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Ion Movement Accompanied by Calcium Uptake of Sarcoplasmic Reticulum Vesicles Studied through the Osmotic Volume Change by the Light Scattering Method

Tadaatsu Kometani and Michiki Kasai
Department of Biophysical Engineering, Faculty of Engineering Science, Osaka University, Toyonaka, Osaka 560, Japan

Summary. The volume change of sarcoplasmic reticulum vesicles was induced by Ca^{2+} uptake. This volume change was measured by the light-scattering method. When vesicles were shrunk beforehand under the condition that anions are more permeable than cations, they swelled during Ca^{2+} uptake due to the concomitant incorporation of anions. On the contrary, they shrank with Ca^{2+} uptake due to the extrusion of cations under the condition that cations are more permeable than anions. From the analysis of the volume change it was concluded that all ions other than Ca^{2+} were transported passively in order to neutralize the membrane potential generated by the Ca^{2+} pump. These results support the idea that the Ca^{2+} pump is electrogenic. By using this technique, it became possible to measure the fast Ca^{2+} uptake rate. The dependence of the Ca^{2+} uptake rate on the Ca^{2+} concentration suggests that the site at which Ca^{2+} inhibits Ca^{2+} uptake is located inside the vesicle. From the osmotic response of the vesicles, the intravesicular concentration of free Ca^{2+} was estimated to be about 15 mM, when Ca^{2+} was fully taken up under the physiological condition.

Sarcoplasmic reticulum (SR) of skeletal muscle controls the Ca^{2+} concentration in muscle cells, and regulates the relaxation-contraction process (Ebashi & Endo, 1968). Mechanisms of Ca^{2+} accumulation have been extensively studied for vesicles prepared from SR. In fact, the major protein in these vesicles is Ca^{2+}-ATPase (MacLennan & Holland, 1975). It is generally accepted that two Ca^{2+} are transported actively into SR vesicles by Ca^{2+}-ATPase using the hydrolysis energy of one ATP (Weber, Herz & Reiss, 1966; Tonomura, 1972). It is important to know how charges are balanced in order to maintain the electroneutrality of the systems during the Ca^{2+} uptake. Tonomura (1972) proposed that $(1+n)$ Mg^{2+} and $2(1-n)$ K^+, with n equaling one or zero, might be released with two Ca^{2+} accumulation. Recently, however, Ueno and Sekine (1978) showed that even at low concentrations of Mg^{2+} and K^+, SR vesicles can accumulate Ca^{2+}. This suggests that these ions are not necessarily counter-transported during Ca^{2+} uptake. If two charges are not actively counter-transported with the uptake of one Ca^{2+}, the Ca^{2+}-pump may work electrogenically and may generate the membrane potential with Ca^{2+} uptake. The electrogenicity of the Ca^{2+}-pump was demonstrated by Zimniak and Racker (1978) by measuring the potential difference of reconstituted vesicles with a fluorescent probe.

To clarify these problems, it is necessary to measure accurately the ion movement during Ca^{2+} uptake. However, the movement of small ions such as Cl^-, K^+, and Na^+ is difficult to study by the usual tracer method, because the membrane of isolated SR vesicles is so permeable for these ions (Hasselbach & Makinose, 1963; Duggan & Martonosi, 1970; McKinley & Meissner, 1978; Miller, 1978). In the previous paper (Kometani & Kasai, 1978; Kasai & Kometani, 1979), we succeeded in measuring the ions' movement through SR vesicles by measuring the fast volume change with the light-scattering method and could determine the permeabilities for various ions and neutral molecules.

In the present paper, ion movement during active Ca^{2+} uptake was followed by measuring the volume change with the light-scattering method. With this method, the volume change coupled with Ca^{2+} uptake was observed and the fast Ca^{2+} uptake rate could be determined. The results showed that any other ions than Ca^{2+} were not transported actively coupled with Ca^{2+} uptake, but diffused in order to maintain the electroneutrality. This phenomenon is

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consistent with the idea that Ca^{2+}-ATPase transports Ca^{2+} electrogenically.

Materials and Methods

Materials

SR vesicles were prepared from rabbit dorsal and hind leg muscle as a microsomal fraction according to Weber et al. (1966) with slight modification (Kasai & Miyamoto, 1976). This was stored in 100 mM KCl and 5 mM Tris-maleate (pH 6.5) at 0 °C. When K^+, Na^+ and Cl^- were to be eliminated from the vesicles, the stored vesicles were diluted 50 times with 10 mM Tris-Hepes (pH 6.8) and centrifuged at 53,000 × *g* for 60 min at 4 °C. This process was repeated twice.

ATP (sodium salt and Tris salt), ADP, and valinomycin were purchased from Sigma Chemical Co. A23187 was donated by Eli Lilly. ^{45}Ca was purchased from International Chemical & Nuclear Co. Other reagents were of analytical grade.

Volume Change Measurement by Light-Scattering Method

The osmotic volume change of SR vesicles was investigated by measuring the light-scattering intensity (450 nm) at the right angle to the incident beam using either a stopped flow spectrophotometer (Union RA-401 and RA-450, Japan) or the combination of a rapid mixing apparatus (Union MX, Japan) and a fluorescence spectrophotometer (Union FS-501, Japan) at 23 °C. In most experiments, SR vesicles were mixed with an equal volume of salt solution after incubating under appropriate conditions, and then change of the scattered light intensity was measured. By this method the net movement of ions could be followed. When ionophores were used, they were added to the incubated suspensions about 30 min before the mixing unless otherwise stated.

Other Measurements

Ca^{2+} uptake was measured by the Millipore method using $^{45}Ca^{2+}$ as described in the previous paper (Kasai & Miyamoto, 1976). After the initiation of the Ca^{2+} uptake reaction in the presence of $^{45}Ca^{2+}$, 1 ml aliquots of the suspension of SR vesicles were filtered through Millipore filters (HAWP 025) at appropriate time intervals and washed with the solvent of 3 ml. Amount of the Ca^{2+} in the SR vesicles was determined by measuring the radioactivity of SR vesicles adhered on Millipore filters using a scintillation counter (Horiba LS-500, Japan).

