Measurement of Steady-state Ca\textsuperscript{2+} Pump Current Caused by Purified Ca\textsuperscript{2+}-ATPase of Sarcoplasmic Reticulum Incorporated into a Planar Bilayer Lipid Membrane

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The electrogenericity and some molecular properties of the sarcoplasmic reticulum Ca\textsuperscript{2+} pump protein were studied by measuring steady-state Ca\textsuperscript{2+} pump currents. Ca\textsuperscript{2+}-ATPase protein was solubilized from rabbit skeletal muscle sarcoplasmic reticulum membrane preparations and purified by liquid chromatography. The purified Ca\textsuperscript{2+}-ATPase molecules were reconstituted into proteoliposomes and then incorporated by fusion into a planar bilayer lipid membrane. Short circuit currents across the planar membrane were detected when the ATPase molecules were activated by addition of ATP under optimal ionic conditions. Thus, the electrogenericity of the Ca\textsuperscript{2+} pump molecules was directly demonstrated. The amplitude of the pump current was dependent on the ATP concentration, and the relation was described by a Michaelis-Menten-type equation. The Michaelis constant was calculated to be 0.69 ± 0.16 mM, which agrees well with the dissociation constant for a low affinity ATP-binding site deduced previously (10, 11).

The Ca\textsuperscript{2+}-dependent ATPase of the sarcoplasmic reticulum (SR) membrane is a Ca\textsuperscript{2+} pump protein translocating Ca\textsuperscript{2+} ions from the sarcoplasm to the SR lumen. The question of whether or not the Ca\textsuperscript{2+} pump of the SR membrane is electrogeneric is quite an important issue for elucidating the mechanism of Ca\textsuperscript{2+} transport and the ATP-hydrolyzing activity of the Ca\textsuperscript{2+}-ATPase molecule.

Indirect evidence suggesting the electrogenericity of the SR Ca\textsuperscript{2+} pump has been provided by various methods. The generation of a membrane potential (inside-positive) upon Ca\textsuperscript{2+} uptake by the intact SR vesicle was claimed on the basis of measurement of ion-pump current of a proton-translocating FIFO-ATPase. The incorporation of ion-pump molecules into a planar bilayer lipid membrane is most desirable, since the interpretation of the results obtained with such a system is straightforward. The planar bilayer membrane system has been providing useful results as a reconstitution system for various ion channels. Since the turnover is much slower in ion pumps than in ion channels, the incorporation of a large number of pump molecules is necessary for measurement of ion-pump currents. Hirata and co-workers (13, 14) devised a new technique for this purpose and succeeded in measuring the pump current of a proton-translocating P\textsubscript{1}O\textsubscript{1}-ATPase.

By applying this technique to the SR Ca\textsuperscript{2+}-ATPase, we succeeded in measuring the steady-state membrane current caused by Ca\textsuperscript{2+}-transporting activity of the Ca\textsuperscript{2+}-ATPase, and directly demonstrated its electrogeneric function.

MATERIALS AND METHODS

Membrane Preparation—A light fraction of SR vesicles was prepared from rabbit white skeletal muscle as described previously (15). A nonionic detergent, dodecyl nonaoxyethyleneglycol monoether (C\textsubscript{12}E\textsubscript{9}), was purchased from Nikko Chemicals, Tokyo. Asolectin (soybean phospholipid, type II-S; Sigma) was partially purified according to the method of Kagawa and Racker (16). Chelex 100 and Bio-Beads SM-2 were from Bio-Rad. EGTA was purchased from Dojindo, Kumamoto, Japan. Other reagents used were of reagent grade. The protein concentration was determined by the biuret method.

The SR vesicles (2-4 mg of protein/ml) were suspended in a solution containing 0.1 M KCl, 5 mM MgCl\textsubscript{2}, 5 mM CaCl\textsubscript{2}, 20%
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short-circuit membrane current of 10–15 pA directed from the cis to the trans compartment was observed, as shown in Fig. 1. This current disappeared after removal of ATP from the cis compartment by perfusion with KMC buffer. This cycle, the significant current flow in the presence of ATP and its disappearance upon ATP removal, was observed repeatedly (Fig. 1).

In order to verify that the current observed above was due to active Ca\(^{2+}\) pump molecules incorporated into the planar bilayer membrane, we carried out several control experiments. Fig. 2 shows the first control experiment with a planar lipid membrane, where the procedure for the incorporation of the Ca\(^{2+}\)-ATPase molecules was omitted. Membrane current was not observed upon application of ATP. This trace also demonstrates the quality of the present current measurements: acceptable signal-to-noise ratio and stability over 150 min.

