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

I. Ca^{2+} OUTFLOW IN THE NON-ENERGIZED STATE OF THE CALCIUM PUMP

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Passive Ca^{2+} permeability of sarcoplasmic reticulum vesicles has been studied after maximal loading with Ca^{2+} (150–200 nmol/mg protein) in the presence of Ca^{2+}, MgATP and an ATP generating system of limited capacity. Outflow of accumulated Ca^{2+} in the non-energized state of the system was studied by depletion of the medium of one of the substrates, either MgATP (by complete consumption) or Ca^{2+} (by complexation with EGTA). It was found that Ca^{2+} outflow under these conditions is relatively slow and independent of the medium concentration of Ca^{2+} ($5 \cdot 10^{-9}$–$5 \cdot 10^{-5}$ M) or MgATP (0.7–730 μM). Outflow curves were steep at the beginning of the outflow phase (30–60 nmol/min per mg protein), and outflow proceeded at a much lower rate below 100 nmol Ca^{2+}/mg protein. Outflow could be completely inhibited by La^{3+}. The Ca^{2+} release curves are not compatible with simple diffusion, and cannot be accounted for by Ca^{2+} binding inside the vesicles. Neither are our observations consistent with permeation mediated via the Ca^{2+} translocation sites involved in active transport. We suggest that non-energized Ca^{2+} outflow may proceed by a process of ion-exchange through negatively charged, water-filled channels in the membrane, the properties of which are altered by a high intravesicular concentration of Ca^{2+}.

Introduction

Isolated vesicles of fragmented sarcoplasmic reticulum from skeletal muscle tissue possess the ability, through the action of a Ca^{2+}-ATPase residing in the membrane, to concentrate calcium in their luminal space, thereby forming a high Ca^{2+} gradient across the membrane [1–7]. The uphill translocation of two calcium ions is stoichiometrically coupled to the splitting by the Ca^{2+}-ATPase of one ATP molecule to ADP by a fully reversible process [4,6,8–12]. In the absence of Ca^{2+} precipitating anions and in the presence of a surplus of calcium available to the sarcoplasmic reticulum vesicles in the medium, a maximal calcium-filling capacity of the vesicles of 100–200 nmol/mg protein is generally found [1,3,5–7], at which efflux of Ca^{2+} balances influx.

It is the purpose of this and the following paper to consider the mechanisms by which Ca^{2+} accumulated by active transport leaves the sarcoplasmic reticulum vesicles. It should be noted from the outset that we are not primarily concerned with the reversal of the transport process, with resynthesis of nucleotide triphosphate, which is the backward reaction in the thermodynamic equilibrium of the Ca^{2+}-pump with ATP, ADP and Ca^{2+} [9,13]. As will become apparent from the

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results presented, other mechanisms also exist for the release of Ca^{2+}, especially at the highest levels of intravesicular accumulation. The present paper describes the passive permeability properties of the vesicles, as measured by Ca^{2+} outflow after cessation of active transport, while the second paper analyzes Ca^{2+} efflux during active Ca^{2+} transport.

The general experimental procedure used in this paper was as follows. Vesicles were loaded with Ca^{2+} in the presence of Ca^{2+}, MgATP and an ATP-generating system, but in the absence of Ca^{2+}-precipitating anions. Ca^{2+} permeability was studied after depletion of the medium for one of the substrates, either by addition of EGTA to chelate Ca^{2+}, or after complete consumption of MgATP. The latter procedure has rarely been used previously (see, however, Refs. 14–18). It permits a complete spectrophotometric record of both Ca^{2+} filling and Ca^{2+} release from the vesicles, by the use of a Ca^{2+}-sensitive indicator dye. We present evidence that, under appropriate conditions, the release phase reflects the Ca^{2+} permeability of the vesicles. The procedures used are more gentle and less likely to lead to experimental artefacts than loading of the vesicles with Ca^{2+} by extended preincubation in media with high Ca^{2+} concentrations.

Materials and Methods

Sarcoplasmic reticulum vesicles were prepared from skeletal muscles of rabbits by the procedure of De Meis and Hasselbach [19], frozen in liquid N_2, and stored at −70°C. When subjected to isopycnic centrifugation according to Meissner [20] most of the protein, Ca^{2+}-ATPase activity and Ca^{2+}-uptake capacity banded in the linear sucrose gradient between 29 and 36% (w/w). The preparation thus belongs to the category of 'light' to 'intermediate' vesicles, which preferentially originate from the longitudinal parts of the sarcoplasmic reticulum [20,21]. As estimated by SDS-gelelectrophoresis [22], the content of proteins other than ATPase was low (10–20%). Ca^{2+}-insensitive ATPase ('basal ATPase') constituted around 15% when measured at 25°C and 1 mM MgATP. The maximal Ca^{2+} uptake in the absence of Ca^{2+}-precipitating anions ranged from 130 to 210 nmol-mg protein (average 163 ± 23 (S.D., $n = 16$)).

Spectrophotometric measurement of Ca^{2+} uptake and release

An Aminco DW-2A spectrophotometer was used at 598–572 nm settings and with arsenazo III at a final concentration of 15–20 μM. The spectrophotometer was equipped with a thermostat, which circulated water through the cuvette holder, and a magnetic stirrer. The stirrer worked at a relatively high speed with a 'Cell-Spinbar' magnet (Bel-Art Products Inc., NJ, U.S.A.) in the cuvette. The complete mixing time when 5–10 μl of reagent was added to a volume of 2.6 ml from a microsyringe was 0.5 s.