K^+, Na^+, and Mg^+ content of SR vesicles were determined by an atomic absorption spectrophotometer (Jarell-Ash AA-845, Japan).

Sarcoplasmic reticulum protein concentration was determined by the Biuret method.

Results

Swelling of SR Vesicles during Active Ca^{2+} Uptake

As in the previous paper (Kometani & Kasai, 1978), SR vesicles, incubated in 2 mM KCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 5 mM Tris-maleate (pH 6.5) were mixed with an equal volume of 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 5 mM Tris-maleate using a stopped flow apparatus, and the change in the scattered light intensity was measured. The obtained curve almost agrees with that of the previous paper where there was no Mg^{2+} and Ca^{2+}. A fast increasing phase in the scattering intensity was followed by a slow decreasing phase as shown in Fig. 1. According to the previous paper, the increase of the scattered light intensity corresponds to the shrinkage of the vesicles due to the outflow of the water caused by the difference of the osmotic pressure of KCl across the membrane, and the decrease of the scattered light intensity corresponds to the swelling of the vesicles due to the water inflow accompanied by the inflow of KCl which is driven by the difference of the chemical potential of these ions.

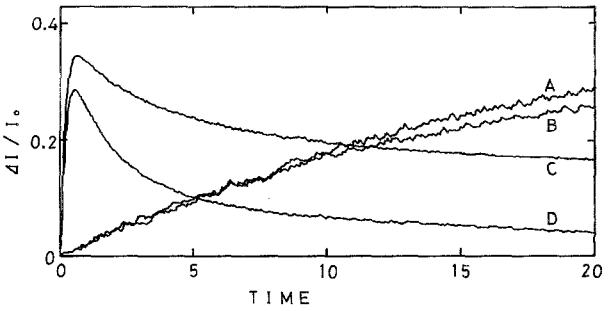

Fig. 1. Acceleration of the swelling of SR vesicles by adding ATP. SR vesicles incubated in 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 2 mM KCl, 5 mM Tris-maleate (pH 6.5) and 0.54 mg SR protein/ml were mixed with an equal amount of salt solution containing 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 100 mM KCl and 5 mM Tris-maleate by a stopped flow apparatus. 1 mM ATP was added only to the salt solution. The ordinate shows the relative change in the light scattering intensity. *A* and *C* are the change of the scattered light intensity without ATP, *B* and *D* are with ATP. Time unit: *A* and *B*, 10^{-2} sec; *C* and *D*, 1 sec

When a similar experiment was carried out with ATP added to the salt solution, the initial rate of increase in the scattered light intensity was the same as that without ATP, but the decreasing phase was faster than that without ATP, as shown in Fig. 1. This enhanced decrease in the scattered light intensity is due to the swelling of the vesicles, and not due to the optical artifact induced by ATP. Because, when ATP was added to SR vesicles incubated in 51 mM KCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 5 mM Tris-maleate, no change in the scattering intensity was observed. This swelling of the vesicles is due to the Ca^{2+} uptake. However, there is a possibility that ATP increases the K^+ permeability as valinomycin does since such a behavior with ATP in the solution resembles that observed when valinomycin is in the solution (Kometani & Kasai, 1978). In order to prove that we can eliminate this possibility, similar experiments

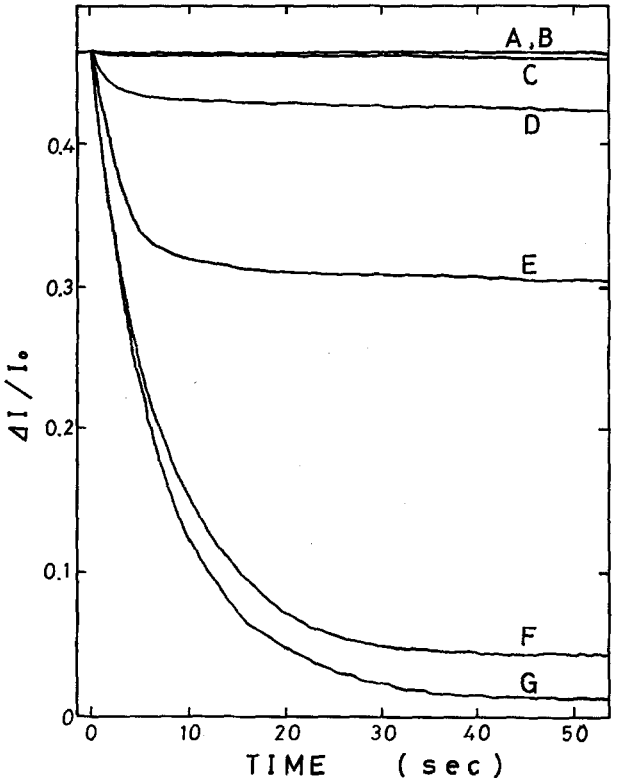

Fig. 2. Effect of ATP on the swelling of shrunken vesicles. After the incubation in 100 mM KCl, 10 mM Tris-maleate, 2×10^{-6} g valinomycin/ml and 0.8 mg protein/ml, SR vesicles shrank by mixing with an equal amount of solution, which contains (in mM): 100, KCl; 40, $MgCl_2$; 1, $CaCl_2$; and 10 Tris-maleate. Five minutes after mixing, the reaction was started by adding an equal amount of solution, which contains the same kinds and amount of salt and various concentrations of ATP. Change in the light scattering intensity was measured by a spectrofluorimeter. ATP concentration was as follows: (*A*) 1.2×10^{-9} M, (*B*) 1.2×10^{-8} M, (*C*) 1.2×10^{-7} M, (*D*) 1.2×10^{-6} M, (*E*) 3.8×10^{-6} M, (*F*) 1.2×10^{-5} M, (*G*) 1.2×10^{-4} M

