VOLTAGE-DEPENDENCE OF Ca^{2+} UPTAKE AND ATP HYDROLYSIS OF RECONSTITUTED Ca^{2+}-ATPASE VESICLES

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ABSTRACT Ca^{2+}-ATPase from sarcoplasmic reticulum was reconstituted into phospholipid/cholesterol (9:1) vesicles (RO). Sucrose density gradient centrifugation of the RO vesicles separated a light layer (RL) with a high lipid/protein ratio and a heavy layer (RH). RH vesicles exhibited a high rate of Ca^{2+}-dependent ATP hydrolysis but did not accumulate Ca^{2+}. RL vesicles, on the other hand, showed an initial molar ratio of Ca^{2+} uptake to ATP hydrolysis of ~1.0. Internal trapping of transported Ca^{2+} facilitated studies over periods of several minutes. Ca^{2+} transport and ATP hydrolysis declined concomitantly, reaching levels near 0 with external Ca^{2+} concentrations ≤ 2 μM. Ca^{2+} uptake was inhibited by the Ca^{2+} ionophore A23187, the detergent Triton X-100, and the metabolic inhibitor quercetin. Ca^{2+} transport generated a transient electrical potential difference, inside positive. This finding is consistent with the hypothesis that the Ca^{2+} pump is electrogenic. Steady state electrical potentials across the membrane were clamped by using potassium gradients and valinomycin, and monitored with voltage-sensitive dyes. Over a range of +50 to −100 mV, there was an inverse relationship between the initial rate of Ca^{2+} uptake and voltage, but the rate of ATP hydrolysis was nearly constant. In contrast, lowering the external Ca^{2+} concentration depressed both transport and ATP hydrolysis. These findings suggest that the membrane voltage influences the coupling between Ca^{2+} transport and ATP hydrolysis.

INTRODUCTION

Despite the broad significance of the energetics of transport processes in biological membranes, the nature of the dependence of these processes on the forces promoting transport is not known. The resolution of this problem has not yet been possible because a suitably simple system, which would permit the simultaneous control and measurement of all pertinent flows and forces, has not been developed (Caplan and Essig, 1983).

One approach to this problem is the study of a single transport mechanism in a reconstituted system. One suitable system is the Ca^{2+}-ATPase of sarcoplasmic reticulum (SR) from skeletal muscle, since this has been intensively studied and characterized with respect to methodology, structure, and function (Tada et al., 1978; Hasselbach, 1978). Previous studies (Hasselbach, 1964; Martonosi and Feretos, 1964; Weber, 1966) have demonstrated that the Ca^{2+}-ATPase couples the hydrolysis of ATP to the translocation of Ca^{2+}. Transport both in SR vesicles and in reconstituted vesicles is associated with generation of an electrical potential difference (inside positive), which appears to influence the rate of transport (Zimniak and Racker, 1978; Akerman and Wolff, 1979; Beeler, 1980). Accordingly, the study of such systems should facilitate understanding of mechanisms of electrogenic active transport. Of particular interest are the changes in relative rates of Ca^{2+} transport and ATP hydrolysis (Ca^{2+}/ATP ratios), which have been reported in different preparations and under differing experimental conditions, e.g., variation of pH and modification of membrane aminophospholipids (Hidalgo et al., 1982; Rossi et al., 1979; Berman, 1982; Navarro et al., 1984). However, it is not yet known to what extent these changes in function reflect a change in the intrinsic mechanism of active Ca^{2+} transport as opposed to effects on membrane leakiness or other as yet poorly defined factors.

Here we describe the preparation and use of a relatively pure and nonleaky reconstituted Ca^{2+}-ATPase vesicle system that should prove useful in the resolution of these issues. Under the conditions of our experiments we find the electrical potential has an important influence on the rate of Ca^{2+} transport but has no demonstrable affect on the rate of ATP hydrolysis.

METHODS

New Zealand white rabbits were obtained from Margaret's Home Farm (Athol, MA). Octylglucoside was a gift of Dr. C. Miller (Brandeis University, Waltham, MA). Egg phosphatidylcholine was obtained from Lipid Products (South Nutfield, England). Cholesterol was purchased from Nu Check Prep, Inc. (Glysian, MN). Phosphoenolpyruvate, lactic dehydrogenase, pyruvate kinase, β-NADH, ATP, ADP, murexide, EGTA, phosphorus standard solution, DL-dithiothreitol, quercetin, Triton X-100, and valinomycin were obtained from Sigma Chemical Co. (St. Louis, MO). A23187 was purchased from Calbiochem-Behring Corp.

(LaJolla, CA). [2-^{3}H(N)]D-glucose and $^{45}CaCl_2$ were obtained from New England Nuclear Corp. (Boston, MA). Oxanol VI was purchased from Molecular Probes, Inc. (Plano, TX); 3,3'-diethoxydicarbocyanine was a gift of Dr. A. Waggoner (Carnegie Mellon University, Pittsburgh, PA). All other reagents were commercial products of guaranteed grade.

Preparation of Ca^{2+}-ATPase

Albino male rabbits (5–6 lbs) were killed and the back and hind leg muscles were removed and placed in ice. SR vesicles were prepared according to the method described by Fernandez et al. (1980). Ca^{2+}-ATPase was prepared by the octylglucoside method as described by Banerjee et al. (1979) SR vesicles were suspended in 20 mM Tris-HCl (pH 8.0), 0.5 M KCl, and 0.2 M sucrose at a final concentration of 5 mg protein/ml. Octylglucoside was added to a final concentration of 22 mM. This mixture was incubated at 0°C for 45 min and centrifuged at 150,000*g* for 30 min. The pellet was suspended in the original volume of extraction medium and centrifuged again. The final preparation was resuspended in 0.25 M sucrose, 0.1 M KCl, 10 mM Tris-HCl (pH 8.0), and the purified Ca^{2+}-ATPase was rapidly frozen under liquid nitrogen and kept at −70°C. The Ca^{2+}-dependent ATP hydrolysis of the purified Ca^{2+}-ATPase at 37°C was 20–30 μmol of phosphate liberated × min^{-1} × mg $protein^{-1}$.