The buffer used in all experiments contained 50 mM maleic acid/100 mM KCl/10 mM $MgCl_2$, titrated to pH 6.70 with Tris. The vesicles were always preincubated 5–10 min in the cuvette together with pyruvate kinase, phospho*enol*pyruvate and adenylate kinase inhibitor at concentrations indicated in the legends to the figures. The adenylate kinase inhibitor, P^1,P^5-di(adenosine-5′) pentaphosphate (Ap_5A), was included to inhibit adenylate kinase present in the vesicle preparations. A concentration of 40–100 μM was found to be sufficient for this purpose, since no ATPase activity and Ca^{2+} uptake took place in the presence of 1 mM ADP, in contrast to the case in the absence of inhibitor. A small volume (12–15 μl) of 20 mM $CaCl_2$ was added in six steps, after the preincubation period, to give a final concentration of 90–120 μM. The absorbance changes recorded after each step of $CaCl_2$ addition were used to construct a standard curve in each experiment from which the Ca^{2+} content of the vesicles could be calculated. The reaction was initiated by the addition of ATP in a small volume (5–15 μl) to give the concentrations indicated in the legends to the figures. Ca^{2+} uptake and release were then followed for the next 5–20 min.

The amount of Ca^{2+} present in the medium was always so high that more than 30% remained in the extravesicular space when the vesicles were maximally filled. Thus the Ca^{2+} concentration of the medium never decreased to such an extent that inhibition of Ca^{2+}-ATPase activity occurred, due to depletion of Ca^{2+} from the medium. The high buffer capacity of the medium ensured that unspecific absorbance changes due to variation of pH did not occur. The high $MgCl_2$ concentration ensured that binding of CA^{2+} to phospho*enol*-pyruvate and ATP was negligible, so that changes in the concentration of these two substrates did not result in changes in the free Ca^{2+} concentration. No changes in the spectrophotometer reading were observed in the absence of arsenazo III or in the presence of dye and enzymatically active sarcoplasmic reticulum vesicles, which had been made completely leaky by addition of dodecyloctaethyleneglycol monoether ($C_{12}E_8$) or deoxycholate.

Radiochemical measurement of Ca^{2+} uptake and release

The experiments were performed under exactly the same conditions as described above, except that the medium contained $^{45}CaCl_2$ (0.5 Ci/mol). At timed intervals, samples were filtered through 0.22 μm Millipore filters. The Ca^{2+} content of the vesicles was calculated from the radioactivity recovered in the filtrates, which were measured by liquid scintillation counting. Alternatively, the filters were washed twice with 1 ml ice-cold buffer without added $CaCl_2$, dried and dissolved in Bray's scintillation liquid. Excellent agreement was found between spectrophotometric and radiochemical measurements.

Ca^{2+} binding experiments

Measurement of Ca^{2+} binding to the interior aspect of sarcoplasmic reticulum vesicles was performed after passive loading with Ca^{2+}. The vesicles (1.5–2 mg protein/ml) were equilibrated with Ca^{2+} after exposure to Ca^{2+} (0.4–18.2 mM), $^{45}Ca^{2+}$, 100 mM KCl, 10 mM Mg^{2+} and 50 mM Tris-maleate buffer (pH 6.7) for 5 h at 20°C. Then an aliquot (0.15 ml) was diluted with 2.5 ml ice-cold 10 mM $LaCl_3$ (in the same buffer without Ca^{2+}) and filtered through Millipore. The filters were washed twice with 2 ml of the same $LaCl_3$ solution, and the $^{45}Ca^{2+}$ retained on the filters was measured by liquid scintillation counting. Unbound $^{45}Ca^{2+}$ present was estimated by Millipore filtration of vesicles which were quenched with $LaCl_3$ immediately after exposure to the $^{45}CaCl_2$ solutions. The vesicle suspensions contained 43 ± 7 nmol/mg Ca^{2+}, as measured by flame photometry, 60% of which sedimented with the vesicles. We have assumed this Ca^{2+} to be exchangeable and therefore not to be of any significance at the low protein concentrations employed in our binding experiments.

Estimation of intravesicular water content

The permeation space of ^3H_2O, relative to that of inulin, was used as a measure of intravesicular water content. A mixture of tritiated water (60 μCi/ml) and [^{14}C]hydroxymethylinulin (8 μCi/ml) was added in a 20 μl volume to a concentrated suspension of sarcoplasmic reticulum (150 μl, 15–20 mg protein/ml), and to control samples containing only Tris-maleate electrolyte solution. After 1 h at 20°C, the vesicles were sedimented by centrifugation on a Beckman Airfuge for 30 min. The change in the ratio of $^{13}H/^{14}C$ was measured by double-channel counting as previously described [23]. The fraction of the sample that represents the water permeation space was calculated as

$$\frac{(^3H/^{14}C)_{\text{sample}} - (^3H/^{14}C)_{\text{control}}}{(^3H/^{14}C)_{\text{sample}}} \qquad (1)$$

where ($^3H/^{14}C$) is the channel ratio, corrected for spill-over of ^{14}C into the ^3H channel. The value obtained was 2.9 ± 0.3 μl/mg (S.D. of four experiments).

