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Energetic state is a strong regulator of sarcoplasmic reticulum Ca^{2+} loss in cardiac muscle: different efficiencies of different energy sources

Malle Kuum[1,2,3], Allen Kaasik[1,2,3], Frederic Joubert[1,2], Renée Ventura-Clapier[1,2], and Vladimir Veksler[1,2]*

[1]*Faculté de Pharmacie, U-769 INSERM, Université Paris-Sud, Châtenay-Malabry F-92296, France;* [2]*Univ Paris-Sud, Châtenay-Malabry F-92296, France; and* [3]*Department of Pharmacology, Centre of Molecular and Clinical Medicine, University of Tartu, Tartu, Estonia*

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Aims Increased diastolic sarcoplasmic reticulum (SR) Ca^{2+} loss could depress contractility in heart failure. Since the failing myocardium has impaired energetics, we investigated whether Ca^{2+} loss is linked to changes in energetic pathways.

Methods and results Leakage from SR in mouse permeabilized preparations was assessed using exogenous ATP, ATP + phosphocreatine (activation of bound creatine kinase, CK), ATP + mitochondrial substrates (mitochondrial activation), or with all of these together (optimal energetic conditions) in Ca^{2+}-free solution. In ventricular fibres caffeine-induced tension transients under optimal energetic conditions were used to estimate SR $[Ca^{2+}]$. In cardiomyocytes, intra-SR Ca^{2+} was monitored by use of the fluorescent marker Mag-fluo 4. In fibres, SR Ca^{2+} content after 5 min incubation strongly depended on energy supply (100%—optimal energetic conditions; $27 \pm 5\%$—exogenous ATP only, $52 \pm 5\%$—endogenous CK activation; $88 \pm 8\%$—mitochondrial activation, $P < 0.01$ vs. CK system). The significant loss with only exogenous ATP was not inhibited by the ryanodine receptor blockers tetracaine or ruthenium red. However, the SR Ca^{2+}-ATPase (SERCA) inhibitors cyclopiazonic acid or 2,5-di(tert-butyl)-1,4-benzohydroquinone significantly decreased Ca^{2+} loss. At 100 nM external $[Ca^{2+}]$, the SR Ca^{2+} loss was also energy dependent and was not significantly inhibited by tetracaine. In cardiomyocytes, the decline in SR $[Ca^{2+}]$ at zero external $[Ca^{2+}]$ was almost two times slower under optimal energetic conditions than in the presence of exogenous ATP only.

Conclusion At low extra-reticular $[Ca^{2+}]$, the main leak pathway is an energy-sensitive backward Ca^{2+} pump, and direct mitochondrial-SERCA ATP channelling is more effective in leak prevention than local ATP generation by bound CK.

1. Introduction

In the heart, sarcoplasmic reticulum (SR) is the central element in excitation–contraction coupling. SR-mediated calcium uptake mechanisms have been described in a number of studies and are well characterized. Much less is known about diastolic SR Ca^{2+} fluxes towards the cytosol, which increase when free intra-SR $[Ca^{2+}]$ rises.[1] These fluxes are the basis for Ca^{2+} loss due to Ca^{2+} movement from the SR under resting conditions, which decreases intra-SR calcium content and thus diminishes the amount of releasable Ca^{2+}, which in turn could alter cardiac contractility. There are two main hypothesis that describe how Ca^{2+} efflux from the SR occurs (see[1,2] and references therein). The first one suggests a leak from the SR, presumably via the cardiac ryanodine receptor (RyR). Theoretically, this leak should increase as the intra/extra-reticular Ca^{2+} gradient increases reaching a maximum in late diastole when the leak equals the rate of Ca^{2+} pumping. Such a leak seems to be associated with considerable energy wastage. The second pathway for Ca^{2+} loss is via the Ca^{2+} pump, due to its reversibility. As in the first case, this flux increases during diastole but the loss of Ca^{2+} does not lead to energy wastage since the reverse flux is coupled to ATP generation. It has been suggested that Ca^{2+} loss through the Ca^{2+} pump is sensitive to cellular energetic state.[1]

SR Ca^{2+}-ATPase (SERCA) function strongly depends on the local ATP/ADP ratio in the pump's vicinity.[3] In living cardiomyocytes, this ratio is maintained by different energetic systems. One is a diffusive exchange of local (near the

* Corresponding author. Tel: +33 1 4683 5763; fax: +33 1 4683 5475.
E-mail address: vladimir.veksler@cep.u-psud.fr

ATPases) adenine nucleotide pools with ATP and ADP in the bulk cytosol. Another system, which is able to efficiently link energy production and utilization, is creatine kinase (CK) structurally associated with SR membranes that can use phosphocreatine (PCr) to rephosphorylate all of the ADP produced by the ATPases. A third system recently described[4] for maintaining regulation of the ATP/ADP ratio is direct energy channelling between mitochondria and cellular ATPases. In the highly oxidative cardiac cells, mitochondria appear to be clustered at sites of high ATP demand and are organized into ordered elongated bundles, which are wrapped around the myofibrils and in contact with SR (see[5,6] and references therein). Mitochondria located close to ATPase sites have preferred access to locally produced ADP and can efficiently sustain calcium uptake. The existence of channelling suggests that in oxidative muscles like heart, mitochondria, myofilaments, and SR are incorporated into functional units or 'intracellular energetic units' around sarcomeres.[7] In addition, such units probably contain glycolytic ATP-producing enzymes.[8]

A number of cardiac pathologies including heart failure are associated with alterations in myocardial energy metabolism.[9] This raises the possibility that compromised energetics might not only decelerate Ca^{2+} pumping into the SR but that it might also favour diastolic SR Ca^{2+} efflux, thus decreasing the amount of releasable Ca^{2+} and thereby altering cardiac contractility. In the present study, we used permeabilized preparations, which provide a unique means to investigate energetic functional regulation of intracellular organelles, in order to test this hypothesis. Indeed, the use of specific membrane permeabilization with detergents enables the study of organelle function while maintaining the cellular architecture and controlling the intracellular milieu. Thus, we have investigated (i) the identity of the main pathway of Ca^{2+} efflux from SR, (ii) the sensitivity of Ca^{2+} efflux to cell energetic state, and (iii) the relative contribution of various energetic pathways in preventing this Ca^{2+} efflux.