Reconstitution of Ca^{2+} ATPase

Ca^{2+}-ATPase was reconstituted into artificial vesicles according to the freeze–thaw-sonication method (Kasahara and Hinkle, 1977). Egg phosphatidylcholine and cholesterol were combined at a weight ratio of 9:1 in a mixture of chloroform/methanol at a volume ratio of 2:1. Lipids were dried under a stream of nitrogen, lyophilized for 10 h, suspended in 0.4 M K^+, Na^+-phosphate buffer (pH 7.5) at 20 mg/ml, and vigorously vortexed. The white suspension was sonicated to clarity with a probe-type sonicator under nitrogen at 2°C. This solution was centrifuged at 150,000*g* for 30 min to remove titanium particles. The phospholipid-cholesterol vesicles were then mixed with the purified Ca^{2+}-ATPase at a weight ratio of 40 in a solution containing 0.4 M K^+, Na^+-phosphate buffer (pH 7.5), quickly frozen in a dry ice/acetone bath, and kept at −70°C until ready for use. (The internal phosphate concentration was set at 0.4 M in an attempt to assure a high capacity for Ca^{2+}, with conversion of transported Ca^{2+} into Ca phosphate, thereby minimizing the generation of large Ca^{2+} gradients across the membrane.) The frozen mixture was then thawed at room temperature and the resulting cloudy solution was sonicated for 15 s to reduce the size of the liposomes. The resultant reconstituted phospholipid/cholesterol vesicles are referred to as RO. Sucrose density gradient centrifugation (15–50% sucrose, 150,000*g* for 2 h) of RO vesicles separates two bands, a light fraction (RL) and a heavy fraction (RH).

ATP Hydrolysis Assay

The standard ATPase assay was carried out at room temperature as described by Møller et al. (1980). ATP hydrolysis was determined by a coupled enzyme system, measuring the oxidation of NADH. The standard incubation system contained 20 mM Tris-MES (pH 7.5), 5 mM $MgSO_4$, 1.5 mM phosphoenolpyruvate, 0.25 mM NADH, 8 mM P_i, 45 μM $CaCl_2$, and 50 units of lactic dehydrogenase; pyruvate kinase was added until the rates of oxidation of NADH became constant (50–150 units). The external medium was adjusted to 1 OsM with sorbitol. The ionic strength (μ) of the external medium was controlled by addition of K-MES or Na-MES. The reaction was started by addition of ATP or enzyme. Except when specified otherwise, the ATP concentration was 50 μM. The complete incubation system contained 10 μg of protein of reconstituted vesicles per 2.5 ml. Ca^{2+}-dependent ATP hydrolysis was determined as the difference between total ATP hydrolysis and ATP hydrolysis in the presence of 1 mM EGTA. Changes in the absorbance of NADH ($A_{400\,nm} - A_{340\,nm}$) were calibrated with ADP. (A unit of activity is defined as the amount of enzyme that hydrolyzes 1 μmol/min of ATP.)

Ca^{2+} Uptake Assay

Ca^{2+} uptake was determined according to the murexide optical method described by Inesi and Scarpa (1972). Ca^{2+} uptake was monitored by following absorbance changes of murexide, ($A_{507\,nm} - A_{540\,nm}$), measured in a double wave-length spectrophotometer (365; Perkin-Elmer Corp., Instrument Div., Norwalk, CT). The standard incubation system was as described above for the ATP hydrolysis assay, supplemented by 100 μM murexide. The protein preparation was preincubated and the reaction was started by addition of ATP. The absorbance of murexide was calibrated with Ca^{2+}-EGTA buffers in the standard incubation system. The sensitivity of this technique limited measurements of Ca^{2+} concentration to the range ≥2 μM. In a few studies of membrane leakiness, Ca^{2+} uptake was determined by using $^{45}CaCl_2$. The extent of incorporation of ^{45}Ca into the vesicles was determined by the Dowex-Tris column method, as described by Gasko et al. (1976). Ca uptake measured by the murexide method was similar to that obtained with the ^{45}Ca incorporation.

Measurement of Electrical Potentials

Voltage-sensitive dyes were used to monitor membrane potentials across the reconstituted membranes. Oxanol VI was used to measure positive (internal) electrical potentials and 3,3'-diethoxydicarbocyanine (di-O-C_2[5]) to measure negative electrical potentials (Waggoner, 1979). The incubation system was essentially the same as the one described for Ca^{2+} uptake or ATP hydrolysis. The concentrations of potassium inside the reconstituted vesicles were 22 and 150 mM for the generation of positive and negative electrical potentials, respectively. A constant ionic strength was achieved by maintaining a total concentration of monovalent ions of 150 mM (Na-MES + K-MES = 150 mM). Oxanol VI and di-O-C_2[5] were added to the incubation system at final concentrations of 1.6–2 and 0.8 μM, respectively. The generation of electrical potentials was achieved by the use of K^+-gradients and valinomycin, or by the operation of the Ca^{2+} pump upon addition of 50 μM ATP to reconstituted vesicles. Changes in the absorbance of Oxanol VI were monitored in a double-wavelength spectrophotometer (365; Perkin-Elmer Corp., Instrument Div.) as $\Delta A \equiv \Delta(A_{650\,nm} - A_{615\,nm})$ and changes in the absorbance of di-O-C_2[5] were monitored as $\Delta A \equiv \Delta(A_{650\,nm} - A_{595\,nm})$. The magnitudes of the electrical potentials were calculated according to the Nernst equation, applied to the ratios of K^+ concentrations.