Other methods

The ATPase activity of the vesicles was assayed by following the liberation of inorganic phosphate by the method of Fiske and SubbaRow [24] after quenching of enzyme activity with 15% trichloroacetic acid. The concentration of acid stable phosphoprotein formed from [γ-^{32}P]ATP was measured by quenching samples of reaction medium with 5 vol. of 15% trichloroacetic acid and 80 mM pyrophosphate, followed by filtration on Millipore filters. The filters were washed four times with 5 ml 5% trichloroacetic acid/40 mM pyrophosphate, dried and dissolved in Bray's scintillation liquid and counted. ATP-concentration was measured after quenching with an equal volume of 10% trichloroacetic acid/4 mM EDTA or directly on Millipore filtrates, using the firefly luciferase assay. A LKB Luminometer 1250 (LKB Instruments, Aps, Uppsala) combined with a potentiometer recorder (LKB 2210) was employed. Protein concentration was determined by the method of Lowry et al. [25], with correction for unequal color development with serum albumin as described by Andersen et al. [26].

Materials

All reagents were of analytical grade. Arsenazo III was purchased from EGA-Chemie (B.R.D.) and purified according to Kendrick [27]. Pyruvate kinase, apyrase, EGTA and Na_2ATP were from Sigma Chem. Co. (St. Louis, U.S.A.) and phospho*enol*pyruvate, hexokinase and P^1,P^5-di(adenosine-5′)pentaphosphate were from Boehringer-Mannheim, F.R.G. Radioactive chemicals were from Amersham International, U.K., and were used without further purtification.

Results

Measurement of Ca^{2+} outflow after the ATP and Ca^{2+} depletion

In the following, we define Ca^{2+} outflow as permeation of accumulated Ca^{2+} from sarcoplasmic reticulum vesicles in the absence of active, inward transport of Ca^{2+}. The vesicles were allowed to accumulate Ca^{2+} by active transport, and outflow was studied by depletion of one of the substrates, i.e., Ca^{2+} or MgATP. An essential part of our initial investigation was to define conditions which permitted us to examine Ca^{2+} outflow by switching the Ca^{2+}-ATPase from Ca^{2+} transport to the non-energized mode of the protein. In the case of Ca^{2+} depletion, this could easily be done by addition of EGTA to lower the free concentration of Ca^{2+} in the medium. However, it was not obvious whether experimental conditions could be established under which ATP depletion, as the result of ATP hydrolysis, occurred at a rate sufficiently rapid to be of any value in the study of Ca^{2+} outflow. Fig. 1 shows results bearing on this issue. Ca^{2+} uptake and release were followed spectrophotometrically in the presence of ATP and an ATP generating system (phospho*enol*pyruvate plus pyruvate kinase). It is seen that the vesicles are capable of accumulating Ca^{2+} to high levels and that the uptake phase is followed by a well-defined release phase. The release occurs rapidly above 100 nmol Ca^{2+}/mg protein, but becomes a very slow process at lower Ca^{2+} loading levels. The onset of the release phase is sharp and occurs at a time which is proportional to the concentration of energy-rich phosphate (see inset). We found in parallel experiments that the transition points exactly coincided with the liberation of one equivalent energy-rich phosphate from the phospho*enol*pyruvate and ATP present in the medium (not shown).

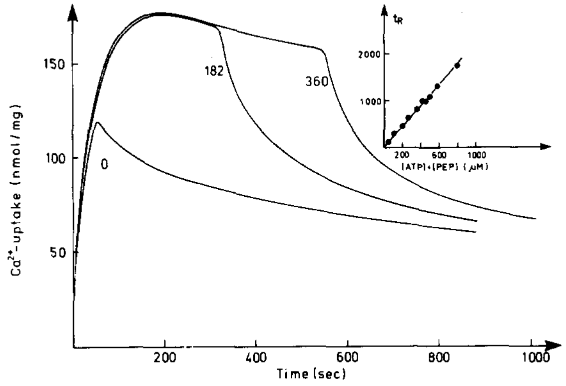

Fig. 1. Ca^{2+} uptake and release in the presence of ATP and an ATP-generating system (phospho*enol*pyruvate and pyruvate kinase). Ca^{2+} uptake was started by addition of a single dose of ATP to the otherwise complete reaction medium. Ca^{2+}-ATPase activity during the period of maximal uptake was constant in all experiments (0.14 μmol/mg per min), whereas no activity was demonstrable at the time of Ca^{2+}-release. The concentration of energy-rich phosphate in different experiments was varied by varying the phospho*enol*pyruvate concentration. Assay conditions: 25°C; 0.33 mg/ml sarcoplasmic reticulum vesicles; 0.2 mg/ml pyruvate kinase; 0, 182 or 360 μM phospho*enol*pyruvate (figures on curves), 40 μM Ap_5A; 36 μM ATP; 109 μM $CaCl_2$; 20 μM arsenazo III. Inset: Demonstration of a linear relationship between t_R (release time) and the sum of the concentrations of ATP and phospho*enol*pyruvate. Assay conditions: 20°C, 0.2 mg/ml sarcoplasmic reticulum vesicle; 0.2 mg/ml pyruvate kinase; 40 μM Ap_5A; 47 μM ATP; 113 μM $CaCl_2$; 19 μM arsenazo III. The phospho*enol*pyruvate concentration was varied from 0 to 750 μM.

