cd^{2+} during detubulation would allow for ‘lighting up’ Rhod-5N to its maximum fluorescence and confirm its presence (or absence) in sealed t-tubules (due to co-trapping with Cd^{2+}). Confocal imaging shows that Rhod-5N indeed is efficiently trapped in sealed t-tubules (Fig. 1A (bottom)). Also, Rhod-5N fluorescence does not appreciably change for extended period of time (<10% decline of fluorescence over 2 hours; data not shown) suggesting both insignificant leak of the dye out of sealed t-tubules and relatively high remaining concentration of t-tubular Cd^{2+} (which may potentially leak out or be transported by some mechanism out of sealed t-tubules). The magnitude of Rhod-5N fluorescence in the absence of extracellular Cd^{2+} is ≈ 15 fold smaller than in the presence of Cd^{2+} : 0.07 ± 0.02 vs 1 ± 0.11 , respectively (the data are from 8 heart preparations, 80 myocytes total, $p < 0.001$).

The quantitative estimation of $[Ca^{2+}]_{st}$ though cannot be achieved until the Ca^{2+} -dependent fluorescence is ‘calibrated’. Unfortunately, Rhod-5N is not a ratiometric dye, and even if it were ratiometric, applying calibrating Ca^{2+} solutions to sealed t-tubules localized inside the cells would be a challenge. However, high sensitivity of Rhod-5N to Cd^{2+} can be employed here again, this time to calibrate the dye. The data in Fig. 1C show that fluorescence of Rhod-5N measured *in vitro* (see Methods) in the presence of 1 mM Ca^{2+} is 50.9 ± 1.4 % of that in the presence of saturating 1 mM Cd^{2+} . Therefore, if one reasonably assumes that the t-tubular fluorescence of Rhod-5N trapped along with 1 mM Cd^{2+} is saturated *in vivo* as well, then in cells trapped without Cd^{2+} half of its value should correspond to that produced by 1 mM Ca^{2+} . The latter allows for transformation of the data in Fig. 1B to $[Ca^{2+}]_{st}$. According to specifications provided by supplier of Rhod-5N (Life Technologies/Molecular Probes) K_d for Ca^{2+} (in the absence of Mg^{2+}) is ~ 320 μM . Assuming that fractional

Rhod-5N fluorescence can be described by Hill equation $R = \frac{1}{1 + \left(\frac{K_d}{[Ca^{2+}]}\right)^h}$ with Hill coefficient $h=1$, the data in Fig. 1B and C yield $[Ca^{2+}]_{st} \approx 36 \pm 10 \mu M$. Since the exact ionic environment in sealed t-tubules is not known, and thus the K_d for Ca^{2+} is also not precisely defined, the above value should only be considered as a good estimation. A simple 'sensitivity' analysis shows, however, that even if K_d for Ca^{2+} would be twice larger, e.g. $640 \mu M$ (in part due to the likely presence of interfering Mg^{2+} ions), the estimation would be $[Ca^{2+}]_{st} \approx 57 \mu M$.

Overall, the data show that sealed t-tubules contain Ca^{2+} at very low concentration, likely below $100 \mu M$.

3.2. Contribution of calcium channels to regulation of $[Ca^{2+}]$ in sealed t-tubules

One of the reasons for low $[Ca^{2+}]_{st}$ values above could be a stress-induced stretch of the membrane of sealed t-tubules leading to their leakiness and shift of ionic homeostasis towards that in the intracellular milieu. Consistent with this, visual analysis of confocal images indicates that significant part of sealed t-tubules appear swollen (data not shown). Alternatively, low $[Ca^{2+}]_{st}$ may just be a result of normal regulation of t-tubular homeostasis by various channels, pumps and transporters which are found in t-tubules at densities quite different from those in the outer sarcolemma. In particular, the density of Ca^{2+} channels is highest in t-tubules [35], and therefore they may present a significant route for Ca^{2+} leak into cytosol. In order to test this hypothesis we measured the $[Ca^{2+}]_{st}$ in the presence of $10 \mu M$ nifedipine, a specific and potent blocker of calcium channels. Accordingly, using hypotonic shock approach nifedipine was trapped in t-tubules along with Rhod-5N, and parallel experiments with Cd^{2+} provided calibration data. The data in Fig. 2 show that blockade of calcium channels leads to ≈ 3.6 fold increase in Rhod-5N fluorescence, from 0.10 ± 0.02 to 0.37 ± 0.04 (2 heart preparations, total $n=20$ myocytes per condition, $p<0.001$; data are normalized to Cd^{2+} -induced fluorescence). Transforming the data to absolute $[Ca^{2+}]$ as described before shows a change from $58 +13/-12 \mu M$ to $389 +94/-75 \mu M$, or ≈ 6.7 fold increase. The fact that restoration of $[Ca^{2+}]_{st}$ towards higher values is strongly affected by specific Ca^{2+} channel blocker directly translates to the idea that Ca^{2+} channels in sealed t-tubules are likely open, which, in turn, is consistent with significant depolarization of t-tubular membrane. It should be noted that detubulation does not lead to significant depolarization of the outer sarcolemma as the myocytes respond to field stimulation by producing Ca^{2+} transients [26].

