Biochimica et Biophysica Acta, 734 (1983) 191–200
Elsevier

BBA 71835

THE Ca^{2+} PERMEABILITY OF SARCOPLASMIC RETICULUM VESICLES

II. Ca^{2+} EFFLUX IN THE ENERGIZED STATE OF THE CALCIUM PUMP

ULRIK GERDES and JESPER V. MØLLER

Institute of Medical Biochemistry, Aarhus University, 8000 Aarhus C (Denmark)

(Received May 9th, 1983)

Key words: Ca^{2+} transport; Ca^{2+}-ATPase; Sarcoplasmic reticulum vesicle; (Rabbit skeletal muscle)

Ca^{2+} efflux from sarcoplasmic reticulum vesicles was studied by measurements of net Ca^{2+} uptake, $^{45}Ca^{2+}$ flux and hydrolysis of energy-rich phosphate. The maximal Ca^{2+} uptake capacity (150–200 nmol/mg protein at pH 6.7, 10 mM $MgCl_2$ and $\mu = 0.26$) was independent of the nature and concentration of the energy-donating substrate (ATP or carbamyl phosphate) and of temperature (15–35°C), suggesting coupling between influx and efflux of Ca^{2+}. In the presence of high concentrations of ATP, this efflux of Ca^{2+} was much higher than the passive Ca^{2+} permeation, measured after ATP or Ca^{2+} depletion of the reaction medium. Ca^{2+} efflux was imperceptible at vesicle filling levels below 35–40 nmol Ca^{2+}/mg protein, and uncorrelated to the inhibition of the Ca^{2+}-ATPase by high intravesicular Ca^{2+} concentrations. Analysis of the data indicated that Ca^{2+} efflux under our conditions probably is associated with one of the Ca^{2+}-ATPase partial reactions, occurring after dephosphorylation, rather than with a reversal of the Ca^{2+} translocation step in the phosphorylated state of the enzyme. Furthermore, passive Ca^{2+} permeation may be concurrently reduced during the enzymatically active state. It is proposed that both Ca^{2+} efflux and passive Ca^{2+} permeation (Ca^{2+} outflow) proceed via the same channels which are closed (occluded) during part of the Ca^{2+}-ATPase reaction cycle.

Introduction

Tight vesicles of membrane fragments of sarcoplasmic reticulum, isolated from skeletal muscle, are able to accumulate Ca^{2+} at the expense of the chemical energy contained in ATP or other energy-rich phosphate compounds. When ATP is added to a suspension of sarcoplasmic reticulum vesicles in a medium without Ca^{2+}-precipitating anions and in the presence of a surplus of Ca^{2+}, the vesicles accumulate Ca^{2+} to a certain maximal filling level, representing balance between the energy-consuming Ca^{2+} translocation (Ca^{2+} influx) and Ca^{2+} efflux. Interesting observations are that identical maximal Ca^{2+}-filling levels are obtained in the vesicle suspensions, despite a wide variation in the turnover rate of the ATPase [1–3]. This is not to be expected if Ca^{2+} accumulation is governed by a 'pump and leak' system, but instead suggests a tight coupling between Ca^{2+} influx and efflux at high intravesicular levels of Ca^{2+}. Recent studies have suggested that the efflux of Ca^{2+} represents reversal of the active transport step, but without resynthesis of ATP [4,5].

In the present paper Ca^{2+} efflux was studied during active Ca^{2+} influx by a combination of net Ca^{2+} uptake, $^{45}Ca^{2+}$ flux and ATPase activity measurements under conditions where reversal of Ca^{2+} transport, with resynthesis of ATP, is negligible. We find that Ca^{2+} efflux at a high turnover of the pump and at high Ca^{2+} load levels is much faster than can be accounted for by passive per-

meation. We obtain evidence that under our conditions Ca^{2+} efflux is associated with one of the latest steps in the enzymatic cycle, rather than with an exchange in the Ca^{2+}-transport step in the phosphorylated state. In addition, a detailed analysis suggests that ATPase activity-dependent efflux and passive permeation of Ca^{2+} are interdependent phenomena.

Materials and Methods

Preparation of sarcoplasmic reticulum vesicles and spectrophotometric measurements of net Ca^{2+} uptake and release were performed as described in the previous paper [6]. The buffer used in all experiments comprised 50 mM Tris-maleate/100 mM KCl/10 mM $MgCl_2$. In addition to a magnetic stirrer, improved mixing of the spectrophotometer cuvette contents was obtained by the use of a motor-driven plastic propeller situated in the upper part of the cuvette. Ca^{2+} uptake was initiated by addition of ATP from a microsyringe mounted in a spring-activated injection device. The time for complete mixing was 300 ms, when 10 μl reagent was shot into a cuvette containing 2500 μl reaction medium.

Ca^{2+}-influx measurements

Measurement of Ca^{2+} influx was made when maximal filling of the sarcoplasmic reticulum vesicles had been obtained with $^{40}Ca^{2+}$ in the presence of ATP or carbamyl phosphate in some experiments. A small volume of $^{45}CaCl_2$ (Amersham International: spec. act. 400–1600 Ci/mol) was added at the appropriate time. The spectrophotometer cuvette with its magnetic stirrer was used as mixing chamber and 250–500-μl samples were taken for Millipore filtration and analyzed as previously described [6]. Influx of Ca^{2+} was calculated either from the initial slope of $^{45}Ca^{2+}$ disappearance from the medium as a function of time, or by logarithmic transformation, assuming a two-compartment model in steady state [7]:

$$\ln\left(\frac{^{45}Ca_o}{^{45}Ca_{o,\infty}} - 1\right) = -V\left(\frac{1}{Ca_{i,\infty}} + \frac{1}{Ca_{0,\infty}}\right) + \ln\frac{Ca_{i,\infty}}{Ca_{o,\infty}} \qquad (1)$$

in which V is Ca^{2+} influx (nmol/ml), $^{45}Ca_o$ is the radioactive concentration remaining in the medium at time t after addition of $^{45}Ca^{2+}$ (cpm/ml) and $Ca_{i,\infty}$ and $Ca_{o,\infty}$ are the equilibrium concentrations of $^{40}Ca^{2+}$ inside and outside the vesicles (nmol/ml).