The second control experiment showed that addition of ADP instead of ATP did not generate any significant mem-
brane current, as shown in Fig. 3. The incorporation of Ca\(^{2+}\)-ATPase molecules into the planar membrane was first confirmed by observing the generation of membrane current upon addition of ATP to the cis compartment. After ATP had been removed by perfusion with KMC buffer, the membrane current dropped to zero. Addition of ADP up to 4.4 mM did not cause any significant increase in the current level. After perfusion to remove the ADP, readdition of ATP again caused the current to increase.

The third control experiment was carried out to demonstrate that the presence of Ca\(^{2+}\) ions was essential for generation of the membrane current. Incorporation of Ca\(^{2+}\)-ATPase molecules into the planar lipid membrane was confirmed by the observation that addition of ATP to the cis compartment produced the membrane current. Then, the cis compartment was perfused with KMC buffer containing 10 mM EGTA to lower the free Ca\(^{2+}\) concentration to below 10\(^{-7}\) M, at which the Ca\(^{2+}\)-ATPase cannot be activated. Addition of ATP (up to 4.4 mM) to the cis solution did not cause any significant change in current level.

**Dependence of Ca\(^{2+}\) Pump Current on ATP Concentration—**

The membrane current caused by the activation of Ca\(^{2+}\)-ATPase molecules incorporated into a planar bilayer lipid membrane was dependent on the concentration of ATP added to the cis compartment. Fig. 4 shows membrane currents generated in the presence of ATP within a concentration range of 0.27–3.2 mM. It also shows that removal of ATP at the end of the experiment brought the membrane current again to zero. The membrane current was about 10 fA at 0.27 mM ATP, increased with the ATP concentration, and became almost saturated at about 25 fA at 3.2 mM ATP.

More precise values for the membrane current at each concentration of ATP were obtained from the amplitude histograms. These were drawn based on microcomputer analysis of the stored data and some of them are shown in Fig. 5. The abscissa represents the amplitude shown in the abscissa was observed. Data were obtained from the same experiments as that in Fig. 4 and had been stored in a digital data recorder. The data were retrieved, fed through a low-pass filter with time constant of 1 s, digitized, stored on floppy disks, and analyzed as described under “Materials and Methods.” The four well-separated peaks, from right to left, correspond to the currents obtained with 0, 0.27, 0.53, and 3.2 mM ATP, respectively.

**FIG. 5. A histogram of current amplitudes obtained with various concentrations of ATP.** The abscissa represents the current amplitude in increments of 0.48 fA. The ordinate represents the relative value of the total period of time for which the current amplitude shown in the abscissa was observed. Data were obtained from the same experiments as that in Fig. 4 and had been stored in a digital data recorder. The data were retrieved, fed through a low-pass filter with time constant of 1 s, digitized, stored on floppy disks, and analyzed as described under “Materials and Methods.”

**FIG. 6. Relationship between ATP induced membrane current (I) and ATP concentration ([ATP]).** Points represent experimental data taken from the same observation as that in Figs. 4 and 5. The solid line was drawn according to the equation 

\[
I = I_{\text{max}}/(1 + K_m/[ATP])
\]

using values of 30.7 fA for \(I_{\text{max}}\) and 0.56 mM for \(K_m\). This set of values was obtained after best fitting using a linear regression method.

![Fig. 4. Effect of ATP concentration on the Ca\(^{2+}\) pump current.](image)

**FIG. 4. Effect of ATP concentration on the Ca\(^{2+}\) pump current.** Purified SR Ca\(^{2+}\)-ATPase molecules were incorporated into a planar bilayer lipid membrane consisting of asolectin as described under “Materials and Methods.” After formation of the Ca\(^{2+}\)-ATPase incorporated membrane, the cis compartment was perfused with KMC buffer. Then, ATP was added to the cis compartment successively. Numerals written below the trace indicate the ATP concentration in millimolar in the cis compartment.
constant \( (K_c) \) were assumed to be 30.7 fA and 0.56 mM, respectively.

Table I summarizes the results of three measurements of the ATP concentration-dependence of the Ca\(^{2+}\) pump current. The value for the maximum current varied considerably from run to run. This is natural because the maximum current depends on the number of Ca\(^{2+}\)-ATPase molecules incorporated into the planar bilayer of lipid, and this in turn varies from run to run. On the other hand, the \( K_c \) values were very close to each other and the average \( \pm \) standard deviation was 0.69 \( \pm 0.16 \) mM.