2. Methods

2.1 Preparation of permeabilizes fibres

Our study conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Male C57BL/6 mice were anesthetized with sodium thiopental according to the recommendations of the Institutional Animal Care Committee (INSERM, Paris, France). Left ventricular fibres were dissected and permeabilized as previously described.[4]

2.2 Solutions

All solutions were prepared using the basic solution. This solution contained (in mM) ethylene glycol-bis(β-aminoethyl ether)*N*, *N*, *N*′,*N*′-tetra-acetic acid 10 (EGTA; except for release solutions, 0.2), *N*, *N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid 60 (BES, pH 7.1), free Mg^{2+} 1, taurine 20, glutamic acid 5, malic acid 2, K_2HPO_4 3, dithiothreitol 0.5, P^1,P^5 diadenosine pentaphosphate 0.04 (to inhibit adenylate kinase activity), MgATP 3.16; ionic strength was adjusted to 160 mM with potassium methanesulfonate. Desired [Ca^{2+}] was obtained by varying the CaK_2EGTA/K_2EGTA ratio. Relaxing solution was made by adding 12 mM PCr. Loading solution was the same as relaxing solution but contained in addition 316 nM [Ca^{2+}]. Leak solutions were the same as basic solution but additionally contained 2 mM NaN_3 (to inhibit mitochondria) and/or 12 mM PCr (to activate CK). In order to study glycolytic support, glycolytic intermediates (4 mM glyceraldehyde-3-phosphate and 4 mM phosphoenolpyruvate) and 4 mM NAD were added to leak solution containing ATP and NaN_3.

Release solution was the same as relaxing solution but additionally contained 2 mM NaN_3 and 0.2 mM EGTA (zero Ca^{2+}). All experiments (except where stated) were carried out in the presence of 10 μM RU360, a specific mitochondrial calcium uniporter blocker, in order to avoid any participation of mitochondria in Ca^{2+} fluxes.

2.3 Estimation of the releasable SR calcium content *in situ*

Isometric force developed by permeabilized fibres was measured as previously described[4] at 22°C. After incubation in relaxing solution, SR loading was performed for 5 min in loading solution at 316 nM Ca^{2+}. After loading, fibres were incubated in leak solutions in the absence of Ca^{2+} or at 100 nM Ca^{2+} under different energetic conditions for various periods of time (30–300 s). Excess EGTA was then washed out in release solution with 0.2 mM EGTA for 1 min before addition of 5 mM caffeine to induce calcium release. This release was always elicited under standard energetic conditions (3.16 mM MgATP+12 mM PCr in the presence of sodium azide) in order to avoid different responses of myofilaments to different energetic conditions. The caffeine-induced tension transient was used to calculate the time course of free [Ca^{2+}] close to the myofibrils during release, using the [Ca^{2+}]/tension dependence as an internal calibration. The [Ca^{2+}]/tension relationship was thus measured at the end of each experiment, in the presence of 5 mM caffeine and under conditions identical to those of Ca^{2+} release, except that 10 mM EGTA was present instead of 0.2 in order to buffer free Ca^{2+} adequately. Using this relationship fitted by Hill equation, the [Ca^{2+}] at each step of the tension–time integral was recalculated to obtain [Ca^{2+}]-time integrals (S_{Ca}), which were used to evaluate the amount of Ca^{2+} released by the SR.

2.4 Intra-reticular [Ca^{2+}] monitoring

To monitor intraluminal SR Ca^{2+} concentration changes, we performed a separated series of experiments using plated onto coverslip mouse or rat ventricular myocytes freshly isolated as described elsewhere.[10] Cardiomyocytes were loaded with the low-affinity calcium indicator Mag-fluo 4 (5 μM, Kd = 22 μM) (Invitrogen) for 45 min at 22°C, washed with indicator-free solution and then permeabilized with saponin (50 μg/mL) for 3 min using relaxing solution (zero Ca^{2+}). Coverslips with mouse cells were attached on the stage of a Carl Zeiss LSM-510 confocal microscope using a recording chamber (Warner Instruments, Hamden, CT, USA). Images were acquired in line scan mode (at intervals of 3.5 or 7 ms) along the longitudinal axis of the cell. Intra-reticular [Ca^{2+}] changes in rat cells were monitored by epifluorescence microscopy (NIKON Eclipse TE300) using a standard FITC filter set.

The SR Ca^{2+} uptake was monitored at a relatively low extra-reticular [Ca^{2+}] (32 nM) to avoid too rapid and large Ca^{2+} accumulation in the SR leading to a saturation of indicator. All the experiments were performed at 22°C.

2.5 Statistical analysis

Values are expressed as mean ± SE. Statistical differences were determined using repeated measures or one-way ANOVA, followed by Bonferroni's multiple comparison test, or *t*-test. $P < 0.05$ was accepted as significant.