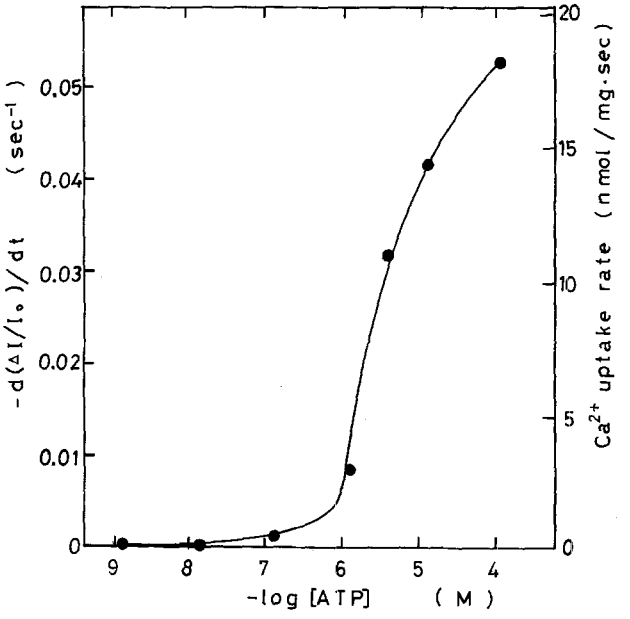

Fig. 3. Relation between the initial swelling rates and ATP concentrations. The values were calculated from Fig. 2. The ordinate shows the relative initial decrease rate of the light scattering intensity, $-d(\Delta I/I_o)/dt$. Ca^{2+} uptake rate was calculated from the relation in Fig. 5

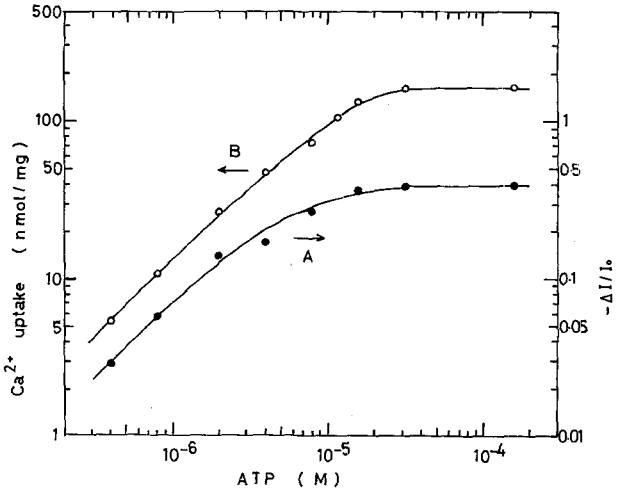

Fig. 4. The change in the scattered light intensity and Ca^{2+} uptake with respect to ATP concentration. Similar experiments in Fig. 2 were carried out. (*A*): SR vesicles incubated in 100 mM KCl, 5 mM Tris-maleate and 5 mg protein/ml were first diluted 50 times with 100 mM KCl, 5 mM Tris-maleate, 20 mM $MgCl_2$, 40 μM $CaCl_2$, 10^{-6} g valinomycin/ml. Four minutes after the dilution, a small amount of ATP was added and the change in the scattered light intensity was recorded. For the magnitude of the intensity change ($\Delta I/I_o$), intensity 3 min after the addition of ATP was used. (*B*): Ca^{2+} uptake in the same condition in *A* was carried out by using $^{45}Ca^{2+}$. 1 ml aliquots of the reaction mixture were filtered with Millipore filters at appropriate time intervals and the amount of Ca^{2+} in the vesicles was determined. Values 3 min after the addition of ATP were used

to Fig. 1 were carried out using choline-Cl, K-methanesulfonate, $MgCl_2$, $CaCl_2$, or Tris-gluconate instead of KCl. In any case the acceleration of swelling of vesicles was induced by ATP. It is not probable, therefore, that ATP increases the permeabilities of all these ions. These results indicate that the swelling induced by ATP is related to Ca^{2+} uptake into the vesicles.

In order to study this phenomenon in detail, vesicles were shrunk beforehand by adding $MgCl_2$, and the whole swelling process, which was induced by ATP, was examined. Since the Mg^{2+} permeability is small, without ATP vesicles remain shrunk for more than 10 min after the mixing with the hypertonic solution of $MgCl_2$. Five minutes after mixing with 40 mM $MgCl_2$, the addition of various concentrations of ATP caused a rapid decrease in the light scattering intensity as shown in Fig. 2. If we examine

the data 3 sec after adding ATP, this curve is in accord with that observed when the experiment was started from the swollen vesicles as in Fig. 1. The experiments in Fig. 2 were carried out in the presence of valinomycin. In such a condition, K^+ and Cl^- are considered to be in equilibrium and do not exert osmotic force since permeabilities for these ions are very large. Accordingly, the volume of the vesicles is determined by the osmotic balance between Ca^{2+} inside the vesicles and Mg^{2+} outside the vesicles. The swelling rate is considered to depend only on Ca^{2+} uptake. At low ATP concentrations, the vesicles swell rapidly for the first 30 sec or so and then slow down. The initial swelling rate increased as the ATP concentration was above 0.1 μM. This critical ATP concentration coincides with the ATP concentration which activates Ca^{2+}-ATPase (Tonomura, 1972). With increasing ATP concentration, the initial swelling rate and the magnitude of the volume change became larger as shown in Figs. 3 and 4. At low ATP concentrations, the magnitude of the swelling rate increased in proportion to the ATP concentration, but at high ATP concentrations it reached a constant value, where the vesicular volume is nearly equal to that before the shrinkage. This swelling was not induced by ADP instead of ATP, and it also disappeared when Ca^{2+} concentration was reduced by adding EGTA. Thus, the swelling induced by ATP is related to the Ca^{2+}-ATPase activity. It must be due to the increase in the concentration of intravesicular Ca^{2+} and other ions transported with Ca^{2+}.