Phospholipid Determination

To determine the amount of phospholipid in reconstituted vesicles and SR vesicles, total lipid phosphate was measured by the ashing method of Ames (1966).

Determination of Vesicle Volume

The volume of reconstituted vesicles was determined according to the method described by Goldin (1977). The intravesicular volume of RO was determined by reconstitution of the Ca^{2+}-ATPase in the presence of (^{3}H)-glucose (0.4 mCi/l). Extravesicular (^{3}H)-glucose was separated from the intravesicular (^{3}H)-glucose by using a Sephadex G-50 column. The reconstituted vesicles elute in the void volume. The intravesicular volume appeared to be ~1.6 μl/mg of phospholipid.

Protein Determination

The protein concentration was determined according to the method of Lowry et al. (1951). Bovine serum albumin was used as a standard.

RESULTS

Purification of Ca^{2+}-ATPase and Reconstitution into Lipid Vesicles

Whereas SR membranes exhibited ~10–15% of Ca^{2+}-independent ATPase, octylglucoside extraction of SR vesi-

cles resulted in almost complete purification of Ca^{2+}-ATPase and negligible Ca^{2+}-independent ATPase activity. The specific activity of the purified protein was similar to that previously reported by Banerjee et al. (1979). The purified protein showed a linear time course of ATP hydrolysis, but was unable to accumulate Ca^{2+}, which suggests that these vesicles were leaky.

Ca^{2+}-ATPase reconstituted into phospholipid/cholesterol vesicles as described by Zimniak and Racker (1978) demonstrated ATP-dependent Ca^{2+} uptake and ATP hydrolysis activities with a mean initial ratio (Ca^{2+}/ATP) of ~0.3 (Table I, fraction RO). Sucrose gradient centrifugation of RO vesicles revealed two bands, consisting of RL and RH vesicles. RH vesicles demonstrated Ca^{2+}-dependent ATPase activity, but did not accumulate Ca^{2+}. RL vesicles demonstrated both Ca^{2+} uptake and Ca^{2+} dependent ATPase activity, with a mean initial ratio of ~1 (Figs. 1 and 2; Table I). These findings suggest that RO vesicles are heterogeneous, being composed of leaky and tight vesicles that can be separated by sucrose density centrifugation. Table II indicates that the lipid-to-protein molar ratio of the transporting RL vesicles is far higher than that of SR and RH vesicles.

Characterization of the Reconstituted Ca^{2+}-ATPase Vesicles

Two agents, the Ca^{2+} ionophore A23187 and the detergent Triton X-100, produced nearly complete inhibition of net Ca^{2+} uptake (Fig. 3). The effect of quercetin, an inhibitor of several ATPases (Suolinna et al., 1975), was much less marked.

Promptly following the addition of ATP, RL vesicles generated an electrical potential, with an overshoot relaxing to a steady state value (inside positive) within <1 min (Fig. 4). Qualitatively this effect was independent of the type of monovalent ion (K^+, Na^+) and the ionic strength of the incubation medium, but was not elicited in the absence

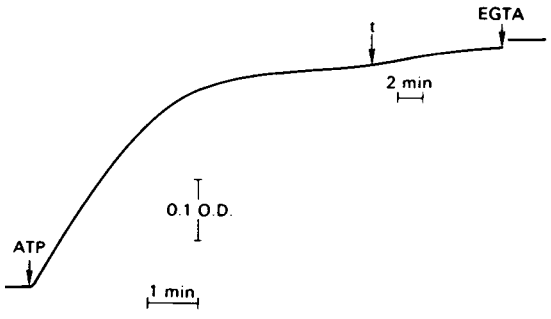

FIGURE 1 Ca^{2+}-dependent ATPase of RL vesicles. The incubation medium was as described in Methods, following ATP hydrolysis by the oxidation of NADH. The ionic strength (μ) was 0.07. The reaction was initiated by the addition of ATP to give a concentration of 0.5 mM and the ATP concentration was maintained constant by the use of a regenerating system. *t* indicates change in time scale. The concentration of EGTA was 1 mM. RL vesicles contained 0.4 M potassium phosphate. 0.1 od = 14 μM.

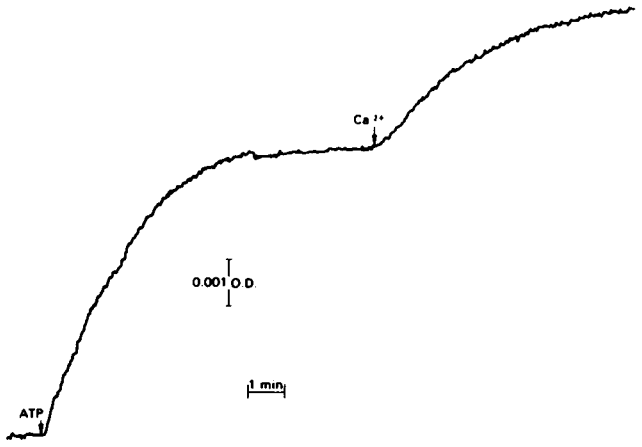

FIGURE 2 Ca^{2+} uptake by RL vesicles. Ca^{2+} uptake was monitored by changes in the absorbancy of murexide (100 μM) [$\Delta A \equiv \Delta(A_{507\,nm} - A_{540\,nm})$]. Otherwise standard incubation conditions were used as in Fig. 1, as described in Methods. The initial outside Ca^{2+} concentration was 45 μM. At the concentrations of Ca^{2+}, ATP, and murexide used, chelation of Ca^{2+} by ATP and association of Ca^{2+} with murexide are negligible. After apparent completion of Ca^{2+} uptake, with decline of Ca^{2+} to levels not accurately measurable by the murexide method (≤2 μM), the concentration was raised by 20 μM (↓), producing a downward deflection. For convenience, the od setting was readjusted at this time. 0.001 od = 7 μM.

of Ca^{2+}. These findings are consistent with previous results of others (Zimniak and Racker, 1978; Akerman and Wolff, 1979; Beeler, 1980), supporting the idea that the generation of the electrical potential results from the uptake Ca^{2+} by the vesicles.

