Adenosine 5′-Triphosphate Dependent Fluxes of Manganese and Hydrogen Ions in Sarcoplasmic Reticulum Vesicles†

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ABSTRACT: Charge displacement induced by the accumulation of Ca^{2+} ions inside sarcoplasmic reticulum vesicles may be counterbalanced by the transport of Mg^{2+} and/or K^+ in the opposite direction [Kanazawa, T., Yamada, S., Yamamoto, T., & Tonomura, Y. (1971) *J. Biochem. (Tokyo) 70*, 95] or by fluxes of monovalent ions through independent channels [Zimniack P., & Racker, E. (1978) *J. Biol. Chem. 253*, 4631]. Alternatively, Ca^{2+} transport may be secondary to the H^+ gradient established by a primary H^+ pump [Madeira, V. M. C. (1978) *Arch. Biochem. Biophys. 185*, 316]. In relation to this problem, we have studied fluxes of two cations which are amenable to direct measurement in conditions relevant to Ca^{2+} transport. We studied a radioactive Mn^{2+} isotope and found that it can be used as an activator of the Ca^{2+} pump in place of Mg^{2+}. We then obtained evidence excluding the participation of Mn^{2+} in a counter transport mechanism. On the contrary, this divalent cation is a weak competitor of Ca^{2+} for vectorial transport. We also confirmed that during active transport H^+ ejection from sarcoplasmic reticulum (SR) vesicles was in excess of the H^+ production caused by dissociation of the ATPase reaction products. However, this phenomenon could not be attributed to a primary H^+ pump, since neither H^+ ejection nor Ca^{2+} uptake is reduced by a H^+ ionophore (FCCP), while both phenomena are reversed by a Ca^{2+} ionophore (A 23187). In addition, in reconstituted SR vesicles, Ca^{2+} uptake is stimulated by FCCP as well as by valinomycin. H^+ ejection and Ca^{2+} uptake occur with parallel kinetics and involve ~ 1 $H^+/1$ Ca^{2+} in the first second of the reaction at pH 6.1. Furthermore, titration of high-affinity Ca^{2+} binding sites of the SR preparation with Ca^{2+} releases H^+ (at pH 6.0 the ratio between Ca^{2+} bound and H^+ liberated is ~ 1). Our experiments indicate that the Ca^{2+} pump does not include countertransport of divalent cations (e.g., Mg^{2+}). Rather, it is likely that H^+ is exchanged for Ca^{2+} in the transport cycle. The charge equivalent ratio of the H^+/Ca^{2+} exchange depends on the pK of the carrier and the intravesicular pH but tends to be less than 0.5 near neutrality. Therefore, Ca^{2+} transport will produce net charge displacement if not compensated for by K^+ and Cl^- fluxes through independent channels or by K^+ countertransport.

One of the most interesting problems in the study of the sarcoplasmic reticulum (SR) calcium pump is whether active transport of Ca^{2+} is coupled to countertransport of another cation or is electrically balanced by fluxes of other ions which are not directly coupled to the pump. In this regard, Kanazawa et al. (1971) proposed that Mg^{2+} and/or K^+ may be exchanged for Ca^{2+} in an antiport system, thereby rendering the pump electrically silent. This proposal was based on the stimulation produced by Mg^{2+} and K^+ on the turnover of the pump. However, fluxes of these cations were never measured directly.

A different view was taken by Zimniack & Racker (1978), who observed an increased calcium uptake by reconstituted SR vesicles in the presence of valinomycin. These authors proposed that the calcium pump is electrogenic and the consequent charge displacement is offset by electrolyte fluxes through independent channels.

Lastly, H^+ fluxes were observed by Carvalho (1972) and later by Madeira (1978). This latter author inferred that the SR ATPase is a primay H^+ pump and Ca^{2+} translocation is secondary to a H^+ gradient.

With the experiments reported here, we have sought to clarify those aspects of this problem which could be studied by direct measurements of cation fluxes. Owing to the availability of a convenient radioactive Mn^{2+} isotope and the ability of Mn^{2+} to activate SR ATPase in place of Mg^{2+}, we have chosen Mn^{2+} as the divalent cation to be studied in place of Mg^{2+}. Furthermore, we have studied H^+ production during transport activity and maximized the information on this phenomenon by the use of rapid kinetics instrumentation.

Materials and Methods

Biological Materials. Sarcoplasmic reticulum vesicles were isolated from rabbit white skeletal muscle as previously described by Eletr & Inesi (1972).

Reconstituted ATPase vesicles were prepared after Triton X-100 solubilization essentially as described by Chiesi et al. (1978). Typically, 30 mg of SR vesicles (10 mg/mL) was partially solubilized at 0 °C by the addition of 1 mg of Triton X-100 per mg of protein in 10 mM Tris-HCl (pH 7.5), 1.5 mM $MgCl_2$, 1 mM EDTA, 0.1 mM $CaCl_2$, 0.02% NaN_3, and 500 mM KCl (reconstitution buffer). After centrifugation, the clear supernatant was combined with 60 mg of crude soybean phosphatidylcholine (commercial grade, Sigma Chemical Co.), which was previously solubilized in 6 mL of the above buffer with the aid of a sonicator and in the presence of 2 mg of triton per mg of lipid. The detergent was removed by passing the mixture through a Bio-Bead SM-2 column (2.5 × 40 cm) equilibrated and eluted at room temperature with reconstitution medium at a rate of 25–35 mL/h. After elution, the reconstituted vesicles were centrifuged for 40 min at 38 000 rpm (Beckman Ti 50 rotor) and resuspended in ice-cold reconstitution buffer (1–2 mg/mL). Vesicles were immediately used after preparation. Overnight storage usually produced loss of Ca^{2+} uptake capacity.

Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard. Phospholipids were determined by using a modification of the procedure of Chen et al. (1956). The phospholipid/protein ratio of reconstituted ATPase vesicles was usually 3.5–4.5.