Similar results were obtained when t-tubules were sealed using formamide treatment [26, 28]. All experiments in this series were performed in the presence of $1.5 mM$ extracellular Ca^{2+} , which is higher than that in experiments using hypotonic detubulation above. Despite this, t-tubular fluorescence of Rhod-5N in the absence of nifedipine was only 0.14 ± 0.03 vs 1 ± 0.11 in the presence of trapped $1 mM Cd^{2+}$, and increased ≈ 2.4 fold to 0.35 ± 0.04 in the presence of $10 \mu M$ nifedipine (3 heart preparations, $n=10-12$ myocytes in each preparation per condition, $p<0.001$). Transforming the fluorescence data to $[Ca^{2+}]_{st}$ gives the following values: $87 +21/-19 \mu M$ to $344 +82/-66 \mu M$ in the absence and presence of nifedipine, respectively, which corresponds to ≈ 4 fold increase.

Overall, the data suggest that calcium channels in sealed t-tubules are functional and contribute to calcium influx during detubulation.

3.3. Effects of caffeine on $[Ca^{2+}]$ in sealed t-tubules

The data above indicate that sealed t-tubules are likely to be fully functional, and therefore, $[Ca^{2+}]_{st}$ should be strongly influenced by the level of intracellular Ca^{2+} , in particular, through the activity of Ca^{2+} pump and due to changes in the driving force for Ca^{2+} ions. First, we tested whether the release of Ca^{2+} from SR during action potential has an effect on $[Ca^{2+}]_{st}$. However, no measurable fast transients of the fluorescence of Rhod-5N trapped in sealed tubules were observed in response to pacing at 1 Hz rate (data not shown). In contrast, application of 10 mM caffeine led to transient ≈ 4 fold increase in t-tubular Rhod-5N fluorescence (Fig. 3A) from 0.13 ± 0.05 to 0.54 ± 0.08 peak values relative to maximal fluorescence in the presence of 1 mM trapped Cd^{2+} (Fig. 3B; 3 heart preparations, $n=11$ myocytes total, $p<0.001$). Recalculation of fluorescence intensities to concentration of $[Ca^{2+}]$ shows that $[Ca^{2+}]_{st}$ is increased ≈ 16 fold from $79 +37/-31 \mu M$ to $1.3 +2.7/-0.6 mM$, before and after application of caffeine, respectively (Fig. 3C). At higher $[Ca^{2+}]$, above 1 mM or so, the results become more variable as the Rhod-5N fluorescence reaches saturation.

Importantly, response to caffeine was quite slow with Rhod-5N fluorescence reaching peak values at 6.2 ± 0.7 sec (Fig. 3 A). This is >20 fold longer than the time to peak for the rise of $[Ca^{2+}]_{in}$ measured using Fluo-3 fluorescence: 0.26 ± 0.11 sec ($n=8$; $p<0.001$; Fig. 3 B).

3.4. Effects of resolution of hyposmotic shock on cytosolic $[Ca^{2+}]$

Since resolution of hyposmotic shock results in low $[Ca^{2+}]_{st}$ (Fig. 1) then almost all Ca^{2+} initially trapped in sealed t-tubules must go inside the cell. The volume of t-tubules is in a similar range as that of sarcoplasmic reticulum (SR) [36], and thus the total amount of Ca^{2+} moved from sealed t-tubules into cytosol may be comparable to the SR load (not assuming Ca^{2+} buffering in SR by, e.g., calsequestrin). However, the exact timing of t-tubular sealing process is not known and thus the rate of influx of t-tubular Ca^{2+} is also uncertain. It is highly unlikely, though, that the sealing process is quick compared to the rate of SR Ca^{2+} release during an action potential or in response to fast application of caffeine, and therefore the influx of Ca^{2+} from sealed t-tubules may be handled to some degree by SR uptake and extrusion to outside by Ca^{2+} pump and NCX. Consistent with this, sealing of t-tubules induced by washout 0.6 Na hyposmotic solution led to sporadic spikes of cytosolic $[Ca^{2+}]$ when measured using intracellular Fluo-3 indicator (Fig. 4A; Insert). The latter was likely due to spontaneous release of Ca^{2+} from SR caused by Ca^{2+} influx through Ca^{2+} channels. Sporadic Ca^{2+} releases were essentially absent at lower concentrations of $[Ca^{2+}]_o$ before and during hyposmotic swelling (data not shown). For example, when 0.6 Na solution contained 0.3 mM $[Ca^{2+}]_o$ but normal washout solution still contained 1.5 mM $[Ca^{2+}]_o$ no intracellular Ca^{2+} transients were observed.