As shown above, by means of sucrose density centrifugation it was possible to isolate a fraction of reconstituted vesicles that showed initial molar Ca^{2+}/ATP ratios of ~1 (Table I). Further analysis of the time course of Ca^{2+}-dependent ATPase and of Ca^{2+} uptake in these RL vesicles revealed, however, that the molar ratios declined progres-

TABLE I
INITIAL Ca^{2+} UPTAKE AND ATPASE ACTIVITY OF RECONSTITUTED Ca^{2+}-ATPASE VESICLES

Fraction	Initial rate of Ca^{2+} uptake	Initial rate of ATPase activity	Ratio (Ca^{2+}/ATP)‡
	$\mu mol \times min^{-1} \times mg\ protein^{-1}$		
RO (3)*	1.09	3.84	0.28
RL (4)*	3.63	3.84	0.96
RH (2)*	0	4.29	0

*Number of experiments.
‡Ca^{2+}/ATP ratios were calculated as the quotient of the initial rates of Ca^{2+} uptake and ATP hydrolysis.
The purified protein was reconstituted into artificial vesicles made of egg phosphatidylcholine/cholesterol (9:1) by the freeze-thaw method (Kasahara and Hinkle, 1977). These vesicles are referred to as RO. Sucrose density centrifugation of RO vesicles separates two bands, a light fraction (RL) and a heavy fraction (RH), comprising ~25% and 75% of the total protein, respectively. Ca^{2+} uptake was measured by using murexide as a Ca^{2+} indicator, and ATP hydrolysis was determined as described under Methods.

TABLE II
MOLAR RATIO OF LIPID TO PROTEIN IN RECONSTITUTED Ca^{2+}-ATPASE VESICLES

Preparation	Phospholipid content	Molar ratio
	mg phospholipid/ mg protein	*mol lipid/ mol ATPase*
SR	0.46	68
RO	40	5,930
RL	80	11,900
RH	2.3	341

The amounts of phospholipids and protein were determined as described in Methods. The average molecular weights for phospholipids and Ca^{2+}-ATPase were taken as 776 and 115,000 daltons, respectively.

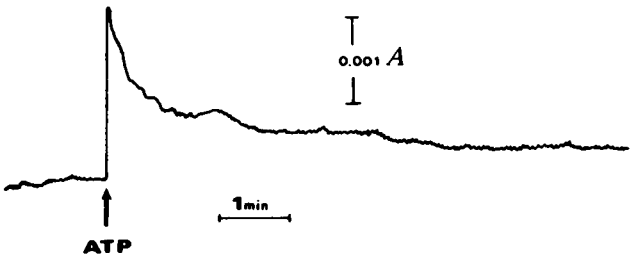

FIGURE 4 The generation of electrical potentials in RL vesicles. The intravesicular concentration of potassium [K_i^+] was 150 mM and the outside potassium concentration [K_o^+] was 3 mM. The concentration of Oxanol VI was 2 μM. Incubation conditions otherwise as described in Methods; external ionic strength μ was 0.07. The arrow indicates addition of ATP (50 μM).

sively to lower values (Fig. 5). When the external Ca^{2+} concentration fell to ≤ 2 μM, where the rate of net transport was not accurately measurable, the rate of ATP hydrolysis was very low, but demonstrably >0. On further lowering the Ca^{2+} concentration by the addition of EGTA, the rate of ATP hydrolysis became indistinguishable from 0 (Fig. 1).

In evaluating the significance of the decline of the molar ratio Ca^{2+}/ATP with time, we first considered the possibility that the vesicle transport system might have become partially inactivated during the 7–10 min of its exposure to ATP. This was ruled out by raising the medium Ca^{2+} concentration from ≤ 2 to 22 μM, after which transport increased promptly to rates observed earlier (Fig. 2).

Another possible explanation for decline of Ca^{2+}/ATP ratios was membrane leakiness. With the establishment of a large electrochemical potential difference $\Delta\bar{\mu}_{Ca}$ across the vesicle membrane, leakiness could permit passive movement of Ca^{2+} out of the vesicle at a rate approximating that of its active uptake. This seemed unlikely in view of the very high lipid-to-protein ratio (Table II), the presence of cholesterol, and the absence of detergent during reconstitution. Nevertheless, to evaluate the possible influence of leakiness more precisely, several control experiments were done. First, external Ca^{2+} concentration was monitored in the absence of ATP, with an initial external Ca^{2+} concentration of 45 μM and with the membrane electrical potential clamped at -100 mV (interior negative). In this situation, in which both concentration and electrical gradients strongly favored Ca^{2+} entry, no Ca^{2+} uptake was