Swelling of Vesicles due to the Increase in the Intravesicular Ca^{2+}

In order to prove that the swelling of SR vesicles induced by ATP is directly related to Ca^{2+} uptake, the amount of Ca^{2+} taken up during the swelling was measured by using radioactive tracer. Figure 4 shows the amount of Ca^{2+} taken up 3 min after the initiation of Ca^{2+} uptake, and the change in magnitude of the scattered light intensity as a function of ATP concentration with a similar condition to Fig. 2. At low ATP concentrations, both increased in parallel with the amount of added ATP. In this condition, about two Ca^{2+} were taken up by the hydrolysis of one ATP. In Fig. 5, the relation between the amount of Ca^{2+} taken up and the light scattering change is shown. When the change in the light scattering is small, it is proportional to the amount of Ca^{2+} taken up, but the light scattering change became saturated with increasing the amount of Ca^{2+} taken up. This deviation may be partly due to the fact that the light scattering change is not necessarily proportional to the volume change of the vesicles, but mainly due to the saturation of the swelling at the maximally swollen state. In this state an increase in the hydrostatic pressure inside the vesicle is expected to occur, which will be discussed in the following section.

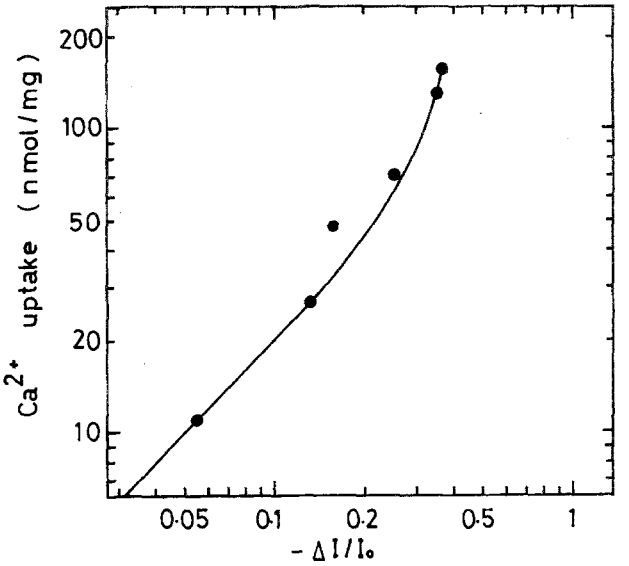

Fig. 5. Relation between the change in the scattered light intensity and Ca^{2+} uptake. Data were taken from Fig. 4

As a result we can calculate the amount of Ca^{2+} taken up from the scattering change in similar conditions. By using this relation, the change in the scattered light intensity was converted into the amount of Ca^{2+} taken up in Fig. 3. A similar experiment to Fig. 4 was carried out without KCl and valinomycin, and a similar relation was obtained.

Next, the effect of a divalent cation ionophore, A23187, was studied by the light-scattering experiment in order to show the increase of the intravesicular Ca^{2+} during the swelling of the vesicles induced by ATP. To begin with, the effect of A23187 on SR membrane was examined. A23187 selectively increased the permeabilities for Ca^{2+} and Mg^{2+} as shown in Table 1. The increment of the Ca^{2+} permeability was proportional to the square of A23187 concentration as shown in Fig. 6. This result is consistent with the fact that two molecules of A23187 contribute to the movement of one Ca^{2+} (Chaney, Jones & Debono, 1976). The following experiments were carried out in the presence of 10^{-6} g A23187/ml.

SR vesicles treated with A23187 were mixed with a hypertonic solution of K-methanesulfonate, and the change of the scattered light intensity was followed. As a control, untreated vesicles were used. As shown in Fig. 7, in the absence of ATP the change of the scattered light intensity was not affected by the treatment with A23187, while in the presence of ATP

Table 1. Effect of A23187 on the permeation time of ions and neutral molecules[a]

Permeants	Final concentration (mM)	Permeation time		Effect
		−A23187 (sec)	+A23187 (sec)	
$CaCl_2$	33	6000	0.45	++
$MgCl_2$	33	1800	3.5	+
KCl	50	10	11	−
NaCl	50	13	14	−
Choline-Cl	50	360	380	−
Tris-gluconate	50	1200	1200	−
Glucose	100	1500	1700	−
Xylose	100	200	200	−

[a] SR vesicles, incubated in 2 mM KCl, 5 mM Tris-Maleate, 2×10^{-6} g A23187/ml and 0.42 mg protein/ml, were mixed with an equal volume of solution containing various salts and 5 mM Tris-maleate. The permeation time was defined as the time that the half value of the maximal increment of the scattering intensity is reached; the maximal increment was estimated by an extrapolation of the swelling phase.

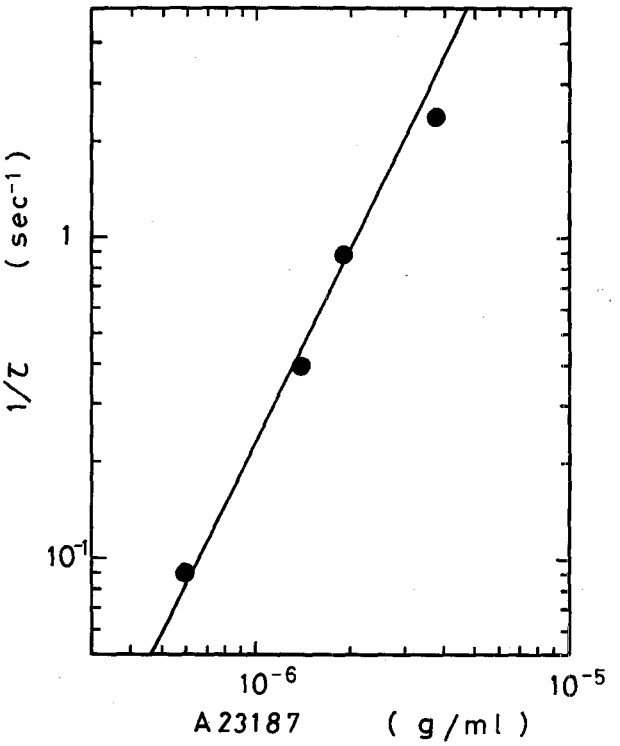

Fig. 6. Effect of A23187 on the permeation time of Ca^{2+}. SR vesicles, incubated in 2 mM KCl, 5 mM Tris-maleate and 0.42 mg protein/ml, were mixed with an equal amount of 67 mM $CaCl_2$ and 5 mM Tris-maleate. A23187 was added to incubated suspension alone 30 min before mixing. The concentration of A23187 given is the final one. The permeation time was defined as the time when the relative light scattering intensity came to 0.25

the swelling, observed in the untreated vesicles, disappeared. Thus, A23187 inhibited the swelling of the vesicles induced by ATP. A similar effect of A23187 was observed using KCl or Tris-Cl instead of K-methanesulfonate. This result is consistent with the fact that the swelling of SR vesicles induced by ATP can be attributed to the increase of the intravesicular Ca^{2+} concentration. This Ca^{2+} accumulation must be accompanied by an incorporation of anions to maintain the electroneutrality. If cations are extruded in order to maintain the electroneutrality, the vesicles cannot swell.