Cation Fluxes. The composition of reaction mixtures for

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cation flux measurements is described in the legends to the figures. If not stated otherwise, biological material (SR or reconstituted vesicles) was preincubated for 5–10 min in the reaction buffer. Reactions were started with addition of substrates [ATP, ITP, or acetyl phosphate (AcP)].

All experiments were carried out at room temperature.

Ca^{2+} uptake by SR vesicles or reconstituted membranes was measured by using the Millipore filtration procedure (Martonosi & Feretos, 1964) and a ^{45}Ca radioactive tracer. Aliquots of the reaction mixture (50–100 μL) were filtered and immediately washed with ice-cold medium containing 2 mM $LaCl_3$ (Chiesi & Inesi, 1979).

Mn^{2+} fluxes were determined similarly to Ca^{2+} fluxes by using a ^{54}Mn radioactive tracer. H^+ fluxes were measured by using proton indicators and a dual-wavelength spectrophotometer. Phenol red (100 μM) was used for pH ranges between 6.5 and 7.5 with wavelength monochromators set at 560 and 600 μm. At lower pH ranges (5.5–6.5), the reaction mixture contained 40 μM chlorphenol red and a wavelength pair of 488 and 577 nm was chosen.

Rapid Kinetic Measurements. Ca^{2+} uptake measurements were carried out in a Dionex D-133 multimixing apparatus as previously described (Verjovski-Almeida et al., 1978). The reaction mixture contained 80 mM KCl, 5 mM $MgCl_2$, 40 μM chlorphenol red, 100 μM $^{45}CaCl_2$, and 1.5 mM Mes,[1] pH 6.1. Syringes A and B were supplemented with SR protein (2 mg/mL) and ATP (500 μM), respectively. Syringe C contained the quench reagent (10 mM EGTA in 80 mM KCl, 5 mM $MgCl_2$, and 20 mM Mops, pH 6.9). Uptake reactions were quenched at serial intervals of time (40–5000 ms), and samples were rapidly filtered. The ^{45}Ca content of the filtrate was measured in a scintillation counter. Proton fluxes were measured under identical conditions with the aid of a Dionex D-137 dual-detector stopped-flow spectrophotometer; 630- and 577-nm filters were chosen. The spectrophotometer was interfaced with a Northstar Horizon computer, which was programmed to store, amplify, and subtract series of data for oscilloscope display. Syringe A contained the SR solution supplemented or not with 0.2% Triton X-100 (leaky and intact vesicles, respectively), and syringe B contained buffer solution supplemented or not with ATP (500 μM). For each curve a base line (in the absence of ATP) was obtained and subtracted from the experimental data.

Measurement of H^+ Release from SR Vesicles during Passive Binding of Ca^{2+}. (*a*) *Removal of Endogenous Ca^{2+}.* The SR vesicles were washed and suspended at a concentration of 10–15 mg of protein per mL in 80 mM KCl, 5 mM $MgCl_2$, and 2 mM MoPS (pH 6.5). Three to five milliliters of the suspension was layered on top of a Chelex X-100 column (1 × 40 cm) which had been preequilibrated at room temperature with the same medium. After elution (150–200 mL/h), the vesicles were collected and diluted to 2–3 mg/mL and the pH was adjusted to 6.0. Total Ca^{2+} contamination in the untreated SR suspension was 15–40 nmol of Ca^{2+} per mg of protein, as determined by atomic adsorption spectroscopy. The removal of endogenous Ca^{2+} was checked by adding radioactive-labeled ^{45}Ca to the vesicles previous the the Chelex treatment. After chromatography the residual Ca^{2+} was always less or equal to 0.5 nmol/mg of protein.

(*b*) *Titration.* Eight to ten milliliters of the Chelex X-100 treated SR vesicles was kept at room temperature under a N_2 atmosphere. A trace amount of ^{45}Ca was added to the stirred suspension, and the pH was kept at 6.0 with the use of a pH stat (Metrohm, Herisau). Known aliquots of $CaCl_2$ (usually 5 or 10 μL of a 5 mM $CaCl_2$ solution) were added sequentially to the suspension. After each addition the H^+ released was titrated automatically with 1 mM NaOH. Thereafter, aliquots of 500 μL were withdrawn and filtered. The radioactivity contained in nonfiltered and filtered suspensions was used to calculate the amount of Ca^{2+} free or bound to the SR vesicles after each addition of Ca^{2+}.

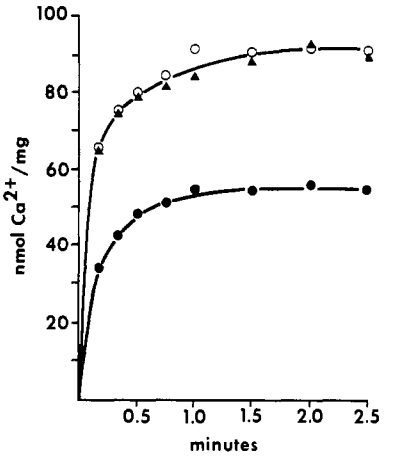

FIGURE 1: Mg^{2+} and Mn^{2+} stimulation of Ca^{2+} translocation by SR vesicles. The reaction mixture contained 80 mM KCl, 20 mM Mops, pH 6.9, 100 μM [^{45}Ca]$CaCl_2$, 220 μg of SR per mL (●), and 0.5 mM $MgCl_2$ (○) or 0.5 mM $MnCl_2$ (△). After 10 min preincubation at room temperature, the uptake reaction was initiated by the addition of 0.5 mM ATP. ^{45}Ca translocation was determined with the Millipore filtration technique.