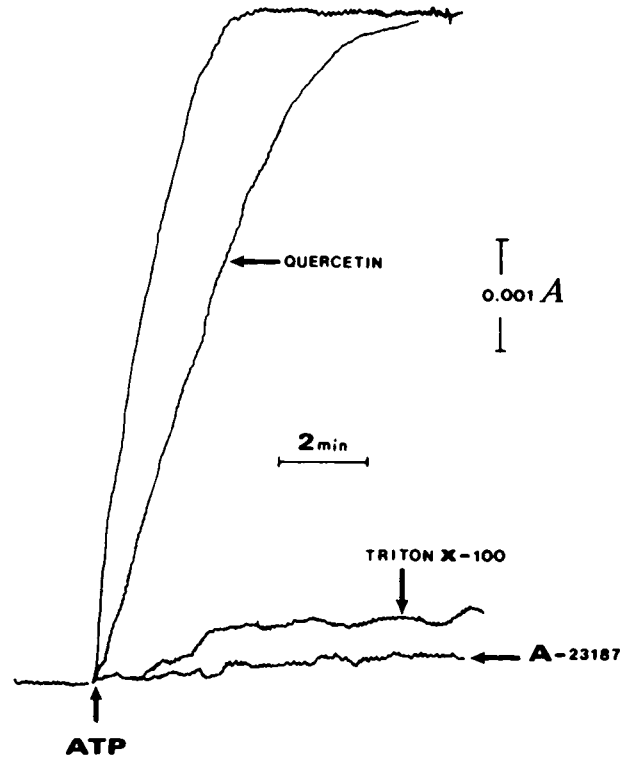

FIGURE 3 Inhibition of net Ca^{2+} uptake in RL vesicles. Ca^{2+} uptake was monitored by changes in the absorbancy of murexide (100 μM). The reaction was initiated by addition of ATP to give a concentration of 50 μM, and the ATP concentration was maintained constant by the regeneration system. The concentrations of A23187, Triton X-100, and quercetin were 5 μM, 0.02%, and 75 μM, respectively. Incubation conditions otherwise as described in Methods. 0.001 ΔA = 8 μM.

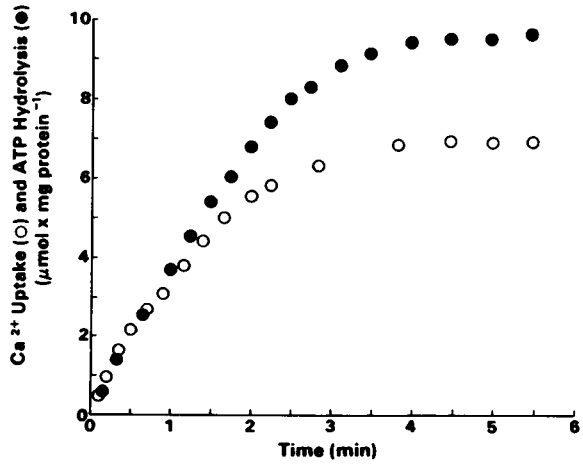

FIGURE 5 Time course of Ca^{2+} uptake and Ca^{2+}-dependent ATPase by RL vesicles. The external concentration of Ca^{2+} was 45 μM initially and ≤ 2 μM at the end of the experiment. Ca^{2+} uptake was measured by using the Ca^{2+} indicator murexide as described under Methods.

demonstrable over a period of 20 min. In the second experiment, reconstituted vesicles were loaded with ^{45}Ca in the presence of ATP. Following loading, the amount of ^{45}Ca remaining in the vesicles was determined at intervals in order to evaluate the efflux of Ca^{2+}. As is shown in Fig. 6, although in the presence of the ionophore A-23187 loss of ^{45}Ca from preloaded vesicles was marked, in the absence of the ionophore loss was slight, both in the absence and presence of ATP (200 μM). In two other studies in the presence of 2 mM ATP, efflux of ^{45}Ca from preloaded vesicles was minimal and uninfluenced by membrane potential over a range from -100 to $+50$ mV (data not shown).

Effect of Electrical Potentials on Ca^{2+} Uptake and ATP Hydrolysis

To investigate the influence of the electrical potential difference across the vesicular membranes systematically, $\Delta\psi$ was fixed by the use of K^+ gradients in the presence of valinomycin and was measured by the absorbance changes of voltage-sensitive dyes. Negative membrane potentials (interior negative) were monitored with the carbocyanine dye di-O-C_2[5]. In an initial control study, the external and internal K^+ concentrations were set at 3 and 150 mM, respectively. Addition of valinomycin elicited an increase in the optical density of the spectrum near the peak at 595 nm, but did not otherwise affect the pattern. A calibration curve was then constructed by changing the external concentration of K^+ over a range of 3 to 150 mM and recording the difference in absorbance ΔA after addition of 0.15 μM valinomycin. Assuming electrochemical equilibrium of K^+ in the presence of valinomycin and near equality of internal and external activity coefficients for K^+, this corresponds to values of $\Delta\psi$ ranging from 0 to -100 mV. As is seen in Fig. 7, a linear relationship between ΔA and $\Delta\psi$ was observed over this range.

To determine if the diffusion potentials generated by K^+ gradients in the presence of valinomycin are clamped, ATP was added to stimulate Ca^{2+} uptake. It was found that the electrical potentials are indeed maintained constant, since the absorbance change generated by the addition of valinomycin was unaffected by addition of ATP (Fig. 8).

Positive electrical potentials were monitored with Oxanol VI. Again addition of 0.15 μM valinomycin elicited an increase in the optical density of the spectrum near the peak (here 616 nm), but did not otherwise modify the spectral pattern. Again a calibration curve was constructed by varying the external K^+ concentration and observing the difference in absorbance ΔA after addition of 0.15 μM valinomycin. In this case the need to establish a well-defined potential while also maintaining constancy of ionic strength limited the range of external K^+ concentrations. Assuming equilibration of K^+, and equality of its internal and external activity coefficients, the range of 22–150 mM used corresponds to values of $\Delta\psi$ ranging from 0 to $+50$ mV. As is seen in Fig. 9, in this range the relationship between ΔA and $\Delta\psi$ was again linear. Again the electrical potential across the membrane appears to be well clamped, since ATP addition to the incubation system did not change the absorbance of Oxanol VI (not shown).

