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Electrical pump currents generated by the Ca^{2+}-ATPase of sarcoplasmic reticulum vesicles adsorbed on black lipid membranes

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Sarcoplasmic reticulum vesicles adsorbed on a black lipid membrane generate an electrical current after a fast increment of the concentration of ATP. This demonstrates directly that the sarcoplasmic Ca^{2+}-ATPase from skeletal muscle acts as an electrogenic ion pump. The increment of the concentration of ATP is achieved by the photolysis of caged ATP (P^3-1-(2-nitro)phenylethyl adenosine 5′-triphosphate) a protected analogue of ATP (Kaplan, J.H. et al. (1978) Biochemistry 17, 1929–1935), which is split into ATP and 2-nitroso acetophenone. The release of ATP leads to a transient current flow across the lipid membrane indicating that the vesicles are capacitatively coupled to the underlying lipid membrane. In addition to this transient signal, a stationary current flow is obtained in the presence of ionophores which increase the conductance of the bilayer system and prevent the accumulation of Ca^{2+} in the lumen of the vesicles. The direction of the transient and the stationary current is in accordance with the concept that Ca^{2+} is pumped into the lumen of the vesicles. The transient current depends on the concentration of ATP, Ca^{2+} and Mg^{2+} as would be the case for a current generated by the sarcoplasmic Ca^{2+}-ATPase. Its amplitude is half-maximal at 10 μM ATP and 1 μM Ca^{2+}. At Ca^{2+} concentrations above 0.1 mM the amplitude of the current signal declines again. The Mg^{2+} concentration dependence of the current amplitude at a constant ATP concentration indicates that the MgATP complex is the substrate for the activation of the current. The pump current is inhibited by vanadate