Results

Mn^{2+} Fluxes. An important suggestion regarding charge displacement by the Ca^{2+} pump of SR is that Ca^{2+} may be exchanged for Mg^{2+} in each cycle of active transport (Kanazawa et al., 1971). This suggestion is based on a Mg^{2+} requirement for hydrolytic cleavage of a phosphorylated enzyme intermediate, which is a recycling step for the ATPase reaction coupled to the Ca^{2+} pump. However, direct evidence is lacking, as measurement of Mg^{2+} fluxes under conditions relevant to Ca^{2+} transport is quite difficult. For this reason, we have replaced Mg^{2+} with Mn^{2+} since the latter is available in the form of a convenient radioactive isotope and is known to activate Ca^{2+} transport in place of Mg^{2+} (Yamada & Tonomura, 1972).

Figure 1 shows the time dependency of the Ca^{2+} translocation by the SR vesicles in the presence of ATP. The contaminant Mg in the reaction mixture is 5–10 μM, as determined by atomic adsorption spectroscopy. When the reaction mixture is supplemented with 0.5 mM $MnCl_2$, the Ca^{2+} uptake reaction occurs within a time and with a magnitude which are similar to those observed in the presence of 0.5 mM Mg. Therefore, Mn^{2+} is a good activator of the Ca^{2+} pump, in analogy with Mg^{2+}.

On the other hand, in the presence of limiting calcium concentrations, an ATP-dependent Mn^{2+} uptake by the SR vesicles can be clearly demonstrated (Figure 2). The maximal level of Mn^{2+} retained by the SR vesicles is 100–150 nmol/mg, and most of it is rapidly released after addition of the ionophore A 23187 (Figure 2), indicating the formation of a Mn^{2+} concentration gradient across the membrane. Figure 2 also presents the passive diffusion of Mn^{2+} into the SR vesicles and shows that a 10-min preincubation time at 25 °C is sufficient

[1] Abbreviations used: Mops, 3-(*N*-morpholino)propanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; FCCP, carbonyl cyanide *m*-fluorophenylhydrazone.

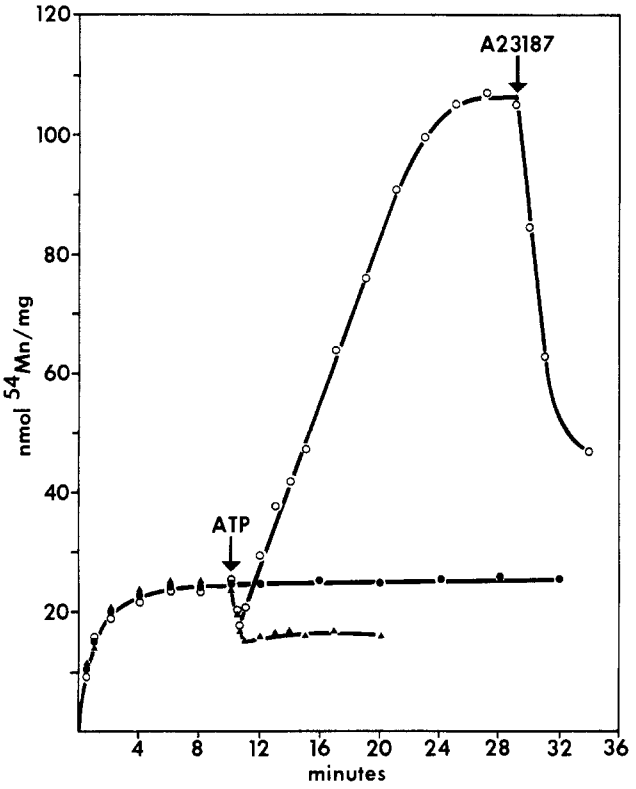

FIGURE 2: ATP-dependent Mn^{2+} fluxes through SR membranes. SR vesicles were added to the reaction mixture at a final concentration of 1.1 mg/mL. The reaction mixture contained 80 mM KCl, 1 mM [^{54}Mn]$MnCl_2$, 20 mM Mops, pH 6.9, and 100 μM added $CaCl_2$ (● and ▲) or no added $CaCl_2$ (○). In the latter case, the total contaminant Ca^{2+} was 14 μM. The figure shows the passive influx of Mn^{2+} into the SR vesicles (●). For the other curves presented, 1 mM ATP was added after 10 min (▲ and ○). The ionophore A 23187 (15 μM) was added where indicated. ^{54}Mn retained by the microsomes was determined by the Millipore filtration technique.

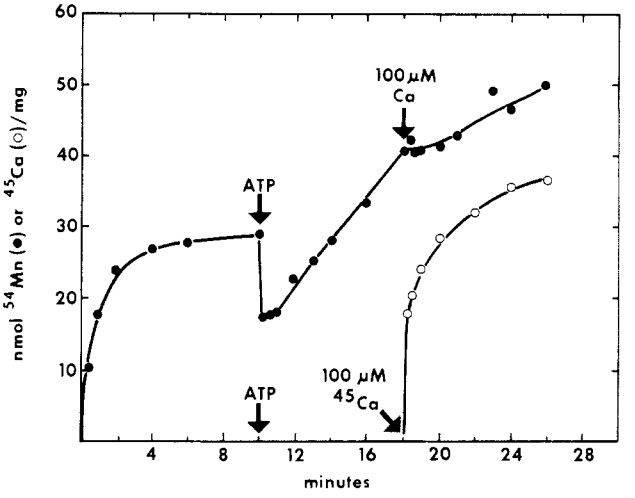

FIGURE 3: Effect of Ca^{2+} uptake on Mn^{2+} content of microsomes. The experiment was carried out essentially as described in Figure 2 with no Ca^{2+} added to the reaction mixture. After 10 min 2.5 mM ATP was added. 8 min after ATP addition, 100 μM cold $CaCl_2$ was added. The ^{54}Mn content of the vesicles was followed throughout the experiment (●). Ca^{2+} movements (○) were followed under identical experimental conditions with no radioactive labeled ^{54}Mn. At the time indicated 100 μM [^{45}Ca]$CaCl_2$ was added.