3. Results

3.1 Demonstration of SR Ca^{2+} loss *in situ*

The first sets of experiments aimed to demonstrate the existence of energy dependent SR Ca^{2+} loss in Ca^{2+}-free

medium by measuring SR Ca^{2+} content in permeabilized cardiac myocytes and in permeabilized fibres. Incubation of Mag-fluo 4 loaded mouse cardiomyocytes at 32 nM Ca^{2+} induced an accumulation of Ca^{2+} in the SR (*Figure 1*). Calcium removal from the external medium led to a decrease in intra-reticular [Ca^{2+}], thus showing that calcium leak occurred. This leak was significantly accelerated, if energy supply was reduced by inhibiting the mitochondria with sodium azide. Importantly, this accelerated calcium loss occurred in the medium still containing rather high ATP and PCr concentrations.

In the next series of experiments, we investigated the time course of the decrease in the amount of releasable Ca^{2+} in permeabilized cardiac fibres. The protocol is shown in *Figure 2A*. SR in permeabilized mouse ventricular fibres was loaded at 316 nM Ca^{2+} for 5 min under optimal energetic conditions (in the presence of 12 mM PCr and activated mitochondria). Afterwards, calcium was washed out with leak solution for 30 s, also under optimal energetic conditions, then fibres were incubated for 1 min in release solution (in order to wash out high EGTA). Finally, 5 mM caffeine was applied to induce release of the sequestered Ca^{2+}, which elicited a relatively high tension transient (*Figure 2B*). Peak tension was ~75% of maximal Ca-induced force. However, as the duration of incubation in leak solution increased, the caffeine-induced tension transient progressively declined (*Figure 2B*). This was not related to a run-down of the fibre state because at the end of experiment the tension transient was as high as at the beginning. Thus, in permeabilized fibres in the absence of extra-reticular Ca^{2+}, the SR *in situ* progressively loses internal Ca^{2+}.

Previously, we have shown[3,11,12] that in the absence of CK and mitochondrial function, 'cytosolic' ATP, even at high concentration, is not able to ensure a high phosphorylation potential for cellular ATPases. Here, under the same energetic conditions, i.e. with ATP as the sole energy source, we found that Ca^{2+} leak was markedly increased.

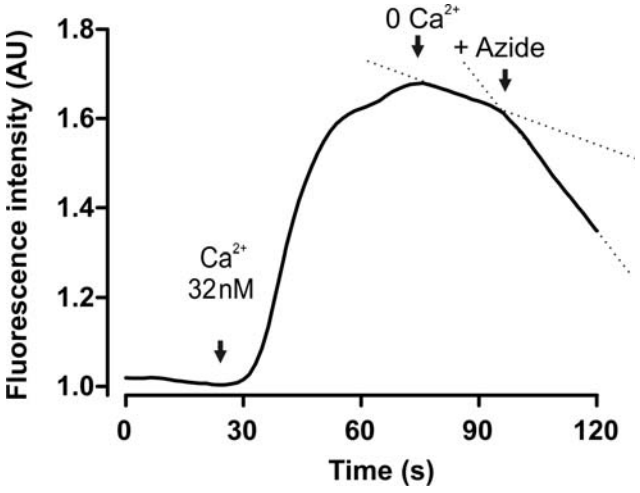

Figure 1 Loss of intra-reticular Ca^{2+} in calcium-free internal medium. Freshly isolated mouse ventricular myocyte loaded with fluorescent Ca^{2+} indicator Mag-fluo 4 was incubated at 32 nM Ca^{2+} in the presence of 3.16 mM MgATP, 12 mM PCr in the absence of mitochondrial inhibitor. Calcium withdrawal from the medium led to a continuous decrease in the fluorescence indicating SR Ca^{2+} loss. Velocity of the loss was markedly increased, if the mitochondria were inhibited by 2 mM sodium azide indicating that leakiness of the SR was energy-dependent.