Ca^{2+}-release curves such as the ones shown in Fig. 1 were stringently dependent on a number of experimental conditions. (i) In the absence of the adenylate kinase inhibitor Ap_5A, Ca^{2+} uptake continued slowly as the result of P_i liberation from ADP. (ii) The ATP concentration (30–100 μM) was critical. Below 10 μM ATP the transition

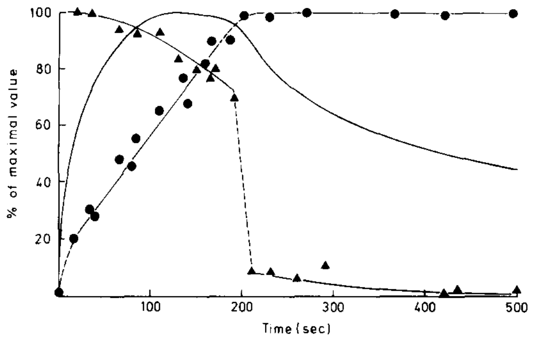

Fig. 2. Ca^{2+} uptake and release after addition of a single dose of ATP in the absence of an ATP-generating system. The symbols denote: ●, inorganic pphosphate liberated and, ▲, concentration of acid-stable phosphoenzyme. All values are expressed in percent of maximally obtained values which were (mean of two experiments): 195 nmol/mg protein, 492 nmol/mg and 2.7 nmol/mg for Ca^{2+} content, P_i liberation and phosphoenzyme concentration, respectively. Assay conditions: 25°C; 0.39 mg/ml sarcoplasmic reticulum vesicles; 90 μM Ap_5A; 90 μM $CaCl_2$; 182 μM ATP. Arsenazo III (17 μM) was included in all assay media.

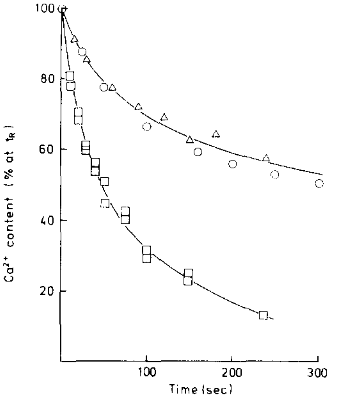

Fig. 3. Comparison between the Ca^{2+} release due to ATP depletion (○), EGTA addition (△) and the combined effect of ATP depletion and EGTA addition (□). The ordinate gives the percent of Ca^{2+} remaining in the vesicles of the Ca^{2+} content at time t_R (○, □; 100% = 147 ± 6 nmol/mg, $n = 8$) or 10 s before the addition of EGTA (△; 100% = 152 ± 6 nmol/mg, $n = 3$). The abscissa is time after t_R (○, □) or EGTA addition (△). Assay conditions: 25°C; 0.44 mg/ml sarcoplasmic reticulum vesicles; 0.2 mg/ml pyruvate kinase; 40 μM Ap_5A; 107 μM $CaCl_2$; 72 μM ATP; 20 μM arzenazo III; 290 μM (○, □) or 2 mM phospho*enol*pyruvate (△). EGTA added to a final concentration of 11 mM (△, □).

point was poorly defined and residual ATPase activity during the release phase was demonstrable. In the absence of phospho*enol*pyruvate (and therefore a higher ATP concentration) the transition was also less sharp, although a clear release phase was demonstrable, see Fig. 2, which also shows that at the transition point there is liberation of one equivalent P_i and a rapid drop in the concentration of phosphorylated enzyme.

Evidence that under optimal conditions Ca^{2+}-release curves represent Ca^{2+} outflow, uncomplicated by ATP supported Ca^{2+} influx, was obtained by the following additional experimental observations. (i) No appreciable uptake of $^{45}Ca^{2+}$ was found when the radioactive isotope was added to the medium during the release phase. (ii) Addition of apyrase (an ATPase/ADPase), or the ATP-consuming system glucose/hexokinase, during the Ca^{2+} release phase had no or negligible effect. (iii) Direct measurements with the luciferase method revealed that the free concentration of ATP dropped to very low levels (under 10^{-7} M) at the start of Ca^{2+} release (see Fig. 4).

Fig. 3 (upper curve) compares Ca^{2+} outflow due to ATP depletion (spectrophotometric assay) with that produced by EGTA addition (Millipore filtration experiments). EGTA was added 200 s after ATP to give a final concentration in the medium of 11 mM, thereby reducing the concentration of free Ca^{2+} from about $3 \cdot 10^{-5}$ M to around $5 \cdot 10^{-9}$ M (calculated from the stability constant given by Harafuji and Ogawa [28]). It is seen that the two types of Ca^{2+} outflow follow each other very closely. Therefore, the Ca^{2+} concentration of the medium does not have any effect on the rate of Ca^{2+} outflow. The EGTA-induced Ca^{2+}-outflow experiments were performed after loading the vesicles in the presence of different ATP concentrations (0.73 μM, 7.3 μM, 73 μM and 730 μM) as well as with carbamyl phosphate (0.96 mM) as an energy-donor. In all cases, the Ca^{2+}-outflow curves were practically superimposable (not shown). Thus ATP seems to be of no importance for Ca^{2+} outflow, either. However, the combined effect of EGTA addition and ATP depletion is a higher rate of Ca^{2+} outflow (lower curve of Fig. 3). But under these conditions we observe resynthesis of ATP (Fig. 4), whereas the free ATP concentration remains at a non-measurable level (under