To study the influence of the electrical potential on pump function, Ca^{2+} transport and ATP hydrolysis were

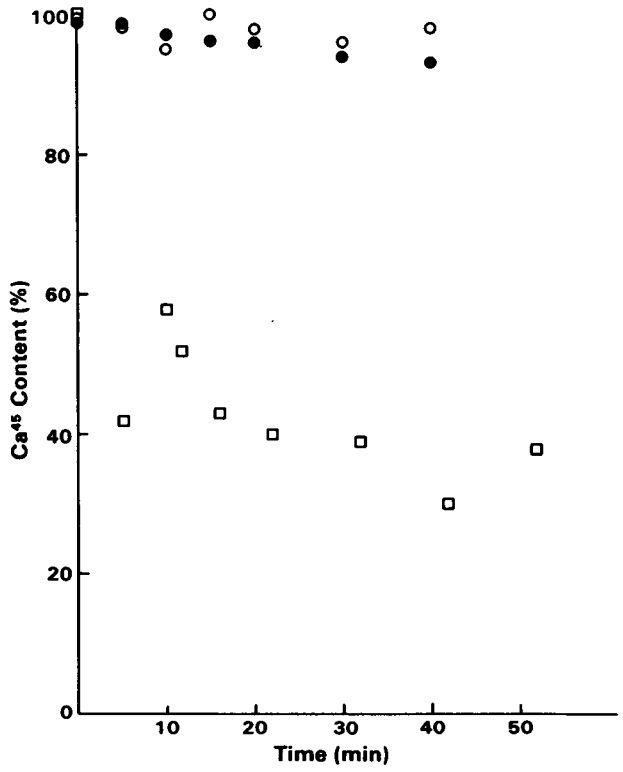

FIGURE 6 ^{45}Ca content of preloaded RL vesicles. Following loading under usual conditions for measurement of uptake, vesicles were exposed to 5 μM A-23187 (□); 200 μM ATP (○); or hexokinase and glucose to remove ATP (●).

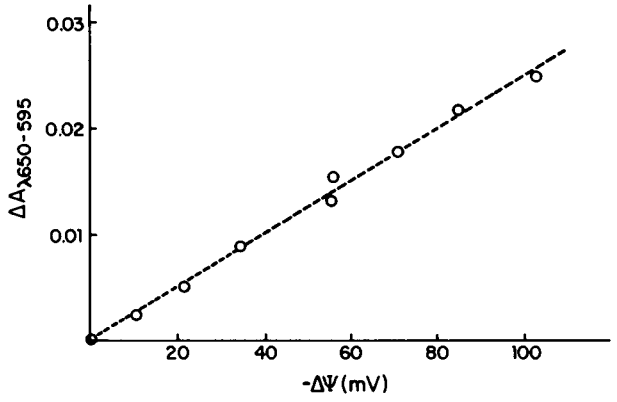

FIGURE 7 Calibration curve for negative (inside) electrical potential measurements. $[K_i^+]$ was 150 mM, $[K_o^+]$ was varied from 3 mM to 150 mM, and external μ was 0.22. The concentration of di-O-C_2[5] was 0.8 μM. Changes in absorbance $[\Delta A \equiv \Delta(A_{650\,nm} - A_{595\,nm})]$ induced by the addition of valinomycin (0.15 μM) were related to values of $\Delta\psi$ calculated from the Nernst equation, assuming equal internal and external activity coefficients.

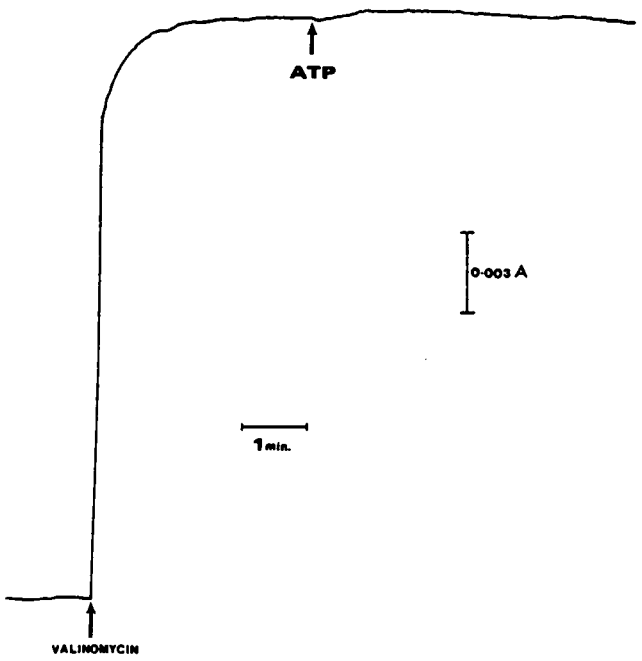

FIGURE 8 Effect of ATP on the negative electrical potential generated by K^+ gradients and valinomycin. Conditions were as in Fig. 7. ATP was added to give a concentration of 50 μM.

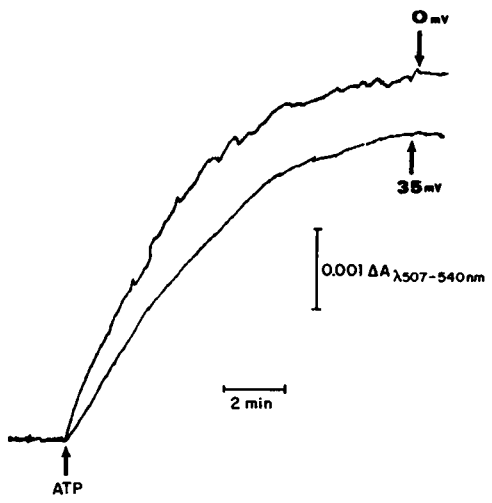

FIGURE 10 Influence of positive electrical potentials upon rate of Ca^{2+} uptake in RL vesicles. Ca^{2+} uptake was monitored by changes in the absorbancy of murexide (100 μM). $[K_i^+]$ was 22 mM, and $[K_o^+]$ was 22 mM for 0 mV, and 84 mM for 35 mV. External μ was 0.22, and the concentration of valinomycin was 0.15 μM. Ca^{2+} uptake was initiated by addition of ATP (50 μM). 0.001 ΔA = 7.5 μM.