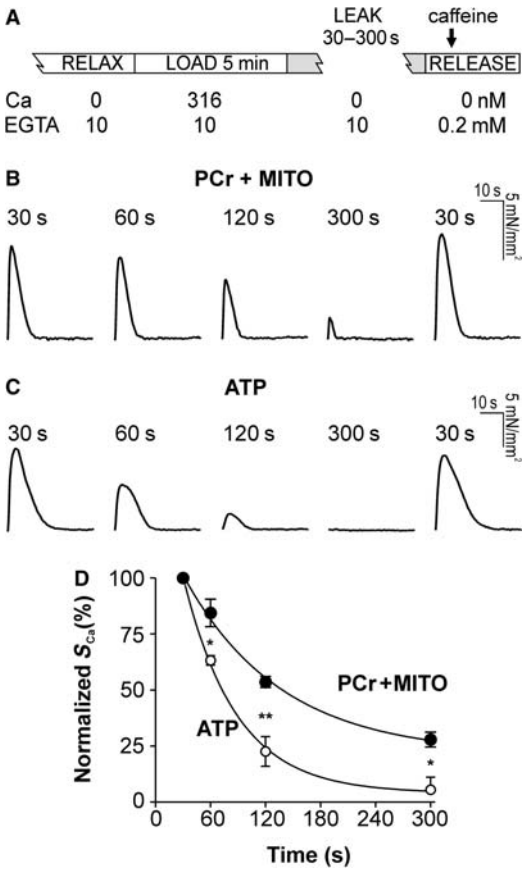

Figure 2 Evidence for energy-dependent SR calcium loss. (*A*) Experimental protocol (see text). (*B* and *C*) Force transients elicited by 5 mM caffeine after 5 min of SR Ca^{2+} loading under optimal energetic conditions followed by 30–300 s incubation in leak solution. Note that each cycle was preceded by standard SR Ca^{2+} loading under optimal energetic conditions. (B) Leak solution contained 3.16 mM MgATP + 12 mM PCr in the absence of mitochondrial inhibitor (PCr + MITO, optimal energetic conditions). (C) Leak solution contained 3.16 mM MgATP in the absence of PCr and in the presence of 2 mM NaN_3 to inhibit mitochondria (ATP only). (*D*) Calcium-time integrals (S_{Ca}) for caffeine-induced transients in the presence of PCr and working mitochondria (optimal energetic conditions, filled circles, $n = 4$) or ATP alone (open circles, $n = 3$). For each fibre, S_{Ca} values were normalized to S_{Ca} obtained after the shortest (30 s) leak (mean values were $2.34 \times 10^{-5} \pm 0.77 \times 10^{-5}$ and $2.36 \times 10^{-5} \pm 0.56 \times 10^{-5}$ mM s for PCr + MITO and ATP, respectively). Experimental points were fitted using a monoexponential decay function. $^{*}P < 0.05$, $^{**}P < 0.01$ between two conditions, *t*-test.

Figure 2C shows that when energy support is compromised, 5 min incubation in the absence of Ca^{2+} leads to an almost complete exhaustion of intra-SR releasable Ca^{2+}.

Figure 2D demonstrates Ca^{2+}-time integral values (S_{Ca}) for caffeine-induced transients normalized to maximal S_{Ca} values obtained at the shortest duration of leak (30 s) for the same fibre. It can be seen that in the absence of mitochondrial and CK support, SR loses Ca^{2+} about two-fold faster than under optimal energetic conditions.

Thus, altogether these data suggest that Ca^{2+} loss from the SR depends on energetic conditions.

3.2 Effects of SERCA and RyR inhibitors

There are two hypothetical pathways by which Ca^{2+} loss from the SR may occur—passive leak via the RyR and calcium pump-mediated backward flux. To determine the relative contribution of each one, we studied the effects of SERCA and RyR inhibitors on Ca^{2+} loss. As already

shown, the SR loses almost all of its Ca^{2+} after 5 min incubation in leak solution without CK and mitochondrial energetic support. However, when leak solution additionally contains 150 μM cyclopiazonic acid (CPA; a SERCA inhibitor), we found that the caffeine-induced force transient after 5 min incubation without Ca^{2+} is rather large (*Figure 3A*). We estimated the amount of released calcium after incubation in leak solution by normalizing the $[Ca^{2+}]$-time integral of the caffeine-induced transient to the maximal $[Ca^{2+}]$-time integral obtained after the shortest (30 s) incubation of the same fibre. *Figure 3C* shows that 5 min incubation without Ca^{2+} leads to a loss of ~90% of SR calcium, whereas after Ca^{2+} pump inhibition by CPA, the SR still retains ~50% of its initial calcium content. Very similar results (*Figure 3B* and *D*) were obtained when the Ca^{2+} pump was blocked with another potent inhibitor, 10 μM 2,5-di(tert-butyl)-1,4-benzohydroquinone (TBQ). It should be noted that both CPA and TBQ were not able to completely inhibit SERCA under our experimental conditions (see inserts in *Figure 3A* and *B*), which could explain their limited efficiency in inhibition of SR Ca^{2+} loss.

In the next series of experiments (*Figure 4*), we asked whether RyR inhibition was able to 'rescue' intra-SR calcium. After SR loading, the fibres were incubated in leak solution in the presence of 1 mM tetracaine, a blocker of the RyR. As for other series, this incubation was followed by a 1 min exposure to release solution in order to wash out the high EGTA concentration. This solution did not contain tetracaine, thus this compound was withdrawn before caffeine challenge. We found that tetracaine was not able to block Ca^{2+} leak such that there was insufficient intra-SR Ca^{2+} to produce a caffeine-induced force transient (*Figure 4A*). Averaged, normalized Ca^{2+}-time integral values for caffeine-induced transients (*Figure 4C*) show the inability of tetracaine to inhibit Ca^{2+} leak in the absence of extra-reticular calcium.

The effect of RyR inhibition on Ca^{2+} leak was tested in another series of experiments using ruthenium red (RR), which inhibits the RyR and mitochondrial calcium uniporter. In this series, caffeine-induced transients were recorded in the same fibres before and after 10 μM RR addition (*Figure 4B*). As can be seen, RR did not increase the size of the caffeine-induced force transient. Interestingly, the force transient elicited by caffeine shortly after Ca^{2+} loading (30 s in leak solution) was higher when loading occurred in the presence of RR. This suggests that Ca^{2+} loading in Ca^{2+}-containing medium is more efficient with RR, probably due to inhibition of calcium-induced calcium release. Averaged, normalized S_{Ca} values (*Figure 4D*) demonstrate that RR is not able to inhibit Ca^{2+} loss from the SR. Thus, in calcium-free medium, Ca^{2+} leak via RyR is not of primary importance.