In order to reveal and quantify a likely hidden influx of trapped t-tubular Ca^{2+} into the cells, we measured the time course of changes in cytosolic Ca^{2+} (using intracellular Fluo-3 indicator) during sealing of t-tubules in myocytes treated with 10 mM caffeine in order to inhibit uptake of incoming t-tubular Ca^{2+} by SR. The data in Fig. 4A show that in the

presence of 1.5 mM extracellular Ca^{2+} sealing of t-tubules leads to a significant and rather sustained (compared to normal clearing time of Ca^{2+}) increase in $[\text{Ca}^{2+}]_{\text{in}}$ which was not associated with visible contractile activity. Fluo-3 fluorescence declines more than two fold within ~ 3 minutes (this was not studied further). For quantification purpose, in order to minimize cell-to-cell variation, the values of stress-induced Fluo-3 fluorescence in individual myocytes were normalized to the corresponding peak values of caffeine response. The magnitude of this relative fluorescence was 0.250 ± 0.01 (Fig. 4B; 5 heart preparations; $n = 11$ myocytes total). The effects were fully abolished in the absence of extracellular (and thus t-tubular) Ca^{2+} . The relative fluorescence of Fluo-3 was only 0.012 ± 0.005 , or ≈ 20 fold smaller than in the presence of 1.5 mM extracellular Ca^{2+} (Fig. 4B; 3 heart preparations; $n = 5$ myocytes total; $p < 0.001$).

In order to further confirm that Ca^{2+} influx originates from sealed t-tubules but, for example, not from outer sarcolemmal membrane (e.g. due to membrane stretch) we have performed similar experiments with myocytes which have been detubulated prior to application of caffeine. In these experiments, relative increase in Fluo-3 fluorescence was only 0.060 ± 0.016 , or >4 fold smaller than in normal myocytes (2 heart preparations; $n = 7$ myocytes total; $p < 0.001$). It should be noted that single osmotic stress is not 100% efficient at sealing t-tubules [27, 28], and therefore, the sealing of remaining t-tubules upon second stress is expected to lead to measurable, although significantly smaller, Ca^{2+} influx.

It can still be argued that the source of Ca^{2+} influx upon resolution of hyposmotic stress is not solely sealed t-tubules but, for example, stressed mitochondria as well. However, this is unlikely, since pretreatment of myocytes with both caffeine and 200 μM DNP (a powerful mitochondrial decoupler and depolarizing agent) does not abolish characteristic increase in Fluo-3 fluorescence upon washout of 0.6 Na hyposmotic solution observed in the presence of caffeine only (Fig. 5). As a standard approach, Fluo-3 fluorescence was measured just before the washout of 0.6 Na solution and 30 sec after washing with normal Tyrode. The difference in fluorescence was then normalized to the peak fluorescence value of caffeine response. Measured in this way, the relative increase in Fluo-3 fluorescence was 0.183 ± 0.019 (3 heart preparations; $n = 8$ myocytes total). This number is likely an underestimation of the true magnitude of the Fluo-3 fluorescence increase due to influx of t-tubular Ca^{2+} because of at least two reasons. First, depolarizing mitochondria with DNP leads to relatively small and gradual release of mitochondrial Ca^{2+} into the cytosol. This effect can also be observed in the absence of extracellular (and thus t-tubular) Ca^{2+} (Fig. 5). Second, on average, Fluo-3 fluorescence at 30 second mark is only about $\sim 80\%$ of the maximum fluorescence increase (this was not quantified in detail). Importantly, in the absence of extracellular Ca^{2+} relative Fluo-3 fluorescence at 30 sec mark is only 0.017 ± 0.002 (2 heart preparations; $n = 6$ myocytes total), or >10 fold smaller ($p < 0.001$) than in the presence of 1.5 mM extracellular Ca^{2+} .

3.5. Effects of extracellular K^+ and ouabain on $[\text{Ca}^{2+}]$ in sealed t-tubules

The data in Fig. 2 strongly suggest the involvement of Ca^{2+} channels in the influx of Ca^{2+} from sealed t-tubules. However, for that influx to occur the membrane potential of t-tubules should be significantly depolarized to allow for opening of Ca^{2+} channels. This

depolarization can potentially be caused by various reasons, including membrane stretch. Therefore, we initially reasoned that stabilization of resting membrane potential should significantly minimize the Ca^{2+} influx from sealed t-tubules. Membrane potential stabilization can be achieved using elevated extracellular K^+ (K_o). In this approach, moderate increase in K_o , such that membrane depolarization is still below activation potential for Ca^{2+} channels opening, would lead to significant stabilization of resting membrane potential due to increase in I_{K1} conductance (strongly K_o -dependent). In order to test this hypothesis, we compared the dynamics of $[\text{Ca}^{2+}]_{\text{in}}$ in response to washout of hyposmotic stress in the presence of 5.4 mM K_o and 20 mM K_o using the same experimental approach as presented in Fig. 4A and Fig. 5A. As before, experiments were performed in the presence of 10 mM caffeine and data analyzed as described in Fig. 2.