Therefore, if the experiment is carried out under the condition that cations are much less permeable than anions, it is expected that the vesicles will reshrink by the difference of the osmotic pressure of the slowly permeable cations used for the preshrinkage when the inner Ca^{2+} is released by A23187. The experiment was carried out in Tris-Cl. As shown in Fig. 8, the vesicular volume increased by adding ATP, and when A23187 was added it decreased to the level which can be obtained when A23187 is added without ATP. This change shows that the reshrinkage of the vesicles is due to the release of Ca^{2+}. Therefore, we can conclude that the swelling induced by ATP is caused by the increase in the intravesicular Ca^{2+}, and that the swelling force is the osmotic pressure due to the increase in the concentration of Ca^{2+} and Cl^- inside the vesicle, where Cl^- is transported into the vesicles so as to maintain the electroneutrality. Similar reshrinkage was observed using choline-Cl instead of Tris-Cl. When Tris-gluconate was used in-

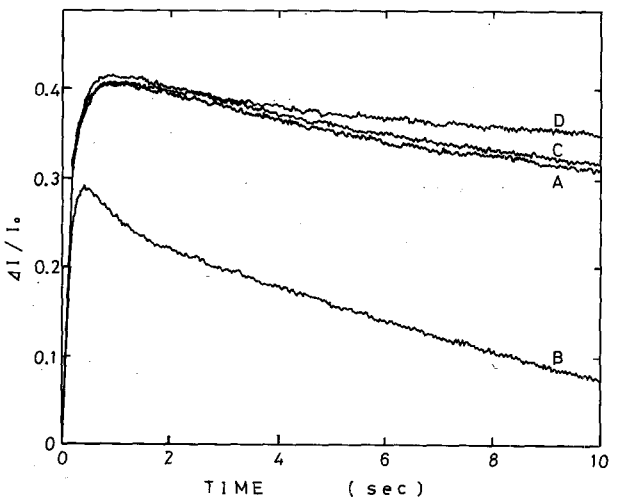

Fig. 7. Effect of A23187 treatment on the SR vesicles' swelling which is induced by ATP. An experiment similar to that in Fig. 1 was carried out by mixing SR vesicles suspended in 2 mM KCl, 4 mM $MgCl_2$, 0.2 mM $CaCl_2$, 5 mM Tris-maleate, 4×10^{-6} g A23187/ml and 0.4 mg protein/ml with salt solution containing 100 mM K-methanesulfonate, 4 mM $MgCl_2$, 0.2 mM $CaCl_2$, 5 mM Tris-maleate and 1 mM ATP. Treatment: (*A*) without A23187 and without ATP, (*B*) without A23187 and with ATP, (*C*) with A23187 and without ATP, (*D*) with A23187 and with ATP

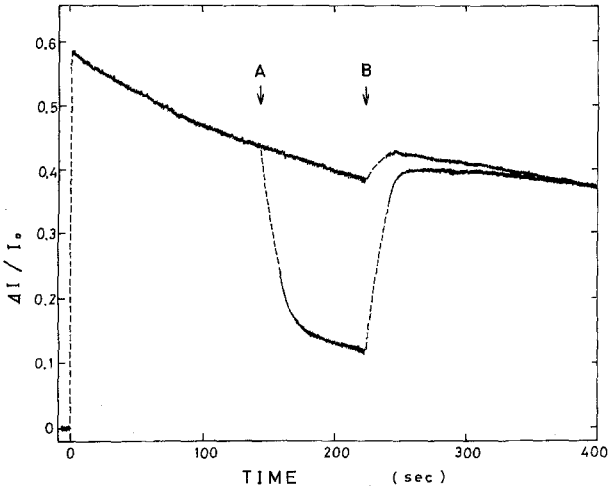

Fig. 8. Reshrinkage of the swollen vesicles by adding A23187. SR vesicles were incubated in 10 mM Tris-Cl, 4 mM $MgCl_2$, 0.2 mM $CaCl_2$, 2 mM KCl, 2×10^{-6} g valinomycin/ml and 0.4 mg protein/ml. They were mixed with an equal amount of 100 mM Tris-Cl, 4 mM $MgCl_2$ and 0.2 mM $CaCl_2$ by the fast mixing apparatus. Each arrow shows the adding of: (*A*) 10 μM ATP, (*B*) 2×10^{-6} g A23187/ml. Upper record, no ATP (control)