ATPase activity

ATPase activity was measured by following the liberation of inorganic phosphate, either chemically [6] or as $^{32}P_i$ from [γ-^{32}P]ATP (Amersham International). In the latter type of experiment, where a high time-resolution was aimed at, a manually operated mixing apparatus with two syringes and a motor-driven Teflon stirrer was used. The reaction was initiated by the addition of ATP and quenched by addition of 250 μl 36% (w/v) trichloroacetic acid, containing 0.5 mM inorganic phosphate as carrier, to 1 ml reaction medium. Remaining [^{32}P]ATP was partly removed from the mixture by addition of 1 ml 2% (w/v) acid-treated charcoal, followed by Millipore filtration [8]. Extraction of $^{32}P_i$ was done by an isobutanol-silicon tungstate procedure [9] and the radioactive content of the extract was counted by liquid scintillation.

Other methods and materials

Determination of the concentration of protein, ATP and of the total Ca^{2+} content in the sarcoplasmic reticulum vesicles (endogenous Ca^{2+}) was performed as described in the previous paper [6]. The Ca^{2+} ionophore X537A (Lasalocid®) was a gift from Hoffman La Roche (Hvidovre, Denmark) and was used from stock solutions (3–4 mM), prepared in dimethylsulfoxide.

Results

Dependence of Ca^{2+} accumulation on Ca^{2+}-ATPase activity and temperature

Fig. 1 shows the time-course of Ca^{2+} uptake by sarcoplasmic reticulum vesicles in the presence of different ATP concentrations. It is seen that, despite widely different initial Ca^{2+} uptake rates, the vesicles ultimately reach the same level of Ca^{2+} content. The constancy of the maximal Ca^{2+} load under the given conditions (pH 6.7, 10 mM $MgCl_2$ and μ-0.26) was demonstrated in different series of experiments, including changes in ATP concentration from 1 μM to 1 mM (not shown) and the use

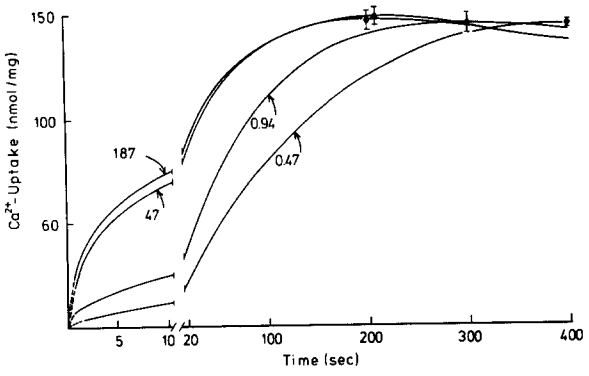


Fig. 1. Net Ca^{2+} uptake by sarcoplasmic reticulum vesicles in the the presence of different concentrations of ATP (figures on curves, μM). Curves are averages of 4–18 experiments and maximal Ca^{2+}-filling levels are indicated by symbol (◆)±S.D. Assay conditions: 20°C; 0.51 mg/ml protein; 0.2 mg/ml pyruvate kinase; 2 mM phospho*enol*pyruvate; ATP as indicated; 113 μM $CaCl_2$ added; 20 μM arsenazo III in all experiments.

of carbamyl phosphate as an energy-donor. In the latter case, the maximal Ca^{2+} load at 930 μM carbamyl phosphate was 160 ± 16 nmol/mg, as compared to 171 ± 17 nmol/mg with 36 μM ATP (a different preparation from that in Fig. 1 was used, which accounts for the somewhat higher accumulation capacity in these experiments). In comparison, the Ca^{2+} influx rate, as measured with $^{45}Ca^{2+}$ during maximal uptake, was 0.85 with carbamyl phosphate as energy-donor and 3.5 nmol/mg per s with ATP. Also, a variation in carbamyl phosphate concentration from 200 to 2000 μM left the maximal Ca^{2+}-filling level unchanged. The constancy of the maximal Ca^{2+} load suggests that Ca^{2+} efflux balancing Ca^{2+} influx is dependent on the activity of the ATPase, or, in other words, coupled to active, inward transport of Ca^{2+}.

Variation in temperature did not significantly change the maximal Ca^{2+} load either (15°C: 189 ± 12; 20°C: 211 ± 9; 25°C: 209 ± 4; 30°C: 199 ± 15; and 35°C: 201 ± 1 nmol/mg; 100 μM ATP and 1 mM phospho*enol*pyruvate: a preparation with an even higher Ca^{2+} accumulation was used). Under the same conditions, the Ca^{2+}-ATPase activity during maximal accumulation increased more than 10-fold from 0.7 nmol/mg per s at 15°C to 9.7 nmol/mg per s at 30°C. As the coupling ratio between Ca^{2+} translocation and Ca^{2+}-ATPase activity is considered to be independent of the temperature over this range [10,11], it follows that the temperature coefficient of Ca^{2+} efflux is identical to that of ATP-supported Ca^{2+} influx, in agreement with a coupling of the two processes.