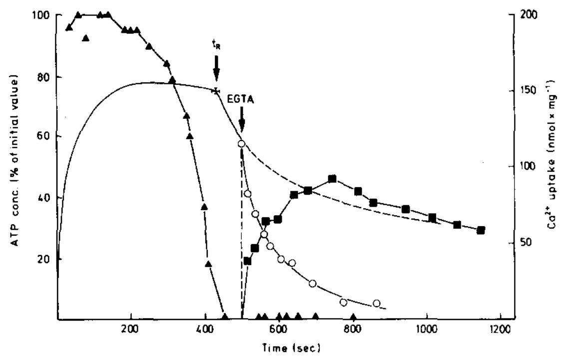

Fig. 4. Demonstration of ATP-synthesis when EGTA is added after ATP-depletion. ATP-concentrations are expressed as percentage of ATP-concentration initially present in the medium (47 μM) (left ordinate). The ATP concentration (▲) declines to non-detectable values at t_R and remains there during normal outflow (broken curve). After addition of EGTA there is resynthesis of ATP (■) and an increased outflow rate of Ca^{2+} (○). Assay conditions: 20°C; 0.48 mg/ml sarcoplasmic reticulum protein; 0.2 mg/ml pyruvate kinase; 40 μM Ap_5A; 47 μM ATP; 112 μM $CaCl_2$; 17 μM arsenazo III; 297 μM phospho*enol*pyruvate; EGTA added (arrow) to a final concentration of 11 mM (■, ○).

10^{-7} M) when Ca^{2+} outflow results from ATP depletion alone. Following the addition of EGTA, the ATP level increases to a value approximately half of that initially present and then slowly declines, probably as the result of resumed basal ATPase activity. During the rapid release phase, the ratio between Ca^{2+} disappearance from the vesicles and ATP resynthesis is approximately 2 : 1. These observations indicate that simultaneous ATP depletion (with concomitant ADP accumulation) and addition of EGTA cause a reversal of ATP driven Ca^{2+} translocation, in agreement with other studies [10,29].

The 'overshoot' phenomenon

Fig. 1 shows the presence of a slow decline in the Ca^{2+} content of the vesicles before the Ca^{2+} outflow phase. This 'overshoot' phenomenon, which is a feature of all Ca^{2+}-uptake curves [30], is associated with an absolutely constant Ca^{2+}-ATPase activity (not shown). Overshoot may be related to time-dependent changes in the Ca^{2+} permeability of the vesicles. This is suggested by a comparison of the three outflow curves of Fig. 1: at comparable levels of intravesicular Ca^{2+} the rate of outflow is the higher the longer Ca^{2+} has been retained by the vesicles. Accordingly, the decline in Ca^{2+} content preceding outflow may reflect increased Ca^{2+} permeability. Alonso et al. [31] have reported a permeability increase of vesicles after exposure to Tris buffer. However, we found that preincubation (5–60 min) of the vesicles with our buffer system had no effect on the subsequent Ca^{2+}-uptake and -outflow curves, even at elevated temperature. Therefore, the observed slow permeability increase with higher concentrations of phospho*enol*pyruvate in the medium presumably is a consequence of exposure of the interior of the vesicles to high concentrations of Ca^{2+}. Fig. 5 shows data from an experiment in which phospho*enol*pyruvate was re-added after one cycle of Ca^{2+} uptake and outflow. Note that the overshoot

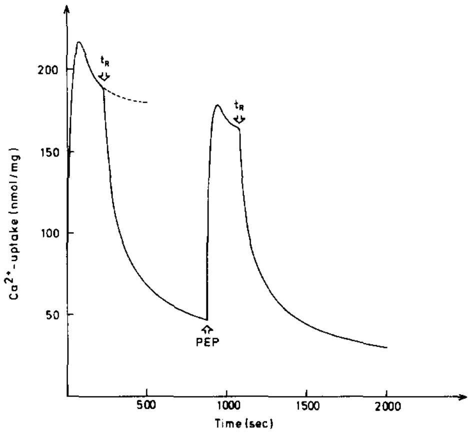

Fig. 5. Reactivation of Ca^{2+} accumulation after one cycle of Ca^{2+} uptake and release. The assay conditions were: 30°C; 0.36 mg/ml sarcoplasmic reticulum protein; 0.2 mg/ml pyruvate kinase; 18 μM arsenazo III; 120 μM Ca^{2+}; 100 μM ATP; 0.405 mM phospho*enol*pyruvate was added twice: at time zero and as indicated by the arrow. Maximal filling of the vesicles is seen after 70 s, followed by a decrease in Ca^{2+} content which, in the case of a higher phospho*enol*pyruvate concentration, follows the broken line. The actual phospho*enol*pyruvate concentration, however, results in spontaneous Ca^{2+} release after 225 s. The vesicles are allowed to release most of the Ca^{2+} before the second dose of phospho*enol*pyruvate is added to the cuvette to initiate renewed Ca^{2+} uptake. Spontaneous Ca^{2+} release follows the consumption of the second dose, too. The high maximal Ca^{2+} content obtained (217 nmol/mg) was a property of the particular sarcoplasmic reticulum preparation employed in the experiment and is not due to the higher temperature, which was used to emphasize the overshoot phenomenon.

is very discernible at the high temperature (30°C) employed, as previously noted by Sreter [32]. The second cycle of Ca^{2+} uptake and outflow has a maximal filling level which is less than corresponding to the Ca^{2+} at the point of release during the first uptake cycle. Furthermore, the rate of Ca^{2+} outflow is higher during the second cycle. These observations suggest that the increased Ca^{2+} permeability as a function of time is at least partially of an irreversible nature.