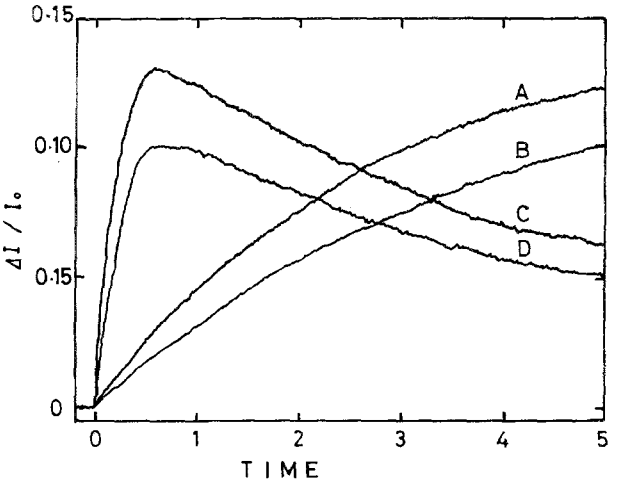

Fig. 9. Shrinkage of SR vesicles due to Ca^{2+} uptake by adding ATP. SR vesicles, washed twice with 10 mM Tris-Hepes (pH 6.8), were incubated in 50 mM K-gluconate, 4 mM Mg-gluconate, 0.2 mM Ca-gluconate, 1 mM Tris-Hepes and 0.4 mg protein/ml. They were mixed with an equal amount of 1 mM ATP which contains the same solution used for the incubation. Valinomycin, 2×10^{-6} g/ml, was added to the incubated vesicles 30 min before mixing. (*A* and *C*): with valinomycin-treated vesicles; (*B* and *D*): with untreated ones. Time unit: (*A* and *B*) 1 sec; (*C* and *D*) 10 sec

stead of Tris-Cl, the reshrunken volume was larger than that when A23187 is added and without ATP. In this case, $Tris^+$ is probably accumulated passively during the reshrinkage because of the low permeability for gluconate.

Ion Movement during Ca^{2+} Uptake and Electrogenicity of the Ca^{2+} Pump

As described in the previous section, Ca^{2+} was accumulated actively into SR vesicles by Ca^{2+}-ATPase and the vesicles were swollen. In this process, anions such as Cl^- or methanesulfonate must be transported into the vesicles so as to maintain the electroneutrality. It is then a problem whether these anions are transported actively or not. If these anions are not transported actively, SR vesicles are expected to shrink accordingly with Ca^{2+} uptake under the condition that monovalent cations in the medium are more permeable than anions, because two monovalent cations must be released during the uptake of one Ca^{2+} so as to maintain the electroneutrality.

After SR vesicles, which were twice washed with 10 mM Tris-Hepes (pH 6.5) to eliminate contaminating Cl^-, were incubated overnight in 50 mM K-gluconate, 4 mM Mg-gluconate, 0.2 mM Ca-gluconate and 1 mM Tris-Hepes, Ca^{2+} uptake was initiated by adding ATP, and the change in the scattered light intensity was recorded. As shown in Fig. 9, SR vesicles shrank with Ca^{2+} uptake. The shrinking rate was about two orders lower than that caused by the water outflow due to the osmotic pressure difference observed at the initial phase in Fig. 1 and was the same order as the swelling rate due to the Ca^{2+} uptake observed in Figs. 1 and 2. This result suggests that two K^+ were released when one Ca^{2+} was taken up and the vesicles shrank due to the decrease in intravesicular osmolarity. If more than one gluconate were actively accumulated when two Ca^{2+} were transported, less than three equivalent monovalent cations must have been released from the vesicles to maintain the electroneutrality and the vesicles should not have shrunk. Therefore, this result indicates that gluconate is not accumulated actively and suggests that any anions are not transported actively. This result also eliminates the possibility of active incorporation of phosphate ions; this was proposed by Martonosi, Lagwinska and Oliver (1974).

In Fig. 9, the result obtained in the presence of valinomycin was also shown. The vesicles shrank more with Ca^{2+} uptake by adding valinomycin. At the same time, Ca^{2+} uptake was measured by the tracer method (the data are not shown). A slight increase in the Ca^{2+} uptake rate (about 10%) was observed by adding valinomycin. However, the change in the scattered light intensity was not parallel to the amount of Ca^{2+} taken up. During the decreasing phase of the scattering intensity, Ca^{2+} content in vesi-

cles kept constant or slightly increased. Detailed analysis is not possible at present.

The shrinkage of vesicles was followed by swelling in Fig. 9. The swelling rate of vesicles is faster than the influx rate of gluconate which was expected from its permeability, and Ca^{2+} release did not take place. This swelling might be due to the influx of phosphate liberated by ATP hydrolysis. Because the shrinkage of vesicles observed in Fig. 9 disappeared when a small amount of permeable anion such as KCl (about 2 mM) was added to the medium. Further experiments have not yet been done.

From the shrinkage coupled with Ca^{2+} uptake in Fig. 9 there is a possibility that the monovalent cations were released actively coupled with Ca^{2+} uptake. However, this possibility can be eliminated as follows. In Fig. 8, the vesicles were swollen by taking up Ca^{2+} in the presence of ATP, and the volume of vesicles, when they reshrunk by the action of A23187, was nearly equal to the volume when they shrank without ATP. If $Tris^+$ was released actively, the reshrunken volume must have been less than that without ATP. This result indicates that $Tris^+$ was not released during Ca^{2+} uptake.

Summarizing these results, it is concluded that all ions except Ca^{2+} move passively in order to maintain electroneutrality, and therefore the Ca^{2+}-pump is electrogenic. This conclusion is consistent with the observation of other researchers (Zimniak & Racker, 1978).

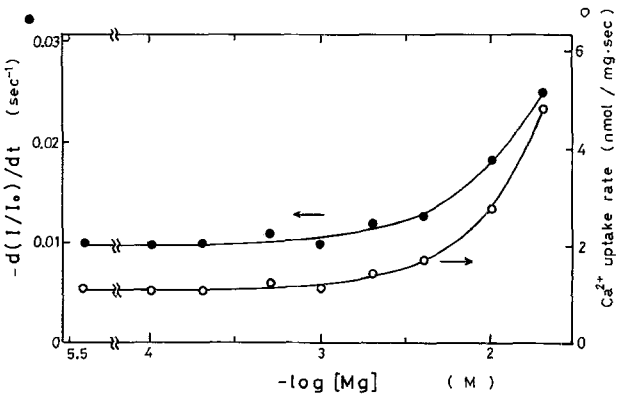

Fig. 10. Effect of Mg^{2+} on the initial Ca^{2+} uptake rate. SR vesicles incubated in 100 mM KCl, 5 mM Tris-maleate, 2×10^{-6} g valinomycin/ml and 1.6 mg protein/ml were preshrunk as in Fig. 2 by adding an equal amount of 100 mM KCl, 40 mM $CaCl_2$, 5 mM Tris-maleate and various concentrations of $MgCl_2$. 0.5 mM ATP was added to the shrunk vesicles 5 min after mixing by the same method as in Fig. 2. (●) change rate in the relative scattered light intensity (○) Ca^{2+} uptake rate calculated as in Fig. 3