The effect of other environmental parameters on Ca^{2+}-accumulation was not studied in any systematic manner. However, we found that a decrease of pH to 6.1 resulted in unchanged maximal Ca^{2+}-filling but decreased Ca^{2+}-ATPase activity and initial Ca^{2+}-uptake rate. Conversely, the maximal Ca^{2+}-filling level decreased to around 80 nmol/mg when pH was elevated to 7.5 and in this case the Ca^{2+}-ATPase activity and the initial Ca^{2+}-uptake rate was increased.

Ca^{2+} efflux during maximal Ca^{2+} accumulation

The disappearance of $^{45}Ca^{2+}$ from the medium, when the isotope is added to the vesicular suspension at the point of maximal uptake (see points in Fig. 1), is shown in Fig. 2A. At the two higher ATP concentrations, $^{45}Ca^{2+}$ partitions rapidly between the medium and vesicles in the same proportion as $^{40}Ca^{2+}$. This ideal behaviour is not fully followed at the two lower ATP concentrations, presumably because the time required for equilibration is too long to maintain intravesicular Ca^{2+} in a steady state (notice the presence of the 'overshoot' phenomenon, which presumably indicates time-dependent changes in the Ca^{2+} permeability of the vesicles [6]). Accordingly, only the data at the two higher ATP concentrations were subjected to compartmental analysis (Materials and Methods), while Ca^{2+} influx was estimated from initial rates in the two other experiments.

The results obtained are compiled in Table I, together with determinations of both steady-state Ca^{2+}-ATPase activity and initial Ca^{2+}-uptake rate. It is seen that Ca^{2+} influx during steady state is lower than the initial Ca^{2+} uptake rate, especially at the higher ATP concentrations. This decrease is attributable to the inhibition of Ca^{2+}-ATPase activity by a high intravesicular concentration of Ca^{2+} [1–3,12]. The apparent coupling ratio in steady state, calculated as the ratio between Ca^{2+} influx and ATPase activity, varies from 1.08 to 1.58 without any systematic variation on the ATP concentration. These values are lower than the generally accepted coupling ratio of 2 for the

ATPase [2,13–16] (but see Ref. 17). It is also lower than the coupling ratio that we have measured during the initial part of the reaction (see Fig. 3) or during reversal of the Ca^{2+} pump [6]. According to the data of Fig. 3, the coupling ratio is 2 up to an intravesicular Ca^{2+} level of 30–35 nmol/mg protein. At higher intravesicular levels there is a relative decrease in net Ca^{2+} uptake, presumably heralding the onset of the Ca^{2+}-efflux phenomenon.

In the experiments of Fig. 2, unidirectional fluxes are measured under conditions approaching steady state, and therefore the coupling ratio might have been expected to be the same as measured in the initial uptake period in Fig. 3. However, as noted by Hasselbach [14], the presence of unsealed vesicles or non-vesicular membrane fragments may be the main cause of apparently low coupling ratios at high Ca^{2+} loads. In contrast to the sealed vesicles, this fraction of the ATPase population is not subject to inhibition by a high intravesicular Ca^{2+} concentation. Even if the 'leaky' vesicles constitute a very small fraction of a preparation, it may contribute considerably to the measured Ca^{2+}-ATPase activity during a maximal Ca^{2+} load and thereby cause an apparent lowering of the measured coupling ratio. For example: 17–34% of the Ca^{2+}-ATPase activity under these conditions will be due to leaky vesicles in a preparation containing 2–5% of these, if the Ca^{2+}-ATPase activity of closed vesicles is 10% of that of the leaky vesicles.

Ca^{2+} efflux at submaximal Ca^{2+} accumulation

At any given time during the approach of Ca^{2+} accumulation to a maximal load level, efflux of