Effect of La^{3+}

Fig. 6 shows the effect of La^{3+} on net Ca^{2+} movement when La^{3+} is added at different times relative to ATP. In Fig. 6A, La^{3+} (0.92 mM) was added 10 s before ATP, which resulted in almost complete inhibition of Ca^{2+} uptake by the vesicles. By addition of La^{3+} at a time when the vesicles are already filled with Ca^{2+} (Fig. 6B) there is no effect on the Ca^{2+} content, in spite of the fact that the rapid exchange of Ca^{2+} in filled vesicles, which is normally seen, is severely inhibited (Fig. 6C). Thus La^{3+} must inhibit efflux as well as influx of Ca^{2+} across the sarcoplasmic reticulum membrane. Fig. 6D, furthermore, shows that La^{3+} is capable of inhibiting the Ca^{2+} outflow that follows ATP-depletion of the medium.

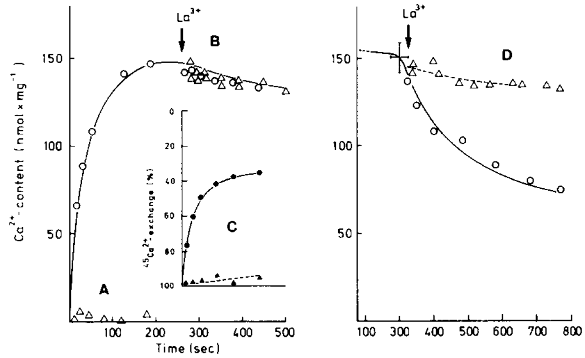

Fig. 6. Effect of La^{3+} on Ca^{2+} content and Ca^{2+} exchange of sarcoplasmic reticulum vesicles. In A, La^{3+} (920 μM) was added 10 s before ATP, in B 250 s after ATP, and in D at t_R. The inset, C, shows Ca^{2+} exchange after addition of $^{45}Ca^{2+}$ 260 s after ATP, with (▲) and without (●) La^{3+} addition. The unbroken curves pertaining to B and D are spectrophotometric records, while the symbols represent Ca^{2+} uptake measured by Millipore filtration in the presence (△) or absence (○) of La^{3+}. Assay conditions for A, B and C: 20°C; 0.52 mg/ml sarcoplasmic reticulum protein; 47 μM MgATP; 0.2 mg/ml pyruvate kinase; 50 μM Ap_5A; 112 μM Ca^{2+}; 750 μM phospho*enol*pyruvate. The spectrophotometric record is the mean of eight experiments, and the symbols are the means of two experiments. Assay conditions for D: As in A, B, and C, except that the phospho*enol*pyruvate concentration was 225 μM ($t_R = 300 \pm 20$ s). The spectrophotometric record is the mean of five experiments, and the symbols are the means of two experiments.

Ca^{2+}-binding

These experiments were performed after passive loading of the vesicles with high concentrations of Ca^{2+} for 5 h at 20°C. We noted that exposure of the vesicles to high Ca^{2+} concentrations (above 1 mM) resulted in marked changes in the properties of the vesicle suspensions as evidenced by increased turbidity and sedimentability, an effect also observed by other workers (Ref. 33 and Hasselbach, W., personal communication). These changes were more obvious at the higher Ca^{2+} concentrations, and were not reversed by subsequent removal of Ca^{2+} by EGTA chelation.

Fig. 7 shows the amount of Ca^{2+} retained by the incubated vesicles after removal of externally bound Ca^{2+} by La^{3+} quenching. We attempted to estimate the free and bound component of the

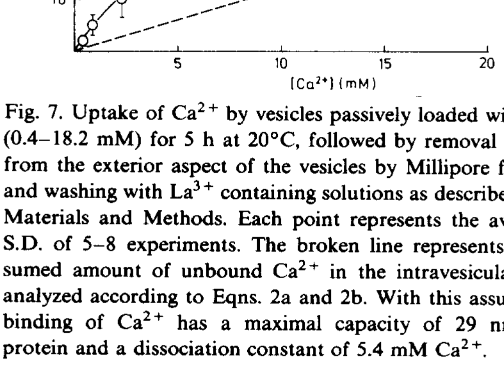

Fig. 7. Uptake of Ca^{2+} by vesicles passively loaded with Ca^{2+} (0.4–18.2 mM) for 5 h at 20°C, followed by removal of Ca^{2+} from the exterior aspect of the vesicles by Millipore filtration and washing with La^{3+} containing solutions as described under Materials and Methods. Each point represents the average ± S.D. of 5–8 experiments. The broken line represents the assumed amount of unbound Ca^{2+} in the intravesicular space analyzed according to Eqns. 2a and 2b. With this assumption, binding of Ca^{2+} has a maximal capacity of 29 nmol/mg protein and a dissociation constant of 5.4 mM Ca^{2+}.