In the presence of elevated K_o the characteristic increase in $[\text{Ca}^{2+}]_{\text{in}}$ was abolished (Fig. 6A). Moreover, Fluo-3 fluorescence 30 sec after washout of 0.6 Na solution was even smaller than before its application, with relative (to the caffeine response) value of -0.070 ± 0.006 (3 heart preparations, $n=3$ myocytes total) vs 0.250 ± 0.01 in the presence of normal K_o .

The above effect would be consistent with extracellular Ca^{2+} being trapped in sealed t-tubules and sustained there at relatively high concentration. In order to test this hypothesis we compared the level of $[\text{Ca}^{2+}]_{\text{st}}$ using Rhod-5N trapped in sealed t-tubules under conditions of normal and elevated K_o using confocal microscopy approach as in Fig. 1. Experiments were performed at 5.4 mM and 20 mM K_o present throughout the whole procedure of hyposmotic shock and imaging. As predicted, the relative (to that in the presence of 1 mM Cd^{2+}) fluorescence of Rhod-5N was increased nearly 6 fold from 0.07 ± 0.02 to 0.41 ± 0.05 , at normal and elevated K_o , respectively (8 heart preparations, $n=10$ myocytes each preparation; $p<0.001$; Fig. 6B). Recalculation of Rhod-5N fluorescence to $[\text{Ca}^{2+}]$ as described before shows the corresponding change of $[\text{Ca}^{2+}]_{\text{st}}$ from $[\text{Ca}^{2+}]_{\text{st}} \approx 36 \pm 10 \mu\text{M}$ (see Fig.1) to $490 + 166/-118 \mu\text{M}$, or ~ 13 fold increase.

It can be argued that high level of $[\text{Ca}^{2+}]_{\text{st}}$ can also be preserved at lowered (2.5 mM; below normal) K_o , a condition which would tend to hyperpolarize the membrane potential and shift it away from activation potential for Ca^{2+} channels. The data show, however, that this manipulation of K_o leads to low level of $[\text{Ca}^{2+}]_{\text{st}}$ similar to that observed at normal K_o ($41 + 10/-9 \mu\text{M}$; 2 heart preparations, $n=10$ myocytes each preparation; $p<0.001$; Fig. 6B). Since lowering K_o would lead to a decrease in stabilizing I_{K1} conductance, the latter results support the idea that during osmotic stress stability of membrane potential may be an important parameter determining the fate of $[\text{Ca}^{2+}]_{\text{st}}$.

The measured increase in $[\text{Ca}^{2+}]_{\text{st}}$ during treatment with 20 mM K_o may also be linked to Na/K ATPase activity. Activation of Na/K ATPase by elevated K_o would lead to increased uptake of cytosolic Na by sealed t-tubules which, in turn, would be followed by activation of t-tubular NCX and associated increase in $[\text{Ca}^{2+}]_{\text{st}}$. To test this hypothesis, we performed detubulation procedure in absence or presence of 1mM ouabain, a potent inhibitor of Na/K ATPase. Treatment with ouabain abolished the characteristic increase in t-tubular Rhod-5N fluorescence observed in myocytes detubulated in the presence of 20 mM K_o alone.

Recalculation of Rhod-5N fluorescence to $[Ca^{2+}]$ shows the corresponding >6 fold decrease of $[Ca^{2+}]_{st}$ from $519 \pm 123/-95 \mu M$ to $33 \pm 6 \mu M$, in the absence and presence of 1mM ouabain, respectively (2 heart preparations, n =10 myocytes each preparation; $p<0.001$; Fig. 6C).

4. Discussion

While the importance of fully functional t-tubular network in the performance of the heart is long and well recognized, the mechanistic understanding of t-tubular remodeling, especially its origin, in various cardiac pathologies is lagging behind. Numerous studies have now provided a wealth of useful information on the topology of t-tubular network in a number of relevant diseases and characterized in significant detail disruptions in various t-tubular related functions (for review) [15]. In particular, it has been well documented that the remodeling or loss of t-tubules in ventricular myocytes from failing hearts is associated with various defects in excitation-contraction coupling. For example, in rat, significant rearrangement of t-tubules in myocytes from failing hearts leads to appearance of orphaned ryanodine receptors and consequent dyssynchronous release of Ca^{2+} from SR [37]. Unfortunately, most of the data are obtained at the late stages of a disease when t-tubules are already remodeled, and therefore it remains unclear what drives what, i.e. whether the origin of disease is the remodeling of t-tubules or *vice versa*. In this regards we find several recent reports very important in resolving the issue.