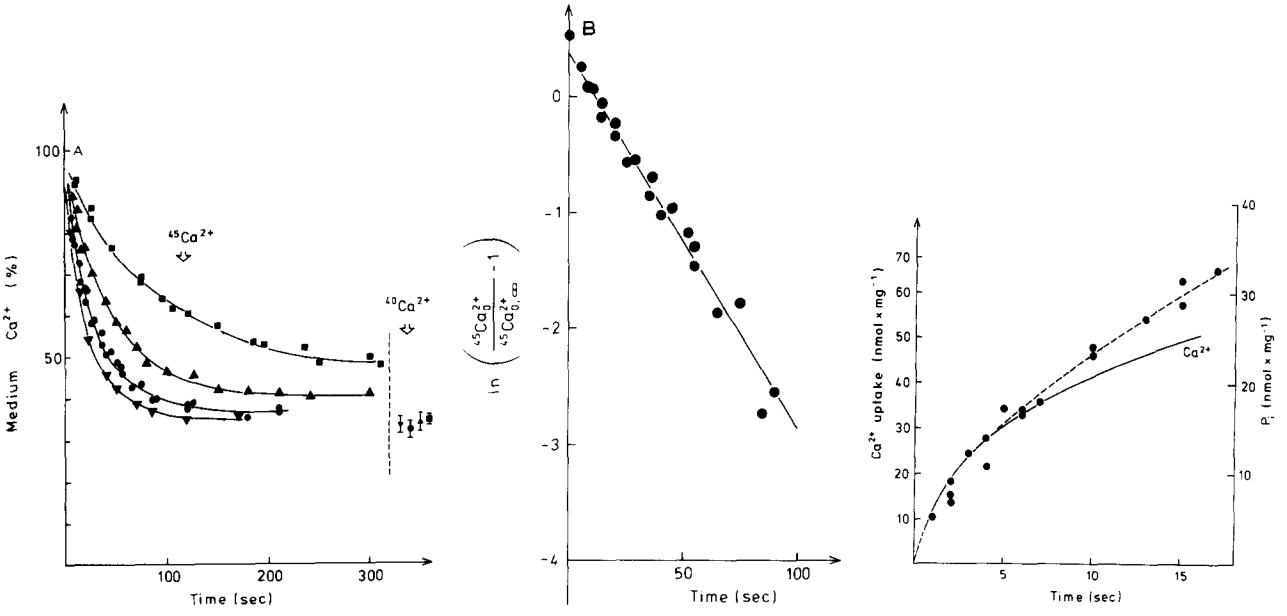


Fig. 2. A. Disappearance of $^{45}Ca^{2+}$ from the medium at different ATP concentrations: 187 μM (▼), 47 μM (●), 0.94 μM (▲) or 0.47 μM (■) ATP. The radioactive isotope was added at a time corresponding to maximal filling of the vesicles with $^{40}Ca^{2+}$ (cf. Fig. 1). This corresponds to a distribution of $^{40}Ca^{2+}$ with 33–35% remaining in the medium (for all ATP concentrations). Assay conditions as in Fig. 1. Part B demonstrates how closely experimental data actually fit the graphical analysis based on a simple two-compartment model (see Methods, data from experiments with 47 μM ATP only).

Fig. 3. Initial net Ca^{2+} uptake (unbroken curve, left-hand ordinate) and inorganic phosphate liberation (broken curve with symbols, right-hand ordinate) in sarcoplasmic reticulum vesicles. P_i liberation was measured using [γ-^{32}P]ATP as described in Materials and Methods. Correction was made for P_i liberation in the presence of 10 mM EGTA (basal-ATPase). The steady-state Ca^{2+}-ATPase activity of the preparation was 0.79 nmol/mg per s. Assay conditions: 15°C; 0.44 mg/ml protein; 575 μM ATP (no regenerating system); 100 μM $CaCl_2$ added; 17 μM arsenazo III in both types of experiment. Note that ADP accumulation, due to the absence of an ATP-regenerating system, cannot be ascribed any significance as only about 3.5% of the ATP is hydrolyzed during the experimental observation period (total ATPase activity).

Ca^{2+} (dCa_e/dt) equals the difference between active Ca^{2+} influx (dCa_i/dt) and net Ca^{2+} uptake (dCa_u/dt) by the vesicles, i.e.

$$\frac{dCa_e}{dt} = \frac{dCa_i}{dt} - \frac{dCa_u}{dt} = \frac{dP_i}{dt}CR - \frac{dCa_u}{dt} \quad (2)$$

where dP_i/dt is the rate of Ca^{2+} dependent ATP hydrolysis and CR is the apparent coupling ratio between Ca^{2+} influx and ATP hydrolysis. The results of calculations based on the data of Fig. 1 and steady-state ATPase activity (Table I) are shown in Fig. 4. It is seen that Ca^{2+} efflux rises steeply at a level of 80 nmol Ca^{2+}/mg. At the lowest ATP concentration (0.47 μM) Ca^{2+} efflux is barely distinguishable from Ca^{2+} outflow obtained by depleting the ATPase of medium substrate. An increase in ATP concentration increases Ca^{2+} efflux from this basal level at all intravesicular Ca^{2+} loads, resulting in highly cooperative efflux curves.

The shaded curve in Fig. 4 depicts the inhibition of Ca^{2+}-ATPase activity. The construction of this curve is based on several measurements of rates of ATP hydrolysis and Ca^{2+} uptake during the initial reaction periods (see legend to Fig. 4). The figure shows that activity is reduced to a minimal level at 80 nmol Ca^{2+}/mg protein. There is thus no correlation between inhibition of Ca^{2+}-ATPase activity and activation of Ca^{2+} efflux by intravesicular Ca^{2+}. In this connection it is appropriate to point out that calculations of Ca^{2+} efflux by Eqn. 2 assume the same Ca^{2+}-ATPase activity as in the steady state (otherwise the apparent coupling ratio, taken from Table I, would be changed). For this reason, the course of Ca^{2+} efflux below 80 nmol/mg has been indicated by broken lines. It seems certain that Ca^{2+} efflux becomes negligible at low Ca^{2+} loads. This conclusion follows from the results of Fig. 3 which showed that up to a Ca^{2+} content of approx. 35 nmol/mg there was no evidence for Ca^{2+} exchange, as might perhaps have been expected at the high Mg^{2+} concentration (10 mM) on the basis of Ca^{2+} exchange results published by Takakuwa and Kanazawa [18].