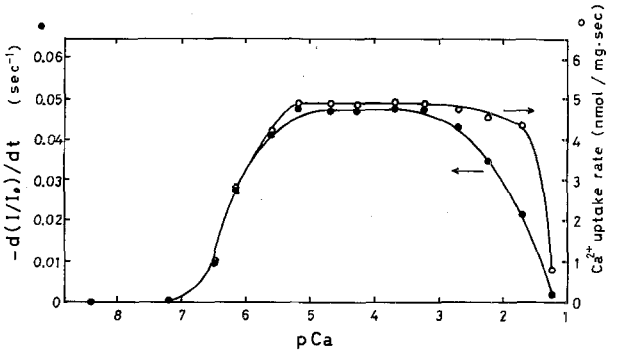

Fig. 11. Effect of Ca^{2+} on the initial Ca^{2+} uptake rate. A similar experiment to that in Fig. 10 was carried out using Ca^{2+} instead of $MgCl_2$. The same analysis as in Fig. 10 was made. Free Ca^{2+} concentration was adjusted by Ca-EGTA buffer (Miyamoto & Kasai, 1978), and the final EGTA concentration was less than 5 mM. Symbols are the same as in Fig. 10

Effects of Mg^{2+} and Ca^{2+} on Ca^{2+} Uptake

Since the change in the light scattering intensity was proportional to the amount of Ca^{2+} taken up when the amount of Ca^{2+} taken up was small in Fig. 5, measurement of the initial Ca^{2+} uptake rate became possible. The effects of Mg^{2+} and Ca^{2+} concentration on the Ca^{2+} uptake rate was examined by using shrunken vesicles. When ATP was added to the vesicles, when shrank in the solution of 20 mM $CaCl_2$ and various concentrations of $MgCl_2$, the initial swelling rate increased with increasing Mg^{2+} concentration as shown in Fig. 10. The swelling was observed even at 4 μM Mg^{2+}. If it is assumed that one Mg^{2+} is counter-transported actively during two Ca^{2+} accumulation in the swelling step as proposed by Tonomura et al. (1972), the amount of Mg^{2+} would be proportional to the volume increment and the initial releasing rate would be about 0.5 nmol/mg/sec. However, the maximal influx of Mg^{2+} is calculated to be only 0.2 pmol/mg/sec from the Mg^{2+} permeability (Nagasaki & Kasai, 1980). The influx is so small that the passive diffusion of Mg^{2+} into the vesicles must not affect Ca^{2+} uptake. Only contaminating Mg^{2+} present at the beginning can be taken into account. Although the amount of the contaminating Mg^{2+} was about 10 nmol/mg from the atomic absorption measurement, the amount of released Mg^{2+} must be 25 nmol/mg from the fast volume change at 100 sec after adding ATP, and the swelling continued further with the influx rate of 0.1 nmol/mg/sec. These facts are contradictory to the above assumption that Mg^{2+} is released actively during the Ca^{2+} accumulation. Thus, these results show that Mg^{2+} may be required for Ca^{2+} uptake from the inside of the vesicles, but is not counter-transported. This is consistent with the observation of Ueno and Sekine (1978).

Effect of Ca^{2+} concentration on Ca^{2+} accumulation was examined using vesicles shrunk by 20 mM $MgCl_2$ in the same way as in the case of Mg^{2+}. Figure 11 shows the initial Ca^{2+} uptake rate as a function of Ca^{2+} concentration. The uptake rate began to increase from about 0.1 μM Ca^{2+}, reached a maximal level at 10 μM, and this level was maintained up to 20 mM. The inhibition effect by Ca^{2+} appeared at concentrations greater than 20 mM; this is one order larger than those required for inhibition of ATPase activity (Weber, 1971). The Ca^{2+} permeability is so small that only extravesicular Ca^{2+} can affect the initial Ca^{2+} uptake. Thus, the result in Fig. 11 is consistent with the observation of other researchers that the inhibition sites for Ca^{2+} uptake by Ca^{2+}, whose dissociation constant is about 1 mM, are located on the inner surface of the vesicles (Weber, 1971).

Intravesicular Ca^{2+} Concentration at Full Uptake

Swelling of SR vesicles occurred during Ca^{2+} uptake in the presence of fast permeable anions. Thus, the intravesicular osmotic pressure must be increased by the Ca^{2+} accumulation if fully swollen vesicles take up Ca^{2+} by adding ATP. The intravesicular osmotic pressure can be determined from the shrinking rate of the vesicles in hypertonic solutions, since the rate of the vesicles' shrinking is linear to the osmotic pressure difference across the membrane when suddenly high osmotic pressure is applied to the vesicles (Kasai, Kanemasa & Fukumoto, 1979).

SR vesicles incubated in 100 mM KCl, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 10 mM Tris-maleate and 2×10^{-6} g valinomycin/ml were fully swollen since no decrease in the light scattering intensity could be observed by mixing with hypotonic solutions. Three minutes after the initiation of Ca^{2+} uptake by adding ATP, osmotic shrinking rate was measured by mixing with a hypertonic solution containing various concentrations of $CaCl_2$ in addition to the same salt solution used for the incubation. The vesicles shrank by the osmotic pressure difference of $CaCl_2$. As shown in Fig. 12, the initial increasing rate in the scattered light intensity was proportional to the concentration difference of $CaCl_2$ in the absence of ATP, but in the presence of ATP the relation shifted in parallel to the higher concentration of $CaCl_2$ above 15 mM $CaCl_2$. From this shift the mean intravesicular Ca^{2+} concentration was estimated to be 15 mM. At lower Ca^{2+} concentrations, however, the curve was not parallel to that obtained without ATP and was proportional to the Ca^{2+} concentration. This may be explained by the distribution of intravesicular Ca^{2+} concentration and it may originate from the heterogeneity of the vesicles.