For example, it has been shown that t-tubular remodeling can be detected before the appearance of clinical signs of heart failure [11, 38]. This is suggestive, although not fully conclusive, of the ‘driving role’ of t-tubular remodeling in the development of disease. The other exciting finding is that by Sacconi et al (2012) [31] who showed that in myocytes isolated from failing hearts action potential propagation may be impaired in some t-tubules which are still connected to the outside solution because they can be labeled with extracellular dye. The latter is indicative of highly constricted t-tubular lumen, an ‘invisible’ step towards its complete sealing, followed by membrane depolarization with a number of easily predictive consequences to EC coupling. The principal point here is that in heart failure t-tubular remodeling may likely be initiated by tight constrictions of t-tubular lumen, followed by further rearrangement, ‘apoptosis’, or some type of long-term disintegration process of sealed t-tubules. This idea is in contrast to other view that the remodeling may be due to retraction of t-tubules from the body of the myocytes in a way opposite to that during the developmental stage [37]. Strong support for ‘constriction hypothesis’ of t-tubular remodeling comes from experiments with cultured adult ventricular myocytes. In particular, it has been shown in late 90s that t-tubules become progressively lost during culture [39-41], although the underlying mechanisms were not disclosed at that time. In 2010 Hammer et al [29] used a combination of membrane labels and provided strong evidence that under culture conditions detubulation likely proceeds as a “pinching off” of the plasma membrane. The idea of t-tubular constrictions and “pinching off” t-tubules in heart failure found strong support in the recent study by Pinali et al (2013) [42]. The authors employed 3D electron microscopy and showed the presence of swollen domains of t-tubules flanked by narrowed (<200 nm) regions (constrictions) in affected tissues. In addition, some swollen t-tubules were shown not to be connected to the outside membrane at all suggesting ‘pinching off’ t-

tubules. The current state of research in this area, however, remains highly controversial. In particular, Wagner et al (2012) [43] showed using high resolution STED confocal imaging the presence of t-tubular dilations but not constrictions in ventricular myocytes isolated from failing hearts. While t-tubular dilations observed in the mentioned study found strong support, a critical analysis of the approach shows, however, that increased spatial resolution of STED microscopy may still not be sufficient to resolve functionally important constrictions which may coexist in close (or distant) proximity to dilated regions. In particular, t-tubule diameters were analyzed only for transverse (“free”) tubules, i.e. running mostly in Z direction. Unfortunately, the resolution of the STED microscope (employed in mentioned study) in Z direction is no better (or even slightly worse) than that in standard confocal microscope. Therefore, short (point-like) constrictions may potentially be overlooked using this approach. In this regard, the existence of very small functional t-tubular constrictions is supported by mentioned above study by Sacconi et al (2012) [31]. It should also be noted that t-tubular remodeling may not be the same in different models (e.g. hyposmotic shock in our study) and relevant diseases. Clearly, more work and new approaches are necessary to determine the mechanisms of t-tubular remodeling in heart failure.

In this regard, we have recently provided further evidence in support of t-tubular constriction hypothesis. Specifically, we showed that fast and mostly hidden t-tubular remodeling characterized by constriction of t-tubular lumens can be observed in isolated mouse ventricular myocytes under conditions which can collectively be characterized as metabolic stress [30]. In our most recent report we showed that in isolated cells resolution of hyposmotic stress of physiologically relevant magnitude also leads to quick and dramatic t-tubular remodeling [27], characterized, in particular, by complete sealing of significant part of t-tubules. Both metabolic and osmotic stresses of various magnitudes are common events during the life time of the organism and therefore it seems highly plausible that sudden (e.g. during infarct) or slowly developing (i.e. during hypertension) stress may lead to t-tubular remodeling through the mechanism of constricting and/or sealing of t-tubules.

The consequences of the loss of t-tubules on EC coupling, sarcolemmal Ca^{2+} influx and uptake has been addressed before using formamide-induced detubulation ([26, 44-46]. In contrast, the process of t-tubular sealing and the role of sealed t-tubules themselves had little attention [33].

Clearly, within the framework of the above hypothesis of t-tubular sealing Ca^{2+} related phenomena stand out most. Therefore, in this study we wanted to first answer the most straightforward question – what is the concentration of Ca^{2+} in sealed t-tubules? Results are not easily predictable. Sealed t-tubules can be considered as ‘inverted microcells’ with small closed ‘extracellular’ space. Immediate answer would be that these t-tubular ‘microcells’ may sustain ionic homeostasis similar to that of the bulk extracellular solution which is characterized by millimolar Ca^{2+} concentrations. However, the closed ‘extracellular’ environment and significantly different densities of various ion channels, pumps and exchanges compared to that in the outer sarcolemma [1, 45] make predictions highly speculative. Computer modeling of these ‘microcells’ will likely follow but at this juncture experimental approach was the only viable option. The central finding is that the $[\text{Ca}^{2+}]_{\text{st}}$ is

low (Fig. 1). Assuming various limitations of the approach, it is also safe to say that $[Ca^{2+}]_{st}$ is likely lower than 100 μM . From this data, it can be concluded that nearly all initially trapped Ca^{2+} is transported inside the myocyte. As mentioned in *Results*, the amount of transported Ca^{2+} is significant, comparable to the full load of SR. However, it seems that a relatively slow sealing process allows the myocytes to handle the massive influx quite safely as most of the myocytes not only survive the resolution of hyposmotic shock but often do it without any visual contractile activity.