Inhibition of Ca^{2+}-ATPase activity by accumulated Ca^{2+}

Independent verification of the inhibitory effect

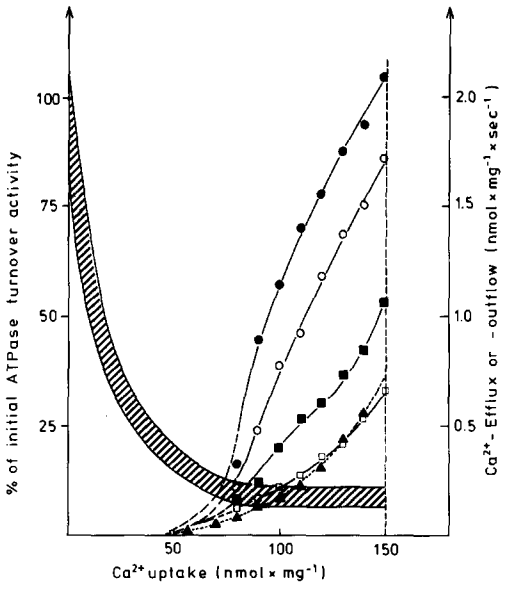


Fig. 4. Ca^{2+} efflux, Ca^{2+} outflow and Ca^{2+}-ATPase inhibition as a function of the vesicular Ca^{2+} content. The Ca^{2+} efflux at different ATP concentrations is calculated from net Ca^{2+} uptake curves and the data of Table I as described in the text (●, 187 μM ATP; ○, 47 μM ATP; ■, 0.94 μM ATP; □, 0.47 μM ATP). The Ca^{2+} outflow experiments (▲------▲) were performed under conditions identical to those of Fig. 1, except that the phospho*enol*pyruvate concentration was lower (225 μM), so that spontaneous Ca^{2+} outflow occurred around 300 s after ATP addition (four experiments). Alternatively, and giving an identical outflow curve [6], EGTA was added to a final concentration of 10 mM at 300 s after ATP (2 mM phospho*enol* pyruvate; three experiments). The quantitative analysis of the inhibitory effect of intravesicular Ca^{2+} on the ATPase turnover activity (hatched curve, left-hand ordinate) is based on several different experimental results. The initial part of the curve was obtained both from fast spectrophotometric measurements of net Ca^{2+} uptake (below 35–40 nmol/mg) and of Ca^{2+}-ATPase activity (up to a Ca^{2+} content of 60 nmol/mg, see Fig. 3). Above 80 nmol Ca^{2+}/mg protein, Ca^{2+}-ATPase activity is assumed to be constant as demonstrated by Fig. 5, and activities in this region are obtained from the data of Table I (47 and 187 μM ATP). As an interesting point, it may be noted that we find it possible to assemble the hatched curve from experimental results obtained under different conditions, i.e., temperature and ATP concentrations in the range 47–575 μM.

of intravesicular Ca^{2+} on Ca^{2+}-ATPase activity was gained from experiments with Ca^{2+} ionophore. By incorporation of varying amounts of X537A in the sarcoplasmic reticulum membrane, a leak of Ca^{2+} could be induced, causing a graded decrease in the steady-state Ca^{2+} content, accord-

TABLE I

EFFECT OF VARIATION IN ATP CONCENTRATION ON Ca^{2+}-EXCHANGE AND Ca^{2+}-ATPase ACTIVITY IN MAXIMALLY Ca^{2+}-FILLED VESICLES AS WELL AS ON PARAMETERS OF NET Ca^{2+} UPTAKE

The Ca^{2+} exchange (= Ca^{2+} influx) was calculated from the data of Fig. 2A as described in Materials and Methods. The Ca^{2+}-ATPase activity is total ATPase activity measured minus Ca^{2+}-independent ('basal') ATPase activity, measured in the presence of 10 mM EGTA. The parameters of net Ca^{2+} uptake were read from spectrophotometric records, and the initial Ca^{2+}-uptake rate was estimated within the first second of the reaction. The figures are averages ± S.D. (number of experiments in parentheses). In the case of exchange measurements, only one calculation was performed for each ATP concentration, experimental data being compiled from two or three experiments, however. Assay conditions as in Fig. 1.

[ATP] (μM)	*a* $^{45}Ca^{2+}$ influx (nmol/mg per s)	*b* Ca^{2+} – ATPase activity (nmol/mg per s)	Ratio *a*/*b*	Maximal Ca^{2+}-filling level (nmol/mg)	Half-time for max. Ca^{2+} filling (s)	Initial net Ca^{2+} uptake rate (nmol/mg per s)
0.47	0.66	0.49 ± 0.03 (3)	1.35	143.9 ± 1.9 (4)	105	1.0 ± 0.11 (7)
0.94	1.06	0.67 ± 0.11 (4)	1.58	145.0 ± 4.6 (10)	49	1.4 ± 0.12 (6)
47	1.72	1.26 ± 0.16 (6)	1.37	148.4 ± 4.3 (18)	13.0	24.2 ± 1.2 (11)
187	2.09	1.93 ± 0.09 (4)	1.08	146.3 ± 4.3 (4)	9.5	28.4 ± 0.0 (3)

ing to the concentration of the ionophore. In this way, the effect of Ca^{2+} load on ATPase activity in the steady state could be estimated under otherwise identical conditions. Fig. 5 shows that the Ca^{2+} content of the vesicles can be reduced to about 80 nmol/mg without any increase in the Ca^{2+}-ATPase activity. This is in perfect agreement with the notion that maximal inhibition is established at this level of Ca^{2+} load. Furthermore, the observed increase in activity that follows a decrease of Ca^{2+} uptake below this level also obeys the relationship depicted by the hatched curve in Fig. 4.

It is to be noted that the Ca^{2+} content of the vesicles indicated in Fig. 5 (as in all other figures) represents uptake from the medium with no correction for endogenous Ca^{2+} (approx. 25 nmol/mg [6]). Also, the 100% turnover activity in Fig. 4 is that exhibited by the enzyme in the presence of endogenous Ca^{2+}, i.e., in the initial 0.5 s or so of the reaction. However, we have experimental results indicating that even endogenous Ca^{2+} inhibits the transport enzyme appreciably. During preincubation of the vesicles with ionophore (8–14 μM ionophore X537A), we recorded spectrophotometrically a loss of 50–75% of the endogenous Ca^{2+} associated with the vesicles in a suspension. The initial net Ca^{2+} uptake rate was found to be increased by a factor 1.3–1.4 after this treatment with ionophore.