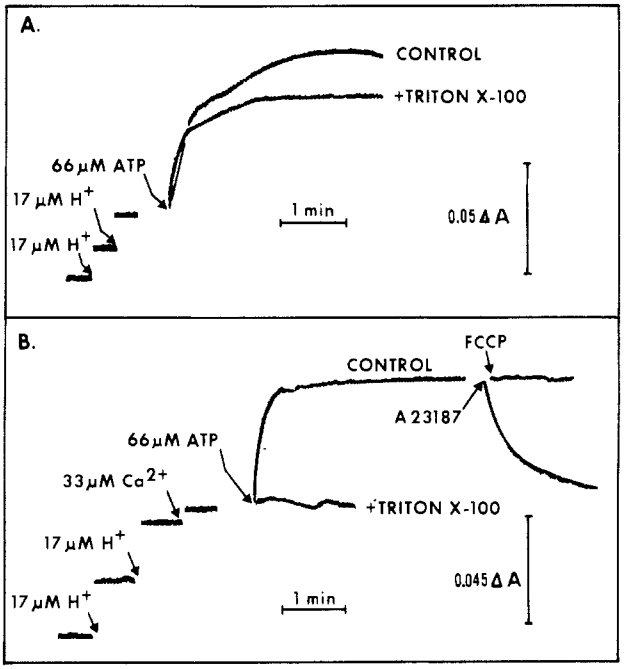

FIGURE 4: pH changes in the reaction medium during Ca^{2+} translocation. (A) Composition of the medium: 80 mM KCl, 2 mM Mops, pH 6.9, 5 mM $MgCl_2$, 100 μM phenol red, 1 mg of SR per mL, 50 μM $CaCl_2$. Additions were made as indicated in the figure. pH changes were detected as described under Materials and Methods with a dual-wavelength spectrophotometer. Wavelength pair was set at 560 and 600 nm. (B) Composition of the medium: 80 mM KCl, 2 mM Mes, pH 6.1, 5 mM $MgCl_2$, 40 μM chlorphenol red, 1 mg of Sr per mL, 50 μM $CaCl_2$. Wavelength pair was set at 488 and 577 nm. When required, the vesicular suspension was preincubated for 5 min in the presence of 0.1% Triton X-100. The final concentration of the ionophores A 23187 and FCCP was 20 and 5 μM, respectively.

to obtain equilibration of the vesicles with Mn^{2+}. It is then apparent that in addition to being an activator of the calcium pump Mn^{2+} is also a weak competitor of calcium for the transport sites and can be actively transported in place of Ca^{2+}, even though at slower rates.

It should be noted that addition of ATP is immediately followed by rapid release of a small amount of Mn^{2+} from the SR vesicles (Figures 2 and 3). Even in the absence of added calcium, addition of ATP produces a similar release of Mn^{2+}, which is followed by the slower uptake of Mn^{2+}. If Ca^{2+} is than added a few minutes after ATP, a burst of Ca^{2+} uptake is noted, which is not accompanied by release of Mn^{2+} (Figure 3). Therefore, the Mn^{2+} release which is observed on addition of ATP is not quantitatively or causually related to the uptake of Ca^{2+}. Rather, it is likely that Mn^{2+} binding by the nucleotide is followed by reduction of the free Mn^{2+} concentration in the medium and dissociation of the divalent cation from weak binding sites on the SR vesicles. The presence of Mn^{2+} binding sites was previously reported by Kalbitzer et al. (1978).

Our experiments with Mn^{2+} show that this divalent cation can replace Mg^{2+} as an activator of the Ca^{2+} pump but that active transport of Ca^{2+} into the vesicles is not accompanied by a corresponding ejection of Mn^{2+}. Furthermore, Mn^{2+} is a weak competitor of Ca^{2+} for the transport sites.

H^+ Fluxes. H^+ production in a reaction mixture containing SR vesicles and the required components for Ca^{2+} transport may be related directly to the hydrolytic ATPase reaction and/or to a transport mechanism including H^+ ejection from the SR vesicles. The amount of H^+ generated by the hydrolytic reaction is dependent on the pH of the medium and the pK for H^+ dissociation from the reaction products (Nishimura et al., 1962). This amount can be estimated empirically by measuring the H^+ production corresponding to hydrolysis of a known aliquot of ATP in the presence of vesicles rendered leaky with Triton X-100. Under these conditions, we found that the H^+/ATP molar ratio is ~0.7, at pH 6.9. However,

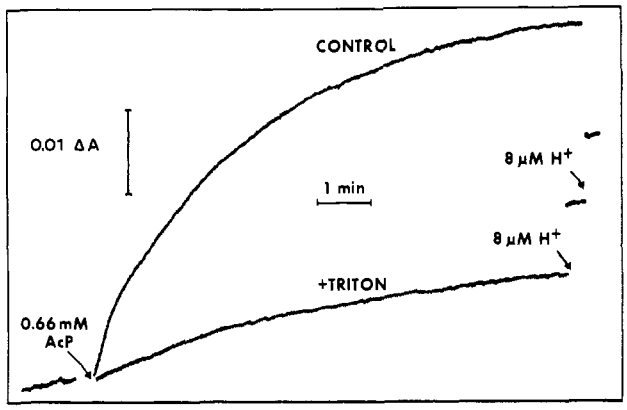

FIGURE 5: H^+ efflux from SR vesicles during AcP-supported Ca^{2+} uptake. The experiment was carried out as described in Figure 4B. Reaction mixture: 80 mM KCl, 2 mM Mes, pH 6.0, 100 μM $CaCl_2$, 0.66 mM $MnCl_2$, 40 μM chlorphenol red, 1 mg of SR per mL. Wavelength: 488 and 577 nm. When present, Triton X-100 was 0.1%.

when intact vesicles are used an excess H^+ production is noted (Figure 4A).