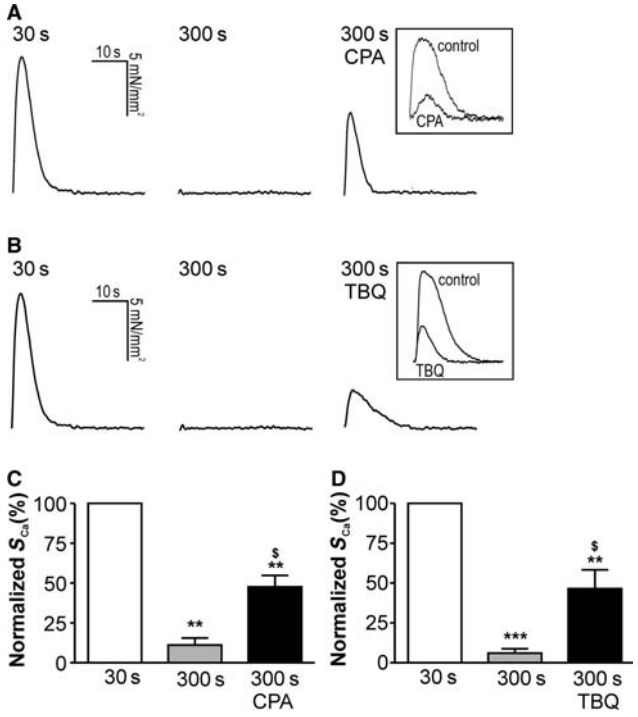

Figure 3 Inhibition of the Ca^{2+} pump decreases SR calcium loss. (*A* and *B*) Force transients elicited by 5 mM caffeine after different leak protocols. The same fibres were subjected to consecutive cycles of 5 min SR Ca^{2+} loading, each followed by incubation in leak solution for various durations—30, 300, or 300 s with or without 150 μM CPA (A) or 10 μM TBQ (B) in the presence of ATP only. Inserts depict force transients elicited by caffeine just after 5 min SR Ca^{2+} loading in the presence or absence CPA or TBQ. (*C* and *D*) Normalized S_{Ca} values of caffeine-induced transients for CPA (C) or TBQ (D). $^{**}P < 0.01$, $^{***}P < 0.001$ ($n = 3$ and $n = 4$ for CPA and TBQ experiments, respectively) vs. S_{Ca} obtained after 30 s incubation in leak solution (taken as 100%). $\$P < 0.05$ vs. normalized S_{Ca} in the absence of SERCA inhibitors.

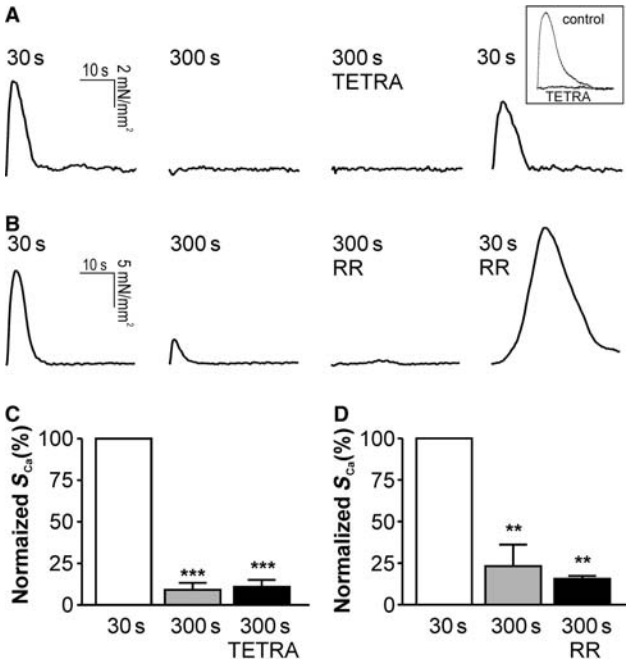

Figure 4 Ryanodine receptor inhibition does not influence SR calcium leak. (*A* and *B*) Force transients elicited by 5 mM caffeine after different leak protocols. The same fibres were subjected to cycles of 5 min SR Ca^{2+} loading, each followed by incubation in leak solution for various durations—30, 300, or 300 s with or without 1 mM tetracaine (A) or 10 μM RR (B) in the presence of ATP only. The last tension transient was obtained in the same fibres in the absence of tetracaine or RR to verify that no run-down of the fibres had occurred. Insert to panel (A) shows that 1 mM tetracaine added to release solution blocks Ca^{2+} release elicited by 2 mM caffeine (positive control). Note that RR was added after eliciting caffeine-induced transients in the absence of RR and then was continuously present in all solutions (*C* and *D*). Normalized S_{Ca} values of caffeine-induced transients for tetracaine (*C*) or RR (*D*). $^{**}P < 0.01$, $^{***}P < 0.001$ ($n = 3$ for each series) vs. S_{Ca} obtained after 30 s incubation in leak solution (taken as 100%).

3.3 Efficiency of different energy sources in inhibiting the backward SR Ca^{2+} leak

We compared the ability of five different energetic pathways to inhibit Ca^{2+} leak in Ca^{2+}-free medium. These pathways are (i) ATP only, (ii) glycolytic support, (iii) mitochondrial support, (iv) CK support, and (v) optimal energetic conditions (mitochondrial and CK support). Caffeine-induced $[Ca^{2+}]$-time integrals recorded after 5 min incubation in leak solution were normalized to corresponding values obtained in the same fibre under optimal energetic conditions (*Figure 5*). As already shown, the efficacy of cytosolic ATP in blocking Ca^{2+} leak was very low. Production of ATP by endogenous glycolytic enzymes bound to intracellular structures did not improve the ability of the SR to keep its Ca^{2+}. However, SR Ca^{2+} content was three- to four-fold higher in the presence of working mitochondria than the content when exogenous ATP was the only energy source. Interestingly, CK-mediated inhibition of Ca^{2+} loss was markedly more efficient than with ATP alone or glycolytic support, but was significantly less efficient than the effect of mitochondrial activity. Taken together, these data show that different energy pathways affecting the phosphorylation potential close to SERCA have very different capacities for inhibiting Ca^{2+} leak.