In this study we purposely used 0.6 Na hyposmotic solution as it leads to strong and lasting (> several hours) sealing of t-tubules allowing for easier quantification of their ionic homeostasis. It is tempting to suggest though that less stronger and repeated hyposmotic shocks may lead to repeated reversible constrictions of t-tubules each associated with translocation of significant amount of Ca^{2+} . Since sealed or partially sealed t-tubules are essentially disconnected from the outer sarcolemmal, this Ca^{2+} transport would actually be independent of the membrane potential of the cell body thus constituting a novel mechanisms of t-tubular-dependent Ca^{2+} entry in ventricular myocytes. Future studies will address this potential phenomenon.

The data in Fig. 2, Fig. 3 and Fig. 6 clearly show that the loss of t-tubular Ca^{2+} is not a result of stress-induced leakiness of t-tubular membrane since $[Ca^{2+}]_{st}$ can be strongly manipulated in μM to mM range with Ca^{2+} channel blocker nicardipine, increases in intracellular Ca^{2+} by caffeine or even by elevation of extracellular K^+ . Moreover, kinetics of $[Ca^{2+}]_{st}$ change in response to caffeine application is very informative. The important finding is that the time to $[Ca^{2+}]_{st}$ peak is significantly longer than the time to peak for $[Ca^{2+}]_{in}$ (Fig. 3A). One explanation would be that the uptake of Ca^{2+} by sealed t-tubules is governed not by bulk cytosolic Ca^{2+} but may rather depend on $[Ca^{2+}]$ in some specialized domain(s). The latter could be, in particular, a junctional space between SR and t-tubule membrane where the local $[Ca^{2+}]$ can be sustained at the levels higher than in the bulk cytoplasm as Ca^{2+} leaves the SR. As mentioned in *Results* many sealed t-tubules appear swollen and, therefore, the junctions may be significantly remodeled as well. The ultrastructural data necessary to convincingly address this issue are not available. However, confocal imaging studies show that in osmotically detubulated rat ventricular myocytes ryanodine receptors remain highly co-localized with t-tubular membrane [47]. Alternatively, a surge in bulk $[Ca^{2+}]_{in}$ can trigger a cascade of signaling pathways with their own kinetics leading, for example, to activation and slow relaxation of t-tubular Ca^{2+} pump which has been shown is highly concentrated in t-tubules [45].

One of the important goals of this study was to show that sealed t-tubules are the major source of Ca^{2+} in stress-induced effects. It is feasible, for example, that mechanical stretch of the sarcolemma may be transduced to intact SR leading to Ca^{2+} release, whether in the presence or absence of extracellular Ca^{2+} . The increase in $[Ca^{2+}]_{in}$ upon resolution of hyposmotic stress, however, can be observed, or even better observed, in the presence of 10 mM caffeine (Fig. 3). It could also be argued that the stretch of outer sarcolemma may lead to opening of Ca^{2+} channels (by yet unknown mechanism) or induce some other Ca^{2+} selective permeability. These scenarios are largely discounted by lack of any significant

effects of hyposmotic stress in experiments using ventricular myocytes which were already detubulated.

Mitochondria is yet another potential source of Ca^{2+} in the observed phenomena. Although the resting concentration intramitochondrial Ca^{2+} is low (in submicromolar range; [48]), a sheer magnitude of total relative volume of these organelles strongly implicates them in various kinds of Ca^{2+} signaling [49]. In particular, depolarization of mitochondria with DNP leads to release of significant amount of Ca^{2+} into cytosol independent of extracellular Ca^{2+} or the load of SR [50]. The data in Fig. 5 obtained in the absence of extracellular Ca^{2+} and presence of 10 mM caffeine are consistent with previous findings. However, sealing of t-tubules in the presence of extracellular Ca^{2+} is associated with significant additional increase in $[\text{Ca}^{2+}]_{\text{in}}$, consistent with t-tubular Ca^{2+} being released into the cytosol (Fig. 5).

The effect of extracellular K_o on $[\text{Ca}^{2+}]_{\text{st}}$ is significant (Fig. 6B) but not easily interpretable. Increase in K_o was not associated with increase in $[\text{Ca}^{2+}]_{\text{in}}$ (due to K-dependent membrane depolarization and potential partial opening of Ca^{2+} channels) and, therefore, the consequent increased pumping of cytosolic Ca^{2+} back to sealed t-tubules was not expected. If anything, upon resolution of hyposmotic shock in elevated K_o the $[\text{Ca}^{2+}]_{\text{in}}$ was even lower than in the resting state (Fig. 6A). Our initial line of thought was that stabilization of resting membrane potential in elevated K_o , in particular due to the presence of I_{K1} channels, during osmotic stress may be one of the important factors in preventing significant opening of Ca^{2+} channels in sealed (or sealing) t-tubules. It is more likely, though, that the increased $[\text{Ca}^{2+}]_{\text{st}}$ is associated with increased pumping by t-tubular Na/K ATPase (due to higher K_o), leading to increase in t-tubular Na and consequent uptake of intracellular Ca^{2+} aided by NCX [33]. Results of experiments using ouabain to inhibit Na/K ATPase strongly support this view (Fig. 6C). We believe that both stability of membrane potential and activity of Na/K ATPase are important factors in determining $[\text{Ca}^{2+}]_{\text{st}}$. However, it is clear that a detailed quantitative investigation of the effects of K_o on $[\text{Ca}^{2+}]_{\text{st}}$ should take into account a combined action of various other contributing ionic pathways, and thus at this stage this work is beyond the scope of this study. Importantly, understanding the major pathways involved in regulation of $[\text{Ca}^{2+}]_{\text{st}}$ will likely be helpful in developing future therapeutic strategies in various relevant disease states including heart failure.