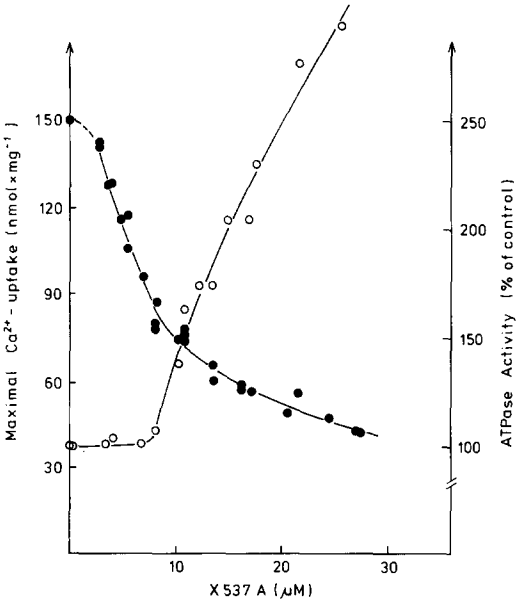


Fig. 5. Effect of the Ca^{2+} ionophore X537A (Lasalocid) on the maximal Ca^{2+}-filling level (●; left-hand ordinate) and the steady-state Ca^{2+}-ATPase activity (○; right-hand ordinate; percent of control without ionophore) of sarcoplasmic reticulum vesicles. Assay conditions: 20 or 25°C; 0.45 mg/ml protein (two different preparations): 110 μM $CaCl_2$ added; 550 μM ATP; 17 μM arsenazo III. Vesicles were preincubated 5 min with ionophore in the reaction medium before Ca^{2+} uptake was initiated by ATP addition.

Discussion

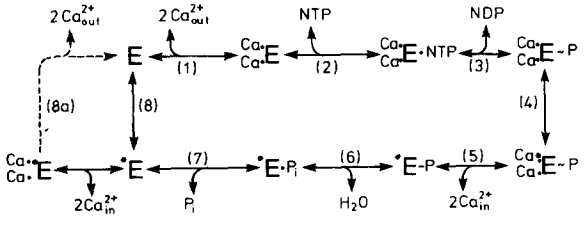
Coupling between Ca^{2+} influx and Ca^{2+} efflux

The present data show that under conditions of a high Ca^{2+} load and ATPase activity, Ca^{2+} leaves sarcoplasmic reticulum vesicles by a process which is much faster than the Ca^{2+} outflow that is observed after stopping Ca^{2+} pumping activity [6]. In contrast to Ca^{2+} outflow, which represents the passive permeability of the vesicles, Ca^{2+} efflux under these conditions presumably is a carrier-mediated process. This can be inferred from the dependence of efflux on the turnover of the enzyme. At the same level of maximal, intravesicular Ca^{2+} load, efflux closely matches influx of Ca^{2+} despite wide variations in ATPase activity, induced by changes in ATP concentration and temperature. Thus Ca^{2+} efflux appears to be closely coupled to ATP-consuming Ca^{2+} influx. This proposal was originally presented by Weber et al. [1], and it has subsequently been supported by various experimental observations [3,5,18–23]. In the further discussion of this phenomenon, it is important to note that Ca^{2+} efflux under our experimental conditions cannot be attributed to reversal of the Ca^{2+} inward translocation process (with ATP synthesis, step 4 of Scheme I, which presents a slightly modified version of schemes for the Ca^{2+}-ATPase reaction sequence previously considered [11,16,24]). This can be inferred from the following facts. (i) The Ca^{2+} concentation in the medium was relatively high (30–100 μM), a circumstance which strongly inhibits reversal, as evidenced by no detectable NTP $\rightleftharpoons$ P_i exchange [25,26]. (ii) ADP was virtually absent due to continuous ATP regeneration. (iii) Ca^{2+} exchange was observed also with carbamyl phosphate as an energy-donor, in which case reversal does not occur.

Feher and Briggs [5], on the basis of data similar to ours, suggest that Ca^{2+} exchange takes place by a dynamic reversal of step 4, not involving resynthesis of ATP. A similar view has been advanced by Takakuwa and Kanazawa [18] in studies on Ca^{2+} exchange at low temperature and in the simultaneous presence of ATP and ADP. However, our data on the low $^{45}Ca^{2+}$-influx/ATPase ratio (Table I) preclude extensive exchange of Ca^{2+} in step 4. It is conceivable that shuttling of Ca^{2+} in step 4 can take place, but if so this process must occur without exchange of the bound $^{45}Ca^{2+}$ with unlabelled Ca^{2+} within the aqueous vesicle compartment. Otherwise we would have observed an increase in the $^{45}Ca^{2+}$/ATP consumption (apparent coupling) ratio, whereas the values obtained under conditions of a high intravesicular Ca^{2+} load actually were low (Table I).

An additional argument against locating Ca^{2+} exchange at step 4 is the lack of correlation between inhibition of the enzyme by intravesicular Ca^{2+} and activation of Ca^{2+} efflux. The inhibitory effect of high intravesicular Ca^{2+} has been attributed to saturation of the Ca^{2+} transport sites in their low-affinity state [11,27,28] and an associated inhibition of step 4 [29] and/or inhibition of step 5 in the reaction sequence [15,30,31]. But in our experiments, significant activation of Ca^{2+} efflux required calcium loads which exceeded the level required to obtain maximal inhibition of enzyme turnover by Ca^{2+} (Fig. 4).