Qualitatively similar results were obtained in experiments on isolated cells where intra-reticular $[Ca^{2+}]$ was monitored using the fluorescent indicator Mag-fluo 4. *Figure 6* shows the rate of $[Ca^{2+}]$ decrease in the SR when the extra-reticular milieu does not contain calcium. We performed the same set of experiments in two different species of rodents and found comparable results. As for permeabilized fibres, under various energetic conditions the Ca^{2+} efflux velocity slows in the order ATP only > ATP + PCr > ATP + mitochondria > ATP + PCr + mitochondria, although the differences between CK and mitochondrial energetic supports did not reach statistical significance.

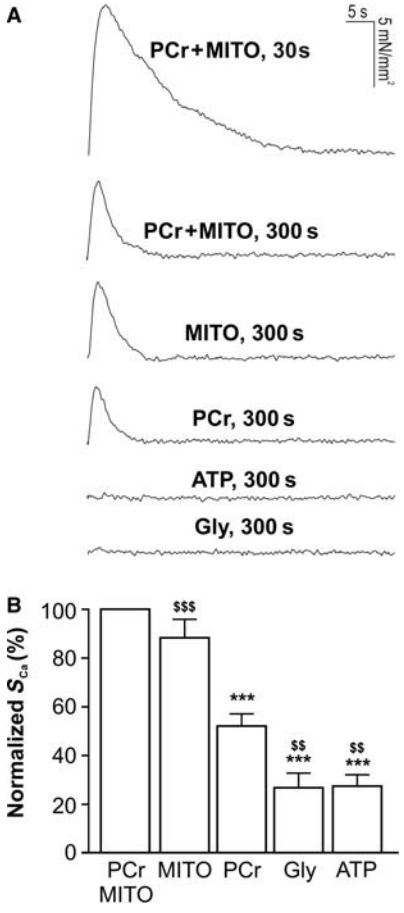

Figure 5 Different effects of various energetic conditions on SR calcium loss via the calcium pump. All solutions contained 3.16 mM MgATP. Energetic conditions were: 12 mM PCr and activated mitochondria (PCr + MITO; optimal conditions); 12 mM PCr and inhibited mitochondria (PCr); activated mitochondria (MITO); glycolytic intermediates and NAD (Gly); ATP alone (ATP). (*A*) Force transients elicited by 5 mM caffeine after 5 min of SR Ca^{2+} loading under optimal energetic conditions followed by 30 or 300 s incubation in leak solution under various energetic conditions. (*B*) Calculated $[Ca^{2+}]$-time integrals after 300 s incubation in leak solution under various energetic conditions normalized to corresponding values obtained under optimal energetic conditions (PCr + MITO). ***$P < 0.001$ ($n = 12$–23) vs. optimal energetic conditions taken as 100%. $$$P < 0.01$, $$$P < 0.001$ vs. PCr.

3.4 Calcium loss from the SR at physiological diastolic $[Ca^{2+}]$ is also energy-dependent

To determine whether Ca^{2+} loss is also energy-dependent at physiological $[Ca^{2+}]$, the SR was loaded for 5 min with Ca^{2+} and then fibres were incubated in leak solution having 100 nM Ca^{2+} for 5 min under either optimal or poor (ATP only) energetic conditions. To block RyR, the experiment was carried out in the presence of tetracaine. Relatively low $[Ca^{2+}]$-time integrals were obtained after incubation in the presence of ATP only (*Figure 7*), showing that compromised energy supply induces considerable Ca^{2+} loss from

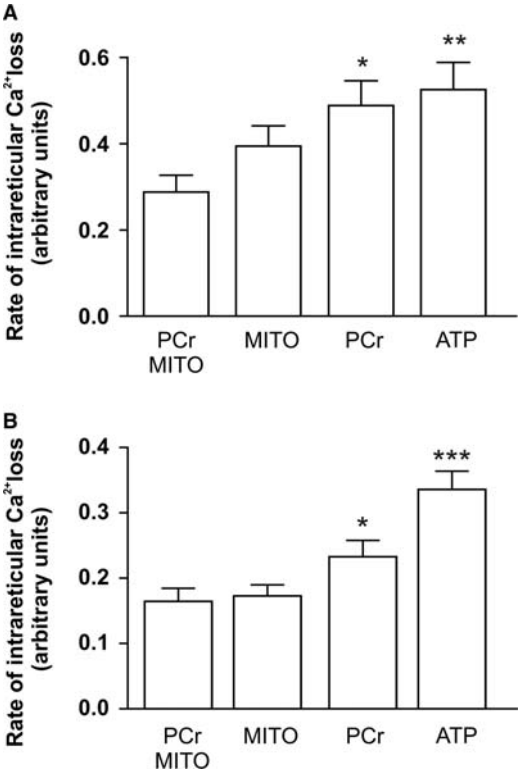

Figure 6 Rate of intra-reticular Ca^{2+} loss under different energetic conditions. Sarcoplasmic reticulum Ca^{2+} concentration was assessed by fluorescent probe as described in Methods section. Mag-fluo 4 loaded mouse (*A*) or rat (*B*) ventricular myocytes were incubated at 32 nM Ca^{2+} and then subjected to leak solution under various energetic conditions (abbreviations as for *Figure 5*). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ [($n = 10$–20 for (A) and 24–30 for (B)] vs. corresponding optimal conditions.