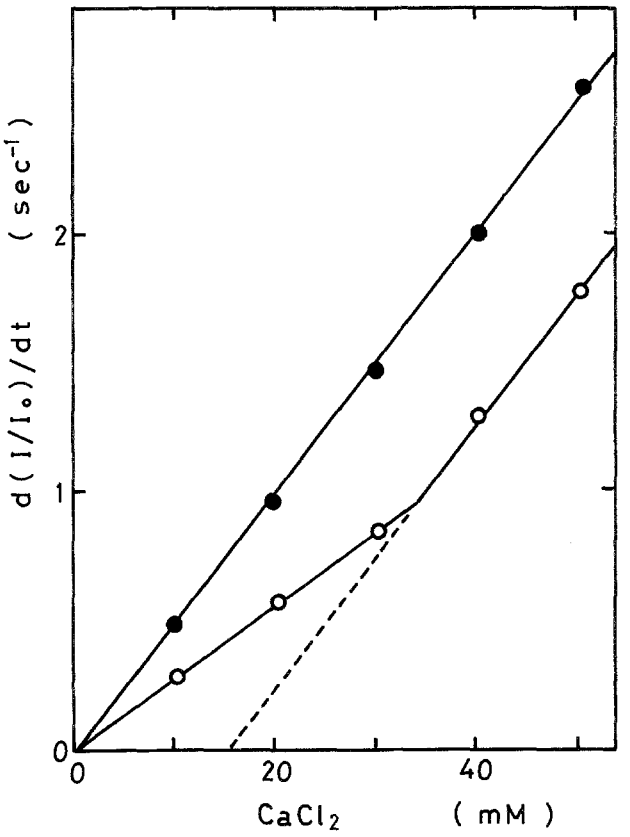

Fig. 12. Increment of intravesicular osmotic pressure at Ca^{2+} uptake. 0.5 mM ATP was added to SR vesicles incubated in 100 mM KCl, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 10 mM Tris-maleate, 10^{-6} g valinomycin/ml and 0.4 mg protein/ml. Three minutes after adding ATP the suspension was mixed with an equal volume of 100 mM KCl, 2 mM $MgCl_2$, 10 mM Tris-maleate and various concentrations of $CaCl_2$ and the change in the scattered light intensity was recorded. The ordinate shows the initial rate of change in the scattered light intensity. (○) ATP added, (●) no ATP

Discussion

In the previous paper (Kometani & Kasai, 1978), it was found that the Cl^- permeability is about 50 times greater than the K^+ permeability, and the permeation time for Cl^- is about 0.4 sec. These facts suggest that if an electrical potential difference is generated across the membrane it will disappear rapidly by the permeation of these ions under physiological conditions. It was, then, difficult to examine ion movement coupled with Ca^{2+} uptake. In this paper,

in order to elucidate the ionic process during Ca^{2+} uptake, fast ionic movement across the membrane was followed through the volume change which is measured by the light scattering method. Turbidity change coupled with Ca^{2+} uptake was once observed by Ohnishi and Terasaki (1967), but the real meaning was not made clear.

The possibilities of the active movement of Mg^{2+} and/or K^+ (Tonomura, 1972) or phosphate (Martonosi et al., 1974) have been proposed. If there is an active movement of these ions, these ions must be transported in a stoichiometric manner. The results obtained in this paper indicate that any ion except Ca^{2+} was not transported actively coupled with Ca^{2+} accumulation and that the Ca^{2+} pump is electrogenic. This conclusion is consistent with the observation of the reconstituted Ca^{2+} pump (Zimniak & Racker, 1978).

In the above discussion, we did not consider movement of the proton. If more than one proton moves actively coupled with one Ca^{2+}, the shrinkage seen in Fig. 9 cannot be observed since only less than one K^+ must be released. At present, however, the possibility of one proton extrusion coupled with 2 Ca^{2+} uptake cannot be eliminated. If this is the case, the remaining 3 charges must be transported in an electrogenic manner and ions move passively so as to maintain the electroneutrality. Thus, this possibility does not affect our main conclusion.

Accordingly, the Ca^{2+} pump may produce an osmotic and electric gradient with Ca^{2+} transport. Since the permeability for Cl^-, K^+ and Na^+ was high (Hasselbach & Makinose, 1963; Duggan & Martonosi, 1970; Miller, 1978), it is considered that, under the physiological salt conditions, Ca^{2+} can be transported readily without osmotic and electric resistance. This conclusion is consistent with the idea of McKinley and Meissner (1978). In physiological conditions, one Ca^{2+} movement may be accompanied by both the passive movement of two K^+ in counter-direction and one Cl^- in codirection to keep the osmotic and electric balance.

In the present paper, it was shown that the fast Ca^{2+} uptake rate could be determined by measuring the swelling of vesicles by light scattering method. This result was consistent with the value determined by the usual tracer method (Hara & Kasai, 1977).

Because Ca^{2+}, which contributes to the volume change, must be free inside the vesicles, the concentration of free Ca^{2+} must be equal to that of impermeable solutes outside the vesicles under the swelling conditions. In the case of Figs. 2 and 4, intravesicular Ca^{2+} concentration is expected to be 20 mM. If the intravesicular volume is assumed to be 4 μl/mg protein (Miyamoto & Kasai, 1978), the amount of free Ca^{2+} becomes 80 nmol/mg protein. Since the amount of total Ca^{2+} taken up was about 160 nmol/mg protein from Fig. 5, about 50% of Ca^{2+} is expected to be free inside the vesicles. This value is much bigger than that estimated by other researchers (less than 1 mM, Ebashi & Endo, 1968). From the experiment in Fig. 12, free Ca^{2+} concentration was estimated to be 15 mM. In this case free Ca^{2+} concentration is calculated to be about 35% of the total Ca^{2+} taken up. Although this value is still high, it is consistent with our previous estimation (Miyamoto & Kasai, 1978). It is possible that the free Ca^{2+} concentration is so high even in the physiological conditions.

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