It therefore seems more attractive to assume that Ca^{2+} efflux is associated with the return of the Ca^{2+} translocation sites to their exterior position, after dephosphorylation of the enzyme (shown as step 8a in Scheme I). Certain aspects of this proposal call for comments. It has been suggested that the relatively low rate at which Ca^{2+} binds to sarcoplasmic reticulum, after preincubation with EGTA-containing media [11,32], represents reaction 8 in Scheme I [11,33]. This would mean that in the presence of a high intravesicular Ca^{2+} concentration alone, step 8a would be expected to occur. However, in the preceding paper we obtained no evidence for a carrier-mediated outflow of Ca^{2+} after addition of EGTA [6]. This could mean that efflux of Ca^{2+} instead is associated with reactions 6 or 7 in Scheme I or that the *E conformation formed after phosphorylation with ATP has somewhat different properties than the conformation formed by gradient-independent phosphorylation with P_i. In agreement with the latter possibility, fluorescence studies suggest that the conversion of E to *E by addition of EGTA is incomplete [34–36]. In particular, Guillain et al. [35,36] have obtained evidence that in the presence of a high Mg^{2+} concentration and pH 7, conditions similar to those used in the present study, one of the Ca^{2+}-binding sites is probably occupied by Mg^{2+} with an outward-facing site.



Scheme I. Partial reactions of Ca^{2+}-ATPase; slightly modified from De Meis [11]. For further explanation, see text.

Previous studies indicate that Ca^{2+} may exchange with intravesicular H^+ [37–41] and K^+ [42–47]. It has been suggested that Ca^{2+} influx occurs by a nonelectrogenic process, i.e., an ATPase mediated counter-movement of H^+ or K^+ [44,48]. If this is the case, efflux of Ca^{2+} mediated by the active pump will occur by competition with K^+ and/or H^+. This could account for the absence of Ca^{2+}-efflux at low Ca^{2+}-filling levels (35–40 nmol/mg protein) of the sarcoplasmic reticulum vesicles, where extrusion of monovalent ions may predominate.

Relation between Ca^{2+} efflux and Ca^{2+} outflow

The data presented here are important in relation to the Ca^{2+} outflow process occurring after cessation of active Ca^{2+} transport, as described in the preceding paper [6]. A stoichiometric relation between Ca^{2+} influx and efflux will only result in a fixed, limiting value for maximal Ca^{2+} uptake, if passive permeation is negligible. However, as shown by Fig. 4, Ca^{2+} outflow is not under all conditions negligible compared to ATP-dependent efflux. This is particularly noticeable at the lowest ATP concentration, where Ca^{2+} efflux is barely

distuinguishable from Ca^{2+} outflow, despite that the ATPase activity is 25–73% of that measured at the higher ATP concentrations (cf. Table I). Thus, Ca^{2+} transport dependent efflux and passive permeation of Ca^{2+} seem to be interdependent phenomena, passive Ca^{2+} permeation being reduced when the transport enzyme is active. A simple explanation for this behaviour is the existence of a common membrane channel, responsible for both Ca^{2+} translocation correlated to enzyme turnover and Ca^{2+} outflow when the enzyme is inactive. There is evidence that Ca^{2+}, at least transiently, is in an occluded state during active transport [33,49,50]. This could mean that the channel is occupied (occluded) under these conditions, and so would result in concomitant blockage of passive Ca^{2+} permeation.

Physiological implications

In the present and companion paper [6] we have dealt with Ca^{2+} efflux (activated by ATP), Ca^{2+} outflow (in the non-energized state of the Ca^{2+} pump), and briefly with time-dependent release of a minor amount of accumulated Ca^{2+} in vesicles ('overshoot'). As noted by Endo [51], sarcoplasmic reticulum is probably only loaded to one-fourth or one-third of its maximum capacity in vivo. A recent electron-probe study has shown that the Ca^{2+} content of the longitudinal sarcoplasmic reticulum in muscle cells is lower than that of the terminal cisternae [52]. The use in the present experiments of a preparation which is derived primarily from the longitudinal sarcoplasmic reticulum may thus indicate that the physiological Ca^{2+} loading level is even lower than proposed by Endo [51], i.e., lower than 40 nmol/mg protein. Under our conditions no Ca^{2+} efflux was detectable at a Ca^{2+} filling level below 35–40 nmol/mg, the Ca^{2+} outflow was very low [6], and 'overshoot' was not demonstrable. Therefore, it is improbable that any of these phenomena is of significance for Ca^{2+} release in vivo. In agreement with this conclusion, theoretical calculations suggest that Ca^{2+} accumulation by muscle cells in the resting state is close to thermodynamic equilibrium [14,53]. Hence, it appears likely that under these conditions, reversal of the Ca^{2+} transport process (with resynthesis of ATP) plays an important role for the balance between Ca^{2+} influx and efflux. In combination with the low ATPase activity, resulting from the low concentration of myoplasmic Ca^{2+}, this would effectively prevent futile, energy-requiring cycling of Ca^{2+} across the sarcoplasmic reticulum membrane during the resting state of the muscle cell.