evaluated in the presence of varying K^+ gradients and valinomycin. Figs. 10 and 11 indicate that positive electrical potentials reduce Ca^{2+} uptake and negative electrical potentials enhance Ca^{2+} uptake. Over a range of +50 to −100 mV, there was an inverse relationship between the rate of Ca^{2+} uptake and $\Delta\psi$. However, ATP hydrolysis was almost unaffected (Fig. 12). Accordingly, the more negative the electrical potential across the reconstituted vesicle membrane, the higher the Ca^{2+}/ATP ratio of the transport system. Since control experiments have indicated that Ca^{2+} uptake requires the presence of ATP and is unaffected by K^+ gradients in the absence of valinomycin, it is suggested that the electrical potential per se affects active Ca^{2+} uptake.

DISCUSSION

Several investigators have studied Ca^{2+} transport and/or ATP hydrolysis in reconstituted Ca^{2+}-ATPase vesicles in an attempt to elucidate the function of the intact structures (Racker, 1972; Knowles and Racker, 1975; Meissner and Fleischer, 1974; Warren et al., 1974; Knowles et al., 1980; Wang et al., 1979; Anderson et al., 1983). The Ca^{2+}/ATP ratios reported in these studies have varied greatly, often reflecting differences in the methods of reconstitution, presence or absence of proteolipids, variability of degrees

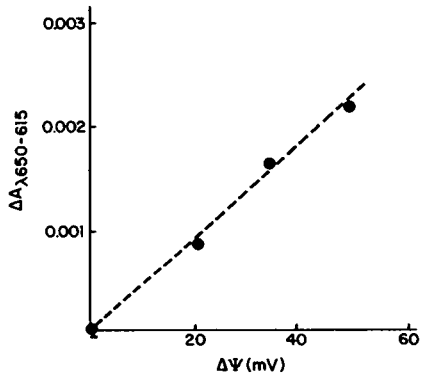

FIGURE 9 Calibration curve for positive (inside) electrical potential measurements. $[K_i^+]$ was 22 mM, $[K_o^+]$ was varied from 22 mM to 150 mM, and external μ was 0.22. The concentration of Oxanol VI was 1.6 μM. Changes in absorbance [$\Delta A \equiv \Delta(A_{650\,nm} - A_{615\,nm})$] induced by the addition of valinomycin (0.15 μM) were related to values of $\Delta\psi$ calculated from the Nernst equation.

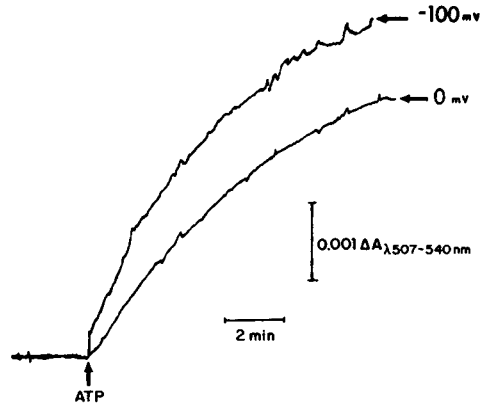

FIGURE 11 Influence of negative electrical potentials upon rate of Ca^{2+} uptake in RL vesicles. Ca^{2+} uptake was monitored by changes in the absorption of murexide (100 μM). $[K_i^+]$ was 150 mM and $[K_o^+]$ was 3 mM for −100 mV and 150 mM for 0 mV. External μ was 0.22, and the concentration of valinomycin was 0.15 μM. Ca^{2+} uptake was initiated by addition of ATP (50 μM). 0.001 ΔA = 10 μM.

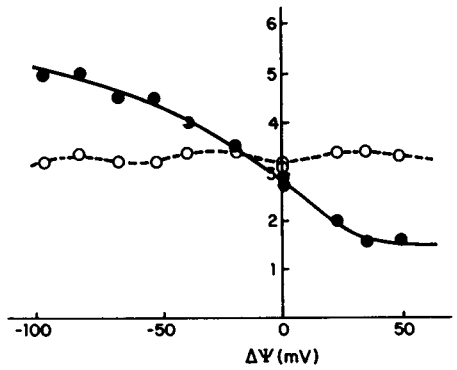

FIGURE 12 Effect of electrical potentials upon rates of Ca^{2+} uptake and ATP hydrolysis in RL vesicles. The initial rates of Ca^{2+} uptake and ATP hydrolysis (μmol $\times$ min^{-1} $\times$ mg $protein^{-1}$) are plotted as functions of the electrical potential ($\Delta\psi$). Ca^{2+} uptake and ATP hydrolysis were monitored as previously. [K_i^+] for positive and negative (inside) electrical potentials was 22 mM and 150 mM, respectively, and [K_o^+] was varied appropriately. External μ was maintained at 0.22, and the concentration of valinomycin was 0.15 μM. The reactions were initiated by addition of ATP (50 μM).

of vesicle leakiness, variable fractions of unreconstituted Ca^{2+}-ATPase, and usually lack of control of either the electrochemical potential difference of the transported Ca^{2+} or the components of the hydrolytic ATP driving reaction.

The procedure used here provided an active preparation of rather well-defined composition, which with further refinements should permit the systematic study of factors regulating Ca^{2+} transport and ATP hydrolysis. In this work our original preparation of the Ca^{2+}-ATPase (RO), prepared according to the freeze-thaw method of Kasahara and Hinkle (1977), showed an initial Ca^{2+}/ATP molar ratio of only ~0.3. Sucrose density centrifugation demonstrated that this low ratio was attributable to vesicle heterogeneity and permitted the isolation of a light fraction (RL) with initial ratios of ~1.