A better demonstration of the difference between *intact* and *leaky* vesicles is obtained by lowering the pH of the medium (pH 6.1), thereby preventing H^+ dissociation from the product of the ATPase hydrolytic reaction. In this case, no significant H^+ production is noted in the presence of *leaky* vesicles on addition of ATP, while 35–40 nmol of H^+ per mg of protein was produced in the presence of *intact* vesicles (Figure 4B). It is noteworthy that the observed pH drop reverts (Figure 4B) to the original base line by addition of a Ca^{2+} ionophore (A 23187) and collapse of the Ca^{2+} gradient formed in the presence of ATP (Scarpa et al., 1972). On the contrary, no reversal is obtained by addition of a H^+ ionophore (FCCP).

These experiments indicate that the observed H^+ ejection is not primary but occurs as a consequence of ATP-dependent Ca^{2+} accumulation by the SR vesicles.

Proton ejection from the SR vesicles can be observed by using a variety of substrates for the ATPase reaction (e.g., ATP, ITP, or AcP) and in the presence of Mg^{2+} or Mn^{2+} as activators as long as the hydrolytic activity is coupled with Ca^{2+} accumulation by the SR vesicles. In fact, if AcP is used in place of ATP, a better time resolution of Ca^{2+} and H^+ fluxes is obtained, owing to the slow rates of AcP utilization. It is noteworthy that during our experimentation with AcP, we found a more rapid utilization of this particular substrate when Mn^{2+}, rather than Mg^{2+}, was used as an activator.

H^+ production following addition of AcP to *leaky* or *intact* vesicles is shown in Figure 5. Here again, H^+ dissociation from the product of the hydrolytic reaction is minimal at pH 6.0. Therefore, H^+ dissociation from SR vesicles which are permitted to accumulate Ca^{2+} can be clearly demonstrated.

The initial rates of H^+ ejection and Ca^{2+} transport in the presence of AcP can be increased by raising the Mn^{2+} concentration (Figure 6). In these experiments we never noted an initial burst or a lag period of H^+ ejection relative to Ca^{2+} transport. Rather, the two fluxes appeared to proceed simultaneously, although the rates of net H^+ ejection were generally lower than those of Ca^{2+} uptake.

Time resolution of H^+ ejection in the presence of ATP required the use of stopped-flow methods, owing to the rapid rates obtained with this substrate. H^+ production (at pH 6.1) following addition of ATP to leaky (a) and intact (b) vesicles is shown in Figure 7. The two curves are then substracted to yield trace c which corresponds to actual ejection of H^+. A comparison of trace c with a time curve of Ca^{2+} uptake reveals that the two phenomena occur simultaneously and the

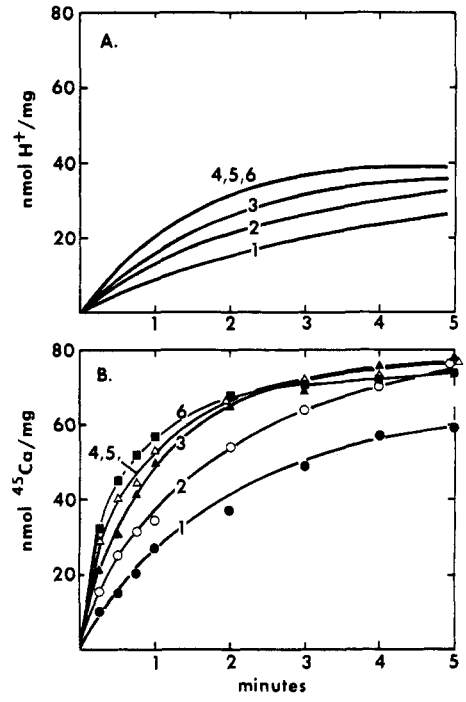

FIGURE 6: Mn^{2+} dependency of H^+ efflux and Ca^{2+} uptake in the presence of AcP. (A) H^+ fluxes were measured as described in Figure 5 in a medium containing 80 mM KCl, 2 mM Mes, pH 6.0, 40 μM chlorphenol red, 0.5 mg of SR per mL, and 77 μM $CaCl_2$. The concentration of $MnCl_2$ was (1) 83, (2) 166, (3) 333, (4) 666, (5) 1330, and (6) 3330 μM. Reactions were started by the addition of 0.66 mM AcP. At each $MnCl_2$ concentration H^+ fluxes were measured in the presence and in the absence of Triton as shown in Figure 5. The difference of the curves thus obtained was calculated and presented in the Figure. (B) The experimental conditions were the same as described under (A). However, ^{45}Ca tracer was added and Ca^{2+} uptake was measured with the Millipore filtration technique.

H^+/Ca^{2+} molar ratio is ~1 at the beginning of the reaction.

Independent of the substrate used, the maximal quantity of ejected H^+ is 35–40 nequiv/mg of protein. It should be noted, however, that when total Ca^{2+} uptake is varied by limiting the amount of calcium which is available in the reaction mixture, the net amount of ejected H^+ also varies (Figure 8). In fact, even the molar ratio between net H^+ ejection and Ca^{2+} uptake is variable, being ~1 at low levels of Ca^{2+} uptake and lower than 1 at higher levels of Ca^{2+} uptake.