total internal Ca^{2+} by assuming that these are represented by a linear and a saturable function of the Ca^{2+} concentration in the medium, according to the following equations:

$$Ca_{i,tot} = [Ca_{i,f}]\delta + Ca_{i,b} \tag{2a}$$

$$Ca_{i,b} = \frac{Ca_{i,bmax}[Ca_{i,f}]}{K_b + [Ca_{i,f}]} \tag{2b}$$

where $Ca_{i,tot}$ represents the total Ca^{2+} load of the vesicles, $[Ca_{i,f}]$ the intravesicular concentration of unbound Ca^{2+}, δ the intravesicular distribution volume of Ca^{2+}, $Ca_{i,b}$ the amount of intravesicular binding of Ca^{2+}, $Ca_{i,bmax}$ the maximal binding capacity, and K_b the apparent binding constant for Ca^{2+} inside the vesicles. In other words, we assume Ca^{2+} binding to take place at a set of identical and non-interacting sites. The best fit of the data to Eqns. 2a and 2b was obtained by an iterative procedure which led to maximal binding capacity of 25–30 nmol/mg and an apparent dissociation constant of 5–6 mM. These results are in agreement with Ca^{2+}-binding data of Prager et al. [34] and Feher and Briggs [18], but different from those produced by Miyamoto and Kasai [35], who found a maximal internal binding capacity around 200 nmol Ca^{2+}/mg. This difference could, at least in part, be due to the presence of a relatively high concentration of Ca^{2+}-binding proteins other than the Ca^{2+}-ATPase in the vesicle preparations of Miyamoto and Kasai [35]. Alternatively, they may represent different degrees of leakiness of the preparations (see below), since Ca^{2+} binding was measured by a washing procedure.

From the slope of the straight line showing the relationship between free internal Ca^{2+} and medium Ca^{2+} (Fig. 7), we obtain a value of 1.2 μl/mg for the distribution volume of unbound Ca^{2+} in the intravesicular space (δ). This value is lower than generally reported in the literature (4–5 μl/mg) for the intravesicular volume from measurements based on the exclusion of compounds like inulin and dextran or trapping of slowly permeating compounds like sucrose [6,39]. It is also lower than the value of 2.9 ± 0.3 μl/mg measured in our laboratory as the ^3H_2O distribution volume (see Materials and Methods) in the absence of added $CaCl_2$. The lower values obtained by analysis of the passive Ca^{2+} uptake curves of vesicles have been noted previously [6], or can be deduced from data presented [34]. It is unclear at present to what extent this difference can be attributed to the existence of leaky vesicles, as proposed by Tada et al. [6], or to shrinkage of vesicles in the presence of high $CaCl_2$ concentrations (over 1 mM), as suggested by Kasai [37] and Miyamoto and Kasai [35]. If the pretreatment with high Ca^{2+} destroys the diffusional barrier of many of the vesicles, this would explain why our binding data are lower than deduced by Hasselbach and Koenig [38] from Millipore filtration experiments (approx. 100 nmol/mg) with a non-washing procedure.

Analysis of Ca^{2+} outflow

Completion of Ca^{2+} outflow is a very slow process, extending over several hours, due to slow permeation of Ca^{2+} below 100 nmol/mg protein. This is in marked contrast to the initial rate with which Ca^{2+} disappears from the vesicles (approx. 30–60 nmol/mg per min, corresponding to an apparent rate constant of 0.2–0.4 min^{-1}). As a result, logarithmic plots of Ca^{2+} load versus time are markedly non-linear. Feher and Briggs [17,18], from data similar to ours, concluded that slow permeation of Ca^{2+} might be attributable to extensive binding of Ca^{2+} at low Ca^{2+} load levels. We corrected our data for binding according to Eqns.

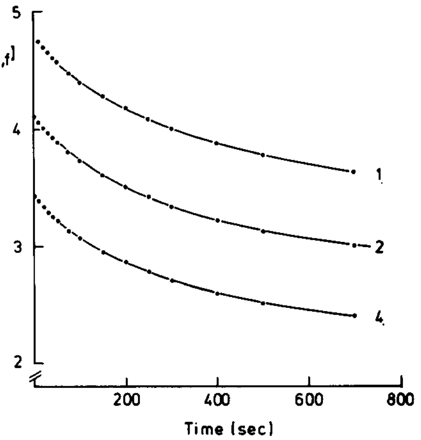

Fig. 8. Semilogarithmic plot of intravesicular Ca^{2+} content as a function of time after ATP depletion. Results were corrected for intravesicular binding of Ca^{2+} as described in the text, assuming different values for the intravesicular distribution volume of Ca^{2+} (1–4 μl/mg protein). Figures on curves are δ values, in μl.

2a and 2b. Assuming the same binding parameters as found by passive Ca^{2+} equilibration in the preceding section, but a range of values for the distribution volume of Ca^{2+} (1, 2, 4 μl/mg), we obtain a set of curves (Fig. 8) which are all non-linear and thus not consistent with a simple diffusion mechanism, in disagreement with Feher and Briggs [17]. It should be noted that non-linearity is observed at Ca^{2+} loads where, according to the view of Feher and Briggs [17], Ca^{2+} binding is saturated and the effect of Ca^{2+} binding on outflow should be minimal. Given the uncertainty which in our opinion pertains to Ca^{2+}-binding data obtained by passive loading of the vesicles, it was also of interest to examine the effect of a range of Ca^{2+}-binding parameters on the corrected Ca^{2+}-outflow curves. Non-linear curves were also obtained by changing the maximal binding capacity from 10–200 nmol/mg and the dissociation constant from 0.5 to 50 mM.