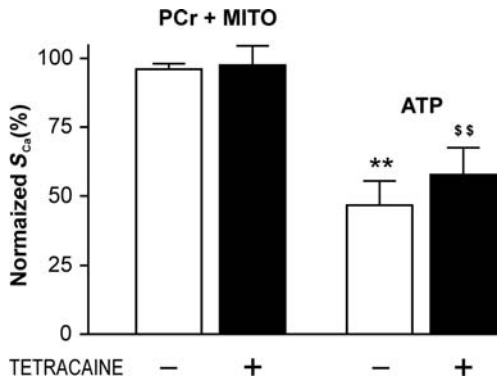

Figure 7 Loss of Ca^{2+} from the SR at diastolic $[Ca^{2+}]$ is energy-dependent. Calculated $[Ca^{2+}]$-time integrals of caffeine-elicited transients after 300 s incubation in leak solution at 100 nM Ca^{2+} under two different energetic conditions normalized to corresponding values obtained after 30 s incubation in leak solution under optimal energetic conditions. ** and $^{\$\$}P < 0.01$ ($n = 4$) vs. optimal energetic conditions in the absence and in the presence of tetracaine, respectively.

the SR even at physiological diastolic $[Ca^{2+}]$. Surprisingly, tetracaine removal during the leak period did not greatly augment Ca^{2+} loss, thus revealing the important role of backward Ca^{2+} flux in SR Ca^{2+} loss at low intracellular $[Ca^{2+}]$.

4. Discussion

In summary, the results presented in this study show that

(1) *in situ*, in the absence of extra-reticular Ca^{2+}, this ion rapidly leaks out of the SR compartment;
(2) this leak occurs mainly by backward flux through the SERCA;
(3) the leak strongly depends on cellular energy state;
(4) direct energy cross-talk between mitochondria and SERCA is more efficient at inhibiting the SR Ca^{2+} leak than local ADP rephosphorylation catalyzed by bound CK;
(5) the leak at physiological diastolic $[Ca^{2+}]$ is also energy-dependent.

Our results confirm that, at least under conditions of high intra/extra-reticular Ca^{2+} gradient, the SR *in situ* is rather 'leaky' such that intra-reticular Ca^{2+} levels start to decrease rapidly if cytosolic $[Ca^{2+}]$ drops. The decrease in SR Ca^{2+} content in Ca-free medium, visualized by fluorescent dye, is concomitant with a reduction in the amount of the Ca^{2+} releasable by the SR. The amount of this 'physiologically active' Ca^{2+}, which is able to activate myofibrils, was estimated from tension transients elicited by caffeine-induced Ca^{2+} release. In order to increase the precision of our analysis, we calculated $[Ca^{2+}]$ in the vicinity of myofibrils using an internal calibration for the Ca^{2+} released by the SR.[3] This is a valid approach because myofibrils are the physiological destination of Ca^{2+} released by the SR. The calibration was performed using the steady state Ca^{2+}/tension relationship for each fibre, from which the $[Ca^{2+}]$ seen by the myofibrils during the transient release was calculated.

An important question is what the main route of this Ca^{2+} leak is from the SR under conditions of high intra/extra-reticular Ca^{2+} gradient. In addressing this question, we inhibited SERCA during the leak period so as to decrease the reverse Ca^{2+} flux via this pump. SERCA inhibition caused a considerable increase in the amount of releasable SR Ca^{2+}. This increase could not result from unspecific effects of CPA or TBQ on ryanodine receptor because these inhibitors do not reduce RyR-mediated Ca^{2+} leak.[13] Thus, these data indicate that the SR Ca^{2+} pump is directly involved in Ca^{2+} leak under our experimental conditions.

In contrast to the effects of SERCA inhibition, RyR antagonists did not reduce SR Ca^{2+} loss. Neither tetracaine nor RR was able to increase the amount of releasable Ca^{2+} under conditions of high Ca^{2+} leak. Some studies[14,15] performed using intact cardiac cells showed that significant diastolic Ca^{2+} leak occurs via the RyR, especially in cardiac pathology.[16–18] However, several other studies support our findings. In voltage-clamped cardiac myocytes, for example, reverse flux through the SR Ca^{2+} pump was found to be the main pathway of diastolic Ca^{2+} flux from the SR.[19] Similarly, using isolated cardiac SR vesicles,[20] showed that at low external $[Ca^{2+}]$ (100 nM), passive leak via the RyR is extremely low relative to backward flux through the Ca^{2+} pump. The reverse mode of the Ca^{2+} pump was also found to be the main contributor to passive leak in SR vesicles isolated from skeletal muscle.[21]

The relevance of these data should of course be considered, given that under physiological conditions diastolic $[Ca^{2+}]$ is $\sim$100 nM rather than zero. Such a physiological concentration would decrease the intra/extra-reticular gradient and thereby inhibit Ca^{2+} loss. Indeed, at 100 nM Ca^{2+} and under optimal energetic conditions, SR Ca^{2+} content was almost the same after 30 or 300 s of leak period. However, in the presence of ATP only, backward Ca^{2+} flux significantly decreased the amount of releasable Ca^{2+}. A rough estimation shows that poor energetic conditions during 5 min incubation could be responsible for a loss of $\approx$50% of Ca^{2+} pumped for a period of the same duration. Interestingly, inhibition of potentially active RyR pathway by tetracaine did not significantly reduce Ca^{2+} loss. Thus, it can be concluded that energetic disturbances are able to activate backward Ca^{2+} flux in the living cell in diastole.