H^+ Released during Titration of the High-Affinity Binding Sites with Ca^{2+}. It has been previously reported that passive binding of Ca^{2+} to various classes of sites in SR vesicles results in the release of as much as 150 nmol of H^+ per mg of protein (Carvalho, 1972). More specifically, it is known that pH can affect Ca^{2+} translocation by shifting the K_m for the Ca^{2+}–enzyme complex (Verjovski-Almeida & de Meis, 1977). In addition, the apparent Ca^{2+} binding constant to the high-affinity binding sites of the ATPase molecules decreases with pH (Meissner, 1973). Therefore, we attempted to investigate if the pH effects are due to actual exchange of H^+ with Ca^{2+} ions in the transport sites. Figure 9 presents the titration of the high-affinity binding sites of the SR vesicles with Ca^{2+} while the pH was kept constant at 6.0. It can be seen that along with the saturation of the binding sites with Ca^{2+} a concomitant release of H^+ occurs. At pH 6.0 the ratio of Ca^{2+} bound to H^+ liberated is ~1. It should be pointed out that the apparent K_d and the total amount of the Ca^{2+} binding sites [(1–2) × 10^{-5} M and 8–11 nmol of Ca^{2+} per mg, respectively] obtained in Figure 9 correspond very closely to those values obtained at pH 6.0 in independent experiments using equi-

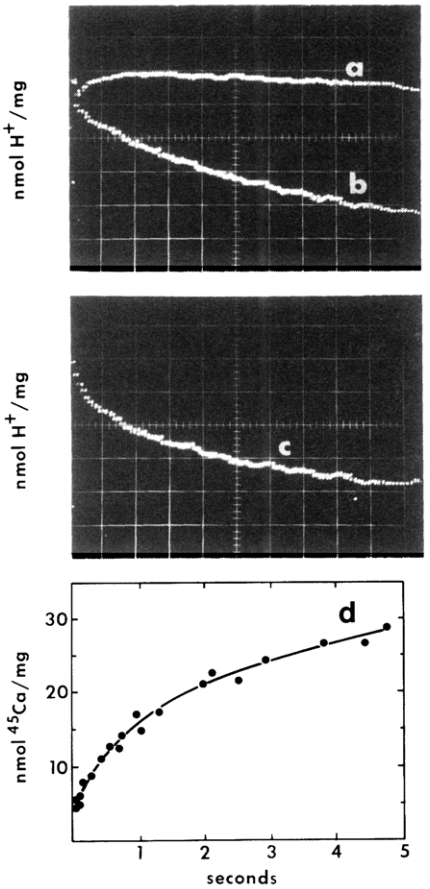

FIGURE 7: Rapid kinetics of Ca^{2+} and H^+ movements through SR membranes upon addition of ATP. The reaction medium contained 80 mM KCl, 5 mM $MgCl_2$. 1.5 mM Mes, pH 6.1, 40 μM chlorphenol red, 100 μM $CaCl_2$, and 1 mg of SR per mL. Final ATP concentration was 250 μM. Details of the experimental procedure are given under Materials and Methods. Proton fluxes (a–c) were carried out with the aid of a Dionex D-137 dual-detector stopped-flow spectrophotometer (wavelenght pair set at 577 and 630 nm) interfaced to a Northstar Horizon computer. ^{45}Ca uptake measurements (d) were performed on a Dionex D-133 rapid mixing apparatus using 10 mM EGTA as the quencher. (a) Proton flux obtained with leaky vesicles (0.2% Triton X-100); (b) proton flux obtained with intact SR; (c) curve obtained by subtracting (a) from (b). Divisions in the abscissa and ordinates correspond to 0.5 s and 8 nmol of H^+ per mg of protein, respectively; (d) Ca^{2+} uptake.

librium binding techniques (not shown here).

Experiments with Reconstituted SR. The functional relevance of H^+ fluxes may be evaluated by measuring Ca^{2+} transport in SR vesicles in which H^+ permeability is modified by the addition of a specific ionophore (e.g., FCCP). However, we found that FCCP does not alter the ability of the SR vesicles to eject H^+ (Figure 4) or to accumulate Ca^{2+} (Scarpa et al., 1972; Madeira, 1978) following addition of ATP or AcP. This indicates that (1) the observed H^+ ejection is not due to a primary H^+ pump since a primary H^+ gradient is expected to collapse following addition of FCCP, (2) the permeability of native SR vesicles is sufficiently high to permit a secondary H^+ ejection, and, therefore, (3) the relevance of such a H^+ ejection to Ca^{2+} transport cannot be tested by increasing the H^+ permeability of native vesicles.

We then used SR vesicles which were reconstituted with high ratios of (hexogenous) phospholipid to protein (Chiesi et al., 1978). The reconstitution process results in loss of much of the protein other than the M_r 100 000 ATPase polypeptide and a minor component of ~50 000, as demonstrated by $NaDodSO_4$ gel electrophoresis. Such reconstituted vesicles have a low passive permeability to solutes (Knowles & Racker,

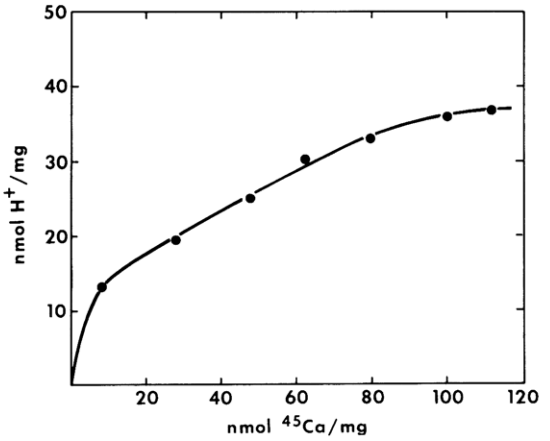

FIGURE 8: Correlation between total amount of Ca^{2+} and H^+ fluxes. The buffer consisted of 80 mM KCl, 0.66 mM $MnCl_2$, 2 mM Mes, pH 6.0, 40 μM chlorphenol red, and 0.8 mg of SR per mL. Total $CaCl_2$ concentration ranged from 8 μM up to 210 μM in separate experiments. Reactions were started by the addition of 0.66 mM AcP. Total Ca^{2+} uptake was determined in the presence of traces of ^{45}Ca with the Millipore filtration technique. Maximal H^+ efflux was calculated from the difference obtained with intact and leaky vesicles (Triton X-100) as described in Figure 6.