We were also unable to resolve the data in terms of a sum of two or three first-order equations with reasonable certainty, i.e., we have no evidence for the existence of subpopulations of vesicles with widely different Ca^{2+}-outflow properties. More definitive evidence against a significant effect of vesicle heterogeneity was obtained from experiments in which the accumulation of Ca^{2+} was reduced to a level of 50–100 nmol/mg^{-1}, by curtailing the supply of Ca^{2+} in the medium. Subsequent addition of EGTA did not produce a rapid outflow phase as would have been expected if there had been marked variations in the Ca^{2+}-permeability characteristics among the Ca^{2+}-accumulating vesicles in the preparation.

Discussion

An essential feature of the present results was the feasibility of measuring the passive permeability characteristics of sarcoplasmic reticulum vesicles for Ca^{2+} after depletion of the medium for energy-rich phosphate. This permits us to examine the hypothesis that the high-affinity Ca^{2+} translocation sites may be involved as a Ca^{2+}-exchanger in the non-energized state of the transport system [37]. We found that the outflow phase was not affected by a medium concentration of 40–100 μM Ca^{2+}, which is sufficient to saturate the translocation sites. Hence, there is no evidence for a Ca^{2+}-Ca^{2+} exchange mediated by the non-energized enzyme, since this presumably would have caused a delay in the outflow of Ca^{2+} after ATP depletion as compared to addition of EGTA to the medium (cf. Fig. 3). Chiu and Haynes [37] after passive loading of sarcoplasmic reticulum vesicles at a high Ca^{2+} concentration and subsequent transfer to media with varying Ca^{2+} concentrations, found an inhibitory effect of extravesicular Ca^{2+} on Ca^{2+} permeation (trans-inhibition). These authors conclude that in the non-energized state there is only shuttling of the translocation site from the outside to the inside in the Ca^{2+} free form. If this phenomenon had been operative in our experiments, it should also have resulted in a lower rate of Ca^{2+} outflow after ATP depletion than after complexation of Ca^{2+} with EGTA. Hence, we conclude that in our experiments there is no evidence for any involvement of the Ca^{2+}-translocation sites in the Ca^{2+}-outflow process. This conclusion is in agreement with similar data on outflow presented by Feher and Briggs [17], and with an examination of the Ca^{2+} permeability properties of reconstituted ATPase [39]. Concerning the discrepant results of Chiu and Haynes [37], we would like to point out that passive Ca^{2+} loading of vesicles with high medium concentrations may lead to significant changes in the Ca^{2+} permeability properties. Thus, Chiesi and Inesi [40] found a much higher Ca^{2+} permeability of vesicles after EGTA addition by passive as compared to active loading *.

Despite the non-involvement of the Ca^{2+} translocation sites, mediation of the Ca^{2+}-outflow process is suggested by the complete inhibitability by La^{3+} (Fig. 6). Mediation of Ca^{2+} outflow is in contrast to the view put forward by Feher and Briggs [17] who suggested permeation by a diffusion mechanism. The main evidence advanced in

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* Chiesi and Inesi [40], following a suggestion by Weber [43], interpreted their results to mean that MgATP increases the passive Ca^{2+} permeability properties. However, this interpretation is not supported by the present data which indicate that EGTA-induced Ca^{2+} outflow is not affected by a variation of MgATP concentrations, ranging from 0.7–730 μM and by the use of carbamyl phosphate as an energy donor instead of ATP.

favor of a diffusion mechanism was proportionality between load level and outflow, after correction for intravesicular binding of Ca^{2+}. This is not in accordance with our data. By contrast, we find that the very low Ca^{2+} permeability, characteristic of intact vesicles, is increased at high load levels. Furthermore, some of the changes induced by exposure of the interior of the vesicles to high Ca^{2+} concentrations are time-dependent and may lead to the overshoot phenomenon (Fig. 5).

Most of the results presented by Feher and Briggs were obtained on cardiac preparations which probably are more leaky than sarcoplasmic reticulum from skeletal muscle. However, a few data were presented referring to sarcoplasmic reticulum preparations of skeletal muscle. Inspection of these data indicates that the permeability of these latter preparations was also higher than in our average preparations. An initially high Ca^{2+} permeability of the vesicles could have obscured the effect of high intravesicular Ca^{2+} concentrations noted in our experiments. In this connection, it is of interest that in the experiments of Feher and Briggs [17,18] there was no evidence of the overshoot phenomenon, which may reflect time-dependent changes in Ca^{2+} permeability (Fig. 4).

The mechanism of Ca^{2+} outflow by a non-diffusion process may be by permeation through water-filled channels, containing fixed negative charges. In that case, permeation may occur by a process of ion-exchange, in competition with La^{3+}, as has been demonstrated to take place in model systems with a phospholipid ion-exchanger membrane [41]. For such a permeation mechanism, the ATPase, perhaps in an oligomeric state [42], would play an important role in the formation of the hydrophilic channels as evidenced by previous comparisons of Ca^{2+} permeability of liposomes and ATPase vesicles [39].

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