**DISCUSSION**

All the findings described above indicate that the membrane current was observed only in the presence of Ca\(^{2+}\)-ATPase molecules activated by appropriate concentrations of ATP and Ca\(^{2+}\) in the presence of K\(^+\) and Mg\(^{2+}\) ions. Therefore, we can safely conclude that the membrane current observed in the present experiments was a Ca\(^{2+}\) pump current caused by the electrogenic Ca\(^{2+}\)-transporting ATPase molecules. This is the first direct demonstration of the electrogenericity of SR Ca\(^{2+}\)-transporting ATPase. It is also noteworthy that this is the first measurement of Ca\(^{2+}\) pump activity obtained in a true steady-state, which was not realized in previous experiments using SR membrane vesicles or reconstituted vesicles. A number of investigators have reported that the ATPase activity of intact SR vesicles or of solubilized preparations depends on the ATP concentration (20). ATP-binding experiments have also been performed (21). Although there is little variance among the reported values, the previous results can be recapitulated as follows (20). The Ca\(^{2+}\)-ATPase molecule has two distinct sites for ATP binding. One with high affinity \( (K_D = 2-3 \, \mu M) \) is the catalytic site, while the other with low affinity \( (K_D = 0.5 \, \text{mM}) \) may be the regulatory site. The latter may be responsible for the increased ATPase activity seen at high substrate concentrations. The present study revealed that the \( K_c \) value \( (0.69 \pm 0.16 \, \text{mM}) \) for ATP dependence of the steady state Ca\(^{2+}\) transporting activity of the purified Ca\(^{2+}\)-ATPase molecules agrees well with the \( K_c \) value for the low-affinity of ATP-binding site. This implies that there is tight coupling between ATP hydrolysis and Ca\(^{2+}\)-transporting activity in the millimolar range of ATP concentration, and that the low-affinity ATP binding site is really responsible for the increased turnover of Ca\(^{2+}\)-transporting ATPase activity.

The number of Ca\(^{2+}\)-ATPase molecules incorporated into a planar bilayer lipid membrane can be estimated in the following way. The ATP-hydrolyzing activity of SR Ca\(^{2+}\)-ATPase has been reported to be about 1 \( \mu \text{mol} \) of ATP/mg of protein/min at 25 °C under optimal conditions (29), similar to those of the present study. The molecular weight of the Ca\(^{2+}\)-ATPase molecule is 1.1 \( \times \) 10\(^{6}\), and it is assumed that two Ca\(^{2+}\) ions are translocated with hydrolysis of one ATP molecule. The orientation of the Ca\(^{2+}\)-ATPase molecule in reconstituted proteoliposomes is reported to be random (23), and hence, is the orientation of the molecule incorporated into a planar lipid membrane. This implies that only half of the Ca\(^{2+}\) pump molecules can be activated upon addition of ATP to the cis compartment. Based on these facts and assumptions, generations of the maximum current of 30 fA requires the incorporation of 5.1 \( \times \) 10\(^4\) active Ca\(^{2+}\)-ATPase molecules into the planar membrane. This value is the same order as that for H\(^+\)-ATPase reconstituted into a planar bilayer membrane (13). It is remarkable that the planar bilayer lipid membrane has such a high resistance (more than 250 GΩ) even after such a large number of protein molecules have been incorporated. This is a great advantage when using the planar bilayer lipid membrane system for measurement of the small steady-state current generated by electrogenic ion-transporting pumps.

In order to investigate the molecular mechanism of the SR Ca\(^{2+}\)-transporting ATPase, both ATPase activity and Ca\(^{2+}\)-pumping activity must be measured and analyzed under strictly controlled conditions. This is difficult to achieve using vesicles. It is not possible to measure the real steady-state activity because the accumulation of Ca\(^{2+}\) ions inside the vesicles inhibits the Ca\(^{2+}\)-ATPase activity. For this reason, steady-state ATPase activity was previously measured in a solubilized system. Solubilization, of course, eliminated the possibility of measuring the corresponding Ca\(^{2+}\)-transporting activity. The present study has thus demonstrated that a planar bilayer lipid membrane system is feasible for the measurement of Ca\(^{2+}\)-transporting activity in a steady-state. The system seems to be a most desirable one, since membrane potential, as well as the ionic conditions on both sides of the membrane, are rigorously controllable.

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