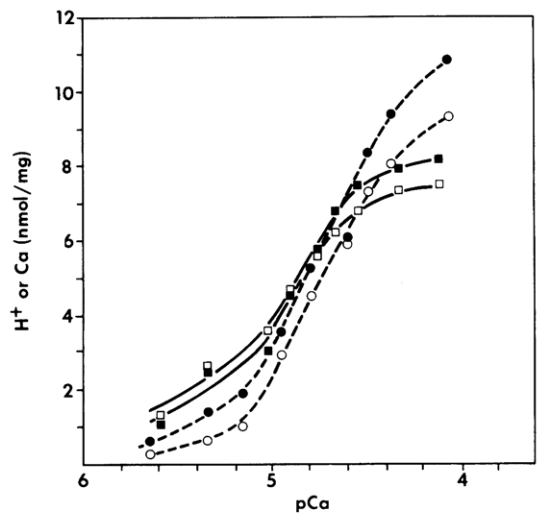

FIGURE 9: Passive Ca^{2+} binding and H^+ release by SR vesicles. The SR vesicles were treated with a chelating resin in order to remove the contaminating Ca^{2+}. The Ca^{2+} binding sites of the preparation were titrated by adding small amounts of $CaCl_2$ to 10 mL of a solution containing 80 mM KCl, 5 mM $MgCl_2$, 2 mM Mops, pH 6.0, and 2 to 3 mg of SR protein per mL. Protons released during the titration were neutralized and measured with a pH stat coupled with a syringe containing 1 mM NaOH. Ca^{2+} bound to the preparation and Ca^{2+}_{free} were determined by using ^{45}Ca and a filtration technique. Further details on the experimental procedure are described under Materials and Methods. The figure presents the results obtained with two different SR preparations (○, ● and □, ■, respectively). Full symbols correspond to Ca^{2+} binding data and empty symbols to H^+ release data.

1975) but are able to accumulate up to 130 nmol of calcium per mg of protein (Figure 10) in reaction mixtures containing ATP and no added calcium precipitating agents (e.g., oxalate). The accumulated calcium is rapidly released by addition of the calcium ionophore A 23187.

Contrary to the experiments with native SR vesicles, it can be shown that ATP-dependent Ca^{2+} uptake by reconstituted vesicles is significantly increased by raising the membrane permeability to H^+ by the addition of FCCP (Figure 10). We also obtained a higher Ca^{2+} uptake by raising membrane permeability to K^+ by the addition of valinomycin. No effect of valinomycin was noted in reaction mixtures containing Na^+

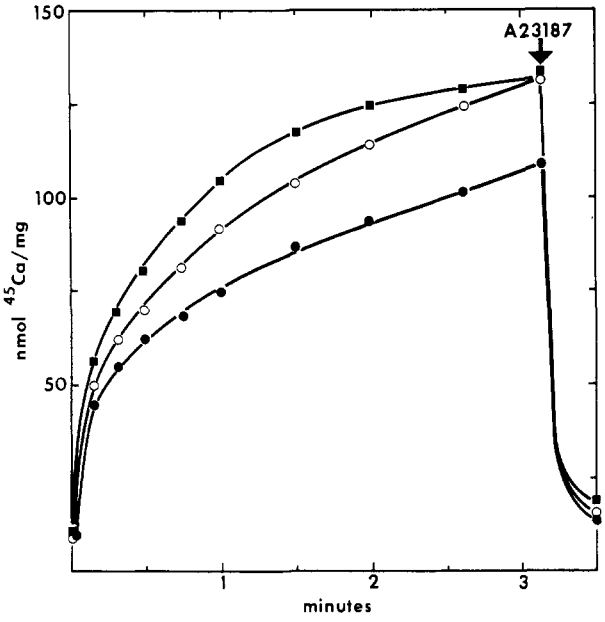

FIGURE 10: Effect of valinomycin or FCCP on Ca^{2+} uptake by reconstituted vesicles. Experiments were carried out in a medium containing 80 mM KCl, 20 mM Mops, pH 6.9, 5 mM $MgCl_2$, 100 μM $^{45}CaCl_2$ (●), and when required 2 μM FCCP (■) or 2 μg/mL valinomycin (○). Reconstituted vesicles were prepared as described under Materials and Methods. 150 μg of protein per mL was preincubated at room temperature for 10 min in the reaction medium. Ca^{2+} uptake was initiated by the addition of 1 mM ATP and determined with the Millipore filtration technique. The concentration of the Ca^{2+} ionophore A 23187 was 20 μM. Valinomycin had no noticeable effect when the medium was supplemented with 80 mM NaCl instead of KCl.

in place of K^+. The valinomycin effect was explained by Zimniak & Racker (1978) by postulating that the Ca^{2+} pump is electrogenic and that the limit imposed by charge displacement is offset by passive K^+ fluxes in the opposite direction.

In agreement with the observations of Zimniak & Racker (1978) and with our own observations on FCCP, we found that the valinomycin effect can be demonstrated much more clearly on reconstituted vesicles than on native vesicles. In this respect, the difference between the two systems may be related to the presence of passive channels for monovalent cations (Miller, 1978; McKinley & Meissner, 1978) in the native membrane which are not incorporated efficiently in the reconstituted vesicles.

Discussion

The experiments reported here were designed to find out whether cation efflux from SR vesicles occurs after addition of ATP in relation to inward Ca^{2+} transport.

First the hypothesis of a Ca^{2+}–Mg^{2+} antiport was tested by measuring fluxes of a radioactive Mn^{2+} isotope. Originally, this hypothesis was based on the Mg^{2+} requirement (Inesi et al., 1970; Kanazawa et al., 1971) for the hydrolytic step of SR ATPase, even though Mg^{2+} fluxes were never measured under relevant conditions.