5. Conclusions

This study shows that in mouse ventricular myocytes sealing of t-tubules in response to hyposmotic stress is associated with influx of trapped t-tubular Ca^{2+} into the cell. Sealed t-tubules remain functional and are capable of significant regulation of their Ca^{2+} content in response to various stimuli. The data further support the hypothesis that various forms of t-tubular remodeling may be associated with their stress-induced sealing leading to aberrant handling of intracellular Ca^{2+} due to active ionic homeostasis of Ca^{2+} in sealed t-tubules.

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Abbreviations

$[\text{Ca}^{2+}]_{\text{in}}$	intracellular $[\text{Ca}^{2+}]$
$[\text{Ca}^{2+}]_{\text{o}}$	extracellular $[\text{Ca}^{2+}]$
$[\text{Ca}^{2+}]_{\text{st}}$	$[\text{Ca}^{2+}]$ in sealed t-tubules
K_{o}	extracellular K^+
SR	sarcoplasmic reticulum
NCX	sodium/calcium exchanger

Highlights

Sealed t-tubules contain Ca^{2+} at low concentration.

T-tubular sealing is associated with translocation of trapped Ca^{2+} into the cell.

Ca^{2+} in sealed t-tubules can be modulated by various stimuli.

Figure 1. Concentration of Ca^{2+} in sealed t-tubules

A. Low affinity Ca^{2+} indicator Rhod-5N ($K_d \sim 320 \mu\text{M}$) was trapped in sealed t-tubules along with 0 mM or 1 mM Cd^{2+} . Myocytes were then imaged using confocal microscope in the absence of extracellular Rhod-5N and Cd^{2+} but in the presence of 1 mM extracellular Ca^{2+} . Small fluorescence in the absence of t-tubular Cd^{2+} (top) is consistent with either low $[\text{Ca}^{2+}]_{\text{st}}$ or low (leaked) Rhod-5N. However, trapped Cd^{2+} (K_d for $\text{Cd}^{2+} \sim 4$ orders less than that for Ca^{2+}) reveals the presence of Rhod-5N in sealed t-tubules (bottom). Inserts at the bottom corners are magnifications of selected areas in the middle of the images. Scale bars: 10 μm . B. Quantification of the data in A. The magnitude of total Rhod-5N fluorescence due to t-tubular Ca^{2+} is ~ 15 fold smaller than that in the presence of t-tubular Cd^{2+} . The data were normalized (F_N) to Cd^{2+} -induced fluorescence. C. *In vitro* comparison of sensitivity of

Rhod-5N to Ca^{2+} and Cd^{2+} . Fluorescence in the presence of 1 mM Ca^{2+} corresponds to ~50% of that in the presence of 1 mM Cd^{2+} (maximum Rhod-5N fluorescence). *D.* Estimation of $[\text{Ca}^{2+}]_{\text{st}}$ using the data in *B* and *C*. The data in *B* were converted to $[\text{Ca}^{2+}]_{\text{st}}$ using Hill equation and assuming $K_d^{\text{Ca}} \sim 320 \mu\text{M}$, hill coefficient $h=1$, and using Cd^{2+} -induced fluorescence as calibration data (~50% of which corresponds to that produced by 1 mM Ca^{2+}).

Figure 2. Effect of blockade of calcium channels on $[\text{Ca}^{2+}]$ in sealed t-tubules

A. Ca^{2+} indicator Rhod-5N was trapped in sealed t-tubules using hyposmotic shock either in control conditions (Ctrl; no agents added), or in the presence of 10 μM nicardipine or 1 mM Cd^{2+} . Myocytes were then imaged using confocal microscope in the absence of extracellular Rhod-5N, nicardipine and Cd^{2+} but in the presence of 1 mM extracellular Ca^{2+} . Inserts at the bottom corners are magnifications of selected areas in the middle of the images. Scale bars: 10 μm . **B.** Quantification of the data in **A**. Blockage of calcium channels leads to ≈ 3.6 fold increase in t-tubular Rhod-5N fluorescence (Ctrl vs Nic) reaching $\sim 37\%$ of that in the presence of Cd^{2+} . The data were normalized (F_N) to Cd^{2+} -induced fluorescence. **C.** Estimation of $[\text{Ca}^{2+}]_{\text{st}}$ using the data in **B** as described earlier in Fig. 1.