Although analysing the mechanism of energy-dependent Ca^{2+} loss, it is important to realize that the driving force for Ca^{2+} flux is determined not only by kinetic parameters of high energy phosphates but also by thermodynamic equilibrium between the substrate and the products of ATPase reaction. This reaction coupled to translocation of two Ca^{2+} for each ATP is fully reversible. The reversal condition depends on the relationship between the free energy of ATP hydrolysis and the energy required to transport Ca^{2+} against the concentration gradient. The SERCA pump will cycle in the forward direction when free energy of ATP hydrolysis (which depends on the ratio of MgATP to its hydrolysis products) is higher than energy of the Ca^{2+} concentration gradient.[22] When ADP and/or inorganic phosphate accumulate, free energy of ATP hydrolysis drops and the pump will stop or even cycle in the reverse direction. Therefore, elimination of the products of ATP hydrolysis in the vicinity of SERCA is a prerequisite for preventing the SR Ca^{2+} uptake inhibition (or preventing the SR Ca^{2+} loss coupled to ATP synthesis). Of course, specific assessment of such a reversibility of the Ca^{2+} pump needs more data concerning the actual magnitude of the phosphorylation potential near SERCA and the intra/extra-reticular Ca^{2+} gradient.

The most interesting result of the present work is the finding of differential sensitivity of backward Ca^{2+} flux to various energy sources *in situ*. This flux is markedly decreased in the presence of various functioning systems able to locally rephosphorylate ADP. Importantly, there are differences in the relative efficiency of these various systems. Our results show that the regeneration of ATP by endogenous glycolytic enzymes had a negligible effect on the reverse mode of the Ca^{2+} pump. This result was unexpected, because in the same model glycolytic support strongly increased Ca^{2+} loading.[11] It is possible, therefore, that glycolytic enzyme activation needs a certain Ca^{2+} concentration. Stimulation of the endogenous CK system caused considerable inhibition of Ca^{2+} loss; however, direct adenine nucleotide channelling mediated by cross-talk between mitochondria and SERCA was even more efficient in inhibiting the backward Ca^{2+} flux from the SR.

Interestingly, the efficacy of CK and mitochondria in supporting SR Ca^{2+} pumping is quite similar.[4] Higher efficacy of mitochondria in blocking the reverse mode of the SR Ca^{2+} pump found in the present work enables us to hypothesize that energetic regulation of the forward and backward flux is not identical. Of possible relevance to such a hypothesis is the activation of backward flux by inorganic phosphate.[23,24] In fact, local ATP regeneration by mitochondria is coupled to equimolar Pi consumption, such that phosphate produced locally by the SERCA is completely eliminated. In contrast, ATP regeneration by the CK system does not involve Pi consumption, so that high ATPase activity even in the presence of effective ATP regeneration at the expense of PCr could create an increased Pi concentration in the vicinity of the SR compartment. This, in turn, would favour the backward Ca^{2+} flux, which could explain the lower efficacy of the CK system in inhibiting Ca^{2+} loss from the SR.

Increased backward Ca^{2+} flux due to perturbations in the SERCA energy supply could contribute to decreased cardiac contractility in pathological conditions, especially heart failure. The failing heart is characterized by multiple alterations in energy metabolism (for review, see[9]). Furthermore, the ultrastructure of failing cardiac cells is profoundly disorganized (see[25,26] and references therein) such that structural relationships between cell compartments are altered. This should affect cross-talks between organelles, including those involved with energetics. Consistent with this, we have previously shown[12] that cell remodelling induced by the muscle LIM protein-null mutation caused a decrease in mitochondrial support for SR Ca^{2+} uptake, despite unchanged mitochondrial content and normal intrinsic mitochondrial function. Thus, direct energy channelling between mitochondria and SERCA, being the main energetic factor inhibiting the reverse mode of the SERCA, seems to be a mechanism which could be easily altered in heart failure due to cell remodelling.[27]

Altogether, our results have shown (*Figure 8*) that at low extra-reticular Ca^{2+}, backward SR flux strongly depends on the cell energy state and the main inhibitor of this Ca^{2+} loss is energy channelling between mitochondria and SERCA rather than CK-catalyzed energy support. This suggests that functional uncoupling between mitochondria and SERCA due to cell remodelling in cardiac pathologies could impair the SR function via increased SR Ca^{2+} loss.

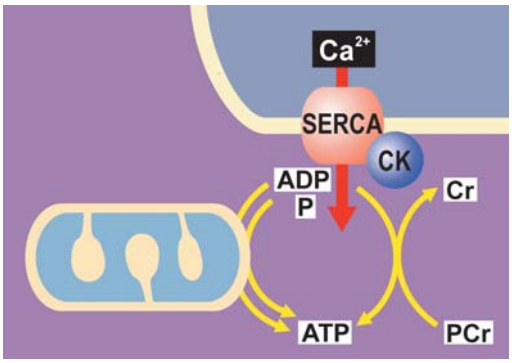

Figure 8 Energetic regulation of backward Ca^{2+} flux inhibition. Sarcoplasmic reticulum Ca^{2+} pump is able to mediate a significant backward flux. This flux strongly depends on local ATP/ADP ratio in the vicinity of SERCA and may be inhibited by two energetic pathways. The first one is mediated by CK bound to SR; the second one is mediated by direct adenine nucleotide channelling between SERCA and juxtaposed mitochondria. Mitochondria seem to be more efficient than CK in inhibiting the flux, probably due to their ability to decrease local inorganic phosphate concentration. Cardiac pathologies, which induce CK down-regulation and/or dissociation between mitochondria and SR due to cell remodelling, could favour SERCA-mediated Ca^{2+} backward flux and compromise contractility.

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