We found that Mn^{2+} is able to function quite adequately as an ATPase activator in place of Mg^{2+}. A hypothetical Ca^{2+}–Mn^{2+} exchange mechanism implies that the Mn^{2+} efflux from the SR vesicles is of the same order of magnitude as the Ca^{2+} translocation in the opposite direction. Measurements of the Mn^{2+} efflux rate and magnitude could be obscured by a rapid recycling of the cation via either passive diffusion or active translocation inside the SR vesicles. However, these possibilities are excluded by the experiment presented in Figure 3 since (a) an inside/outside concentration gradient of Mn^{2+} was produced by active transport *before* the addition of Ca^{2+}, so that Mn^{2+}–Ca^{2+} exchange would be favored during Ca^{2+} transport, and (b) ATP-dependent Mn^{2+} uptake by the microsomes was negligible in the presence of 100 μM Ca^{2+} in the extravesicular medium (Figure 2), especially if compared with the rapid rate of the Ca^{2+} translocation (Figures 1 and 3). The results presented in Figure 3, therefore, clearly show that ejection of Mn^{2+} from the SR vesicles does not occur concomitantly with ATP-dependent Ca^{2+} uptake.

If the experiments on Mn^{2+} can be extrapolated to Mg^{2+}, our observations do not support the hypothesis of a Ca^{2+}–Mg^{2+} antiport in the Ca^{2+} pump of SR.

A second line of experimentation was directed to study H^+ fluxes. Ejections of H^+ from SR vesicles following addition of ATP was first reported by Madeira (1978, 1979), who attributed this phenomenon to a primary H^+ pump and implied that Ca^{2+} accumulation by SR is secondary to a H^+ gradient.

With our experiments we were able to confirm that, in addition to H^+ dissociation from the products of the hydrolytic reactions, a significant amount of H^+ is ejected from SR vesicles during Ca^{2+} transport. However, kinetic resolution of H^+ ejection shows that this phenomenon never precedes Ca^{2+} uptake. Furthermore, addition of FCCP, which is expected to increase passive membrane permeability to H^+ and to collapse a primary H^+ gradient, does not alter either H^+ ejection or Ca^{2+} accumulation in the presence of ATP. On the other hand, collapse of the Ca^{2+} gradient by addition of a Ca^{2+} ionophore reverses the H^+ ejection. Therefore, our observations indicate that H^+ efflux from SR vesicles cannot be attributed to a primary H^+ pump but is rather a secondary phenomenon occurring as a consequence of ATP-dependent Ca^{2+} uptake.

The increased Ca^{2+} uptake observed with reconstituted vesicles in the presence of FCCP suggests that H^+ ejection plays some role during active transport of Ca^{2+}. In this regard, the analogous effect of valinomycin (Zimniak & Racker, 1978) indicates that efflux of K^+ and/or H^+ may counterbalance, at least in part, charge displacement by the Ca^{2+} pump. It is also likely that anion (Cl^-) influx also contributes to offsetting charge displacement by the pump, since the SR membrane appears to be significantly permeable to monovalent cations and anions (McKinley & Meissner, 1978; Kometani & Kasai, 1978).

An intriguing question is whether H^+ ejection occurs through a specific mechanism directly linked to the Ca^{2+} pump in addition to diffusion passively through independent channels. In this regard, the parallel kinetics and constant stoichiometry observed within the first second of reaction (Figure 7) suggest that H^+ is involved in an exchange reaction with Ca^{2+}. In fact, H^+ exchange with Ca^{2+} is commonly observed in Ca^{2+} binding reactions of several organic molecules. Specifically, Figure 9 shows that the binding of Ca^{2+} to the activating (e.g., transport) sites of SR ATPase displaces H^+.

To account for a cyclic mechanism of vectorial transport, H^+ should be acquired by the transport site when Ca^{2+} is released and the site is in a low-affinity state which is exposed to the inner side of the vesicles. H^+ would then be released when the site is in a state of high affinity for Ca^{2+} on the outer surface of the vesicles.

On the assumption that the binding sites acquire H^+ upon dissociation inside the vesicles, the stoichiometry of protonation depends on the pK of the sites and the intravesicular pH. On the basis of the measurements obtained within the first second of reaction (Figure 7) and also supported by the titration

results (Figure 9), it appears that even at pH 6.1 ~1 H^+ exchanges for 1 Ca^{2+}, and therefore the pump is still electrogenic if not compensated for by passive fluxes of permeable electrolytes such as K^+ (Zimniak & Racker, 1978) and/or Cl^-.

As Ca^{2+} accumulation proceeds, the stoichiometry of *net* H^+ ejection decreases (Figure 8), probably due to reentry of H^+ into the vesicles through independent channels.

Under conditions permitting maximal Ca^{2+} uptake (120–180 nmol of calcium per mg of protein), maximal (*net*) ejection of H^+ is 35–40 nmol/mg of protein. On the assumption that before addition of ATP the intravesicular pH is approximately equal to that of the medium (pH 6 to 7) (Nomura & Nakamaru, 1976) and that the intravesicular volume is 5–7 μL/mg of protein (Duggan & Martonosi, 1970), the amount of free H^+ inside the vesicles should not exceed a few picomoles. Therefore, H^+ ejection of the observed magnitude requires H^+ dissociation from buffering systems or divalent cation binding sites (Carvalho, 1972). The observed reduction of H^+/Ca^{2+} ratios in the latter part of the reactions implies that a significant amount of H^+ is recycled after reentering the vesicles through independent channels. This explains the stimulating effect of FCCP on the Ca^{2+} translocation of reconstituted vesicles which have a limited permeability for reentry of H^+ and are deprived of calcium binding proteins (other than ATPase) which may be a significant source of H^+ in native vesicles.

A remarkable feature of the proposed mechanism is that it provides H^+ for a H^+–Ca^{2+} antiport, while a rapid reduction of the intravesicular pH is avoided by buffering systems and recycling pathways.

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