Although it is unclear why in the absence of voltage clamping our Ca^{2+}/ATP ratios are ~1, whereas those of native SR vesicles are ~2, RL vesicles prepared in the manner described showed several other characteristics indicative of normal Ca^{2+} transport. Thus, addition of ATP to the medium resulted in prompt onset of Ca^{2+} uptake and ATP hydrolysis, with gradual decline of external Ca^{2+} concentration; on restoration of Ca^{2+} to nearly initial levels, there was prompt restoration of both Ca^{2+} transport and ATP hydrolysis, indicating that the enzyme had not been denatured. The possibility that an appreciable amount of Ca^{2+} uptake was the result of adsorption to the surface of the vesicles appears to have been ruled out by the finding that Ca uptake was completely inhibited by the Ca^{2+}-ionophore A23187. Triton X-100 and quercetin, an inhibitor of several ATPases (Suolinna et al., 1975), markedly depressed the ATP-dependent Ca^{2+} uptake. (The high concentration of quercetin [75 μM] required here for the partial inhibition of uptake may well be due to the high lipid-to-protein ratio of our preparation.) Furthermore, the operation of the Ca^{2+} pump in the RL vesicles generated a positive membrane potential, with a very fast rise followed by a slow decay, despite the fact that Ca^{2+} uptake was still taking place. The generation of a positive potential is consistent with the idea that the Ca^{2+}-ATPase of sarcoplasmic reticulum operates as an electrogenic pump, as previously reported (Zimniak and Racker, 1978; Akerman and Wolff, 1979; Beeler, 1980).

Clamping electrical potentials across the reconstituted vesicles by the use of K^+ gradients and valinomycin has allowed us to explore the effects of voltage on the coupling of the Ca^{2+} pump. Our results indicate that negative and positive potentials, respectively, enhance and decrease the initial rate of Ca^{2+} uptake without apparent effect on the rate of ATP hydrolysis (Fig. 12). Beeler (1980) has reported that electrical potentials (inside negative) generated by K^+ gradients and valinomycin in SR vesicles enhanced both Ca^{2+} uptake and ATP hydrolysis. However, due to the high permeability of SR (Meissner, 1981), it was not possible to clamp the electrical potential, so that its magnitude during transport was not well defined. In our studies the Ca^{2+}/ATP ratio is ~1 at 0 mV, increases to 1.5 at −100 mV, and decreases to 0.5 at +50 mV. K^+ gradients in the absence of valinomycin do not affect uptake. This indicates that the K^+ gradients do not act by directly influencing Ca^{2+}-K^+ exchange (Haynes, 1983) but because of their influence on membrane potential.

A priori the effects of electrical potential on Ca^{2+} uptake might be attributed to membrane leakiness, changes in Ca^{2+} permeability, or direct effects on the active transport mechanism. We believe that the first and second possibilities are unlikely. With the use of K^+ gradients and valinomycin so as to make the interior of the vesicles markedly negative, no Ca^{2+} uptake was detectable in the absence of ATP. Nor was efflux significant, either in the absence or presence of ATP. With 2 mM ATP, efflux of ^{45}Ca from preloaded vesicles was minute over a range of potentials from −100 to +50 mV. Factors possibly accounting for this high degree of impermeability include the large lipid-to-protein ratio (~150 times higher than in SR vesicles), the presence of cholesterol, and reconstitution in the absence of detergents.

Our data are consistent with the idea that electrical forces can modulate the coupling mechanism of the Ca^{2+} pump. Although the significance for function of native SR is unclear, we feel that these observations are relevant to an eventual understanding of the pump mechanism. Modulation of coupling has been demonstrated on modification of aminophospholipids of SR membranes (Hidalgo et al., 1982). We have recently shown that phosphatidylethanolamine and monogalactosyldiglyceride, lipids tending to adopt nonbilayer structures (Cullis et al., 1983), are required for optimal coupling of the Ca^{2+}-ATPase reconstituted into lipid vesicles (Navarro et al., 1984). These data suggest that the nature of lipid-protein interactions

modulates coupling of the pump. It is thus possible that the electrical potential affects lipid-protein interactions of the Ca^{2+}-ATPase, which in turn results in changes in the tightness of coupling.

A fundamental problem in the study of active transport processes is the definition of the relationship between rates of transport and metabolism and driving forces. Such a characterization is not readily carried out in intact tissue, owing to its physical and chemical complexity. The results above suggest the utility of reconstituted Ca^{2+}-ATPase vesicles for this purpose. Here activities of ATP and P_i were nearly constant (ATP levels were regulated by an ATP-regenerative system; the near constancy of P_i was assured by initial incubation medium P_i content orders of magnitude greater than the amounts released by subsequent net ATP hydrolysis.) ADP levels, however, initially very low, were presumably increasing slowly.[1] Thus, the free energy of ATP hydrolysis was both very large and uncontrolled.

In future studies it should be possible to control or monitor all pertinent concentrations and forces. With the use of phosphate (or oxalate) as the major intravesicular anion, it would be feasible to observe extended periods of active transport, while controlling the internal Ca^{2+} concentration as well as the membrane potential, and monitoring external Ca^{2+} concentration. ATP, ADP, and P_i concentrations may be varied over a physiological range, using radioactive ATP turnover to monitor the rate of ATP hydrolysis. The combination of these approaches would allow a systematic determination of the dependence of the rates of transport and metabolism on reactant concentrations and thermodynamic forces.

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[1]Initially the ADP concentration was nominally zero. The amounts released by ATP hydrolysis were almost entirely reconverted to ATP by the ATP regeneration system.

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