Figure 3. Effects of caffeine application on $[\text{Ca}^{2+}]$ in sealed t-tubules

A. Overlaid examples of the time courses of t-tubular Rhod-5N fluorescence (black trace) and cytosolic Fluo-3 fluorescence (gray trace) in response to application of 10 mM caffeine ($\uparrow$). F – fluorescence, in arbitrary units, is adjusted to match baseline and peak values for both traces. Note longer time to peak for Rhod-5N fluorescence ($\blacktriangledown$). All recordings were performed in the presence of 1 mM extracellular Ca^{2+} . Myocytes were electrically stimulated at 1 Hz before application of caffeine in order to load SR. B. Quantification of peak times for Fluo-3 and Rhod-5N fluorescence for the data as in A. C. Quantification of steady-state (Ctrl) and peak (Caff) Rhod-5N fluorescence for the data as in A. The data were corrected for average background (no trapped Rhod-5N) and normalized to average fluorescence of Rhod-5N trapped along with 1 mM cadmium (Cd^{2+}). Correction data were obtained in separate experiments using identical settings. D. Estimation of steady-state and peak $[\text{Ca}^{2+}]_{\text{st}}$ using the data in C as described earlier in Fig. 1.

Figure 4. Effects of resolution of hyposmotic shock on intracellular $[Ca^{2+}]$

A. Ventricular myocytes loaded with Fluo-3 Ca^{2+} indicator were first exposed for 7 min to 0.6 Na hyposmotic solution in the presence of 1.5 mM extracellular Ca^{2+} and 10 mM caffeine was added in order to empty sarcoplasmic reticulum. ~30 sec after the caffeine application 0.6 Na solution was washed out using Tyrode solution (Tyr) containing 0 mM or 1.5 mM extracellular Ca^{2+} and 10 mM caffeine. For presentation purposes Fluo-3 fluorescence (F , arbitrary units) from two representative myocytes was scaled such that the peaks of caffeine response (●) and the steady-state levels of fluorescence just before the washout of 0.6 Na solution (○) match each other. *Insert:* Example of sporadic Ca^{2+} transients observed in the presence of 1.5 mM Ca^{2+} and intact SR (0 mM caffeine). Extended time scale. B. Quantification of the data in A. Absolute levels of Fluo-3 fluorescence (corrected for ~6% background fluorescence) were measured ~30 sec after application of Tyr solution (▼; ▽) and the data normalized to the peak of caffeine response. Accordingly, F_R stands for relative fluorescence.

Figure 5. Contribution of mitochondria to Ca^{2+} influx during sealing of t-tubules

A. Ventricular myocytes loaded with Fluo-3 Ca^{2+} indicator were first exposed to 0.6 Na hypotonic solution in the presence of 1.5 mM extracellular Ca^{2+} and 10 mM caffeine was added along with supplemented with 200 μM 2,4-dinitrophenol (DNP) in order to both empty sarcoplasmic reticulum and depolarize mitochondria. ~ 90 sec after the caffeine application 0.6 Na solution was washed out using Tyrode solution (Tyr) containing 0 mM or 1.5 mM extracellular Ca^{2+} and in continuing presence of caffeine and DNP. For presentation purposes Fluo-3 fluorescence (F , arbitrary units) from two representative myocytes was scaled such that the steady-state levels of fluorescence just before the washout of 0.6 Na solution ($\circ$) match each other. A spike of Fluo-3 fluorescence of unknown origin is indicated ($\blacklozenge$). **B.** Quantification of the data in A. Absolute levels of Fluo-3 fluorescence (corrected for $\sim 6\%$ background fluorescence) were measured ~ 30 sec after application of Tyr solution ($\blacktriangledown$; $\triangledown$) and the data normalized to the peak of caffeine response. Accordingly, F_R stands for relative fluorescence.

Figure 6. Effects of extracellular potassium on Ca^{2+} movements during sealing of t-tubules

A. Ventricular myocytes were treated as described in Fig. 4 in the presence of 5.4 mM and 20 mM extracellular K^+ (K_o) throughout the whole procedure. Increase in relative (to caffeine response; F_R) cytosolic Fluo-3 fluorescence observed in the presence of 5.4 K_o was changed to a decrease below resting levels in the presence of 20 mM K_o . B. $[Ca^{2+}]_{st}$ was measured in the presence of 2.5, 5.4 mM and 20 mM extracellular K^+ (K_o) throughout the whole procedure using Rhod-5N as described in Fig. 1. C. Effect of 1 mM ouabain on $[Ca^{2+}]_{st}$ in the presence of 20 mM K_o throughout the detubulation procedure using Rhod-5N as described in Fig. 1. Experiments in A, B and C were performed in the presence of 1.5 mM extracellular Ca^{2+} .