References

1 Weber, A., Herz, R. and Reiss, I. (1966) Biochem. Z. 345, 329–369
2 Weber, A. (1966) in Current Topics in Bioenergetics (Sanadi, D.R., ed.), Vol. 1, p. 203, Academic Press, New York
3 Waas, W. and Hasselbach, W. (1981) Eur. J. Biochem. 116, 601–608
4 Feher, J.J. and Briggs, F.N. (1982) J. Biol. Chem. 257, 10191–10199
5 Feher, J.J. and Briggs, F.N. (1983) Biochim. Biophys. Acta 727, 389–402
6 Gerdes, U., Nakhla, A.M. and Møller, J.V. (1983) Biochim. Biophys. Acta 734, 180–190
7 Francis, G.E., Mulligan, W. and Wormall, H. (1959) Isotopic Tracers, 2nd Edn. University of London, The Athlone Press
8 Verjovski-Almeida, S., Kurzmack, M. and Inesi, G. (1978) Biochemistry 17, 5006–5013
9 Lindberg, O. and Ernster, L. (1956) in Methods of Biochemical Analysis (Glick, D., ed.), Voll. 3, pp. 1–22, Wiley Interscience New York
10 Vale, M.G.P. and Carvalho, A.P. (1980) Biochem. J. 186, 461–467
11 De Meis, L. (1981) in Transport in the Life Sciences, Vol. 2 (Bittar, E.E., ed.), J. Wiley & Sons, New York
12 Makinose, M. and Hasselbach, W. (1965) Biochem. Z. 343, 360–382
13 Hasselbach, W. (1964) Prog. Biophys. Mol. Biol. 14, 167–222
14 Hasselbach, W. (1981) in Membrane Transport (Bonting, S.L. and De Pont, J.J.H.H.M., eds.), Ch. 7, pp. 183–208, Elsevier/North-Holland Biomedical Press, Amsterdam
15 Tada, M., Yamamoto, T. and Tonomura, Y. (1978) Physiol. Rev. 58 (1), 2–66
16 De Meis, L. and Vianna, A.L. (1979) Annu. Rev. Biochem. 48, 275–292
17 Rossi, B., De Assis Leone, F., Gache, C. and Lazdunski, M. (1979) J. Biol. Chem. 254 (7), 2302–2307
18 Takakuwa, Y. and Kanazawa, T. (1982) J. Biol. Chem. 257, 10770–10775
19 Balzer, H., Makinose, M. and Hasselbach, W. (1968) Naunyn-Schmiedebergs Arch. Pharmak, U. Exp. Path. 260, 444–455
20 Katz, A.M., Dunnet, J., Repke, D.I. and Hasselbach, W. (1976) FEBS Lett. 67 (2), 207–208
21 Katz, A.M., Repke, D.I., Dunnet, J. and Hasselbach, W. (1977) J. Biol. Chem. 252 (6), 1950–1956
22 Katz, A.M., Repke, D.I., Fudyma, G. and Shigekawa, M. (1977) J. Biol. Chem. 252 (12) 4210–4214
23 Kirchberger, M.A. and Wong, D. (1978) J. Biol. Chem. 253, 6941–6945

24 Carvalho, M.G.C., De Souza, D.G. and De Meis, L. (1976) J. Biol. Chem. 251, 3629–3636
25 Makinose, M. (1973) FEBS Lett. 37, 140–143
26 De Meis, L. and Carvalho, M.G.C. (1974) Biochemistry 13, 5032–5037
27 Ikemoto, N. (1975) J. Biol. Chem. 250 (18), 7219–7224
28 Ikemoto, N. (1976) J. Biol. Chem. 251 (22), 7275–7277
29 Shigekawa, M. and Dougherty, J.P. (1978) J. Biol. Chem. 253, 1458–1464
30 Yamamoto, T., Takisawa, H. and Tonomura, Y. (1979) Curr. Top. Bioenerg. 9, 179–236
31 Yamada, S. and Tonomura, Y. (1972) J. Biochem. (Tokyo) 72, 417–425
32 Rauch, G., Chak, D. and Hasselbach, W. (1978) FEBS Lett. 93, 65–68
33 Dupont, Y. (1978) Biochem. Biophys. Res. Commun. 82, 893–900
34 Pick, U. and Karlish, S.J.D. (1982) J. Biol. Chem. 257, 6120–6126
35 Guillain, F., Gingold, M.P. and Champeil, P. (1982) J. Biol. Chem. 257, 7366–7371
36 Champeil, P., Gingold, M.P., Guillain, F. and Inesi, G. (1983) J. Biol. Chem. 258, 4453–4458
37 Madeira, V.M.C. (1980) Arch. Biochem. Biophys. 200 (2), 319–325
38 Meissner, G. and Young, R.C. (1980) J. Biol. Chem. 255, 6814–6819
39 Chiesi, M. and Inesi, G. (1980) Biochemistry 19, 2912–2918
40 Ueno, T. and Sekine, T. (1981) J. Biochem. (Tokyo) 89, 1239–1246
41 Ueno, T. and Sekine, T. (1981) J. Biochem. (Tokyo) 89, 1247–1252
42 Chiu, V.C.K. and Haynes, D.H. (1980) J. Membrane Biol. 56, 203–218
43 Chiu, V.C.K. and Haynes, D.H. (1980) J. Membrane Biol. 56, 219–239
44 Haynes, D.H. (1982) Arch. Biochem. Biophys. 215, 444–461
45 Meissner, G. (1981) J. Biol. Chem. 256, 636–643
46 Kometani, T. and Kasai, M. (1980) J. Membrane Biol. 56, 159–168
47 Koshita, M. and Hotta, K. (1981) Jap. J. Physiol. 31, 109–120
48 Bennett, N. and Dupont, Y. (1981) FEBS Lett. 128, 269–274
49 Takisawa, H. and Makinose, M. (1981) Nature 290, 271–273
50 Nakamura, Y. and Tonomura, Y. (1981) J. Biochem. (Tokyo) 91, 449–461
51 Endo, M. (1977) Physiol. Rev. 57 (1), 71–103
52 Somlyo, A.V., Gonzalez-Serratos, H., Shuman, H., McClellan, G. and Somlyo, A.P. (1981) J. Cell. Biol. 90, 577–594
53 Tanford, C. (1981) J. Gen. Physiol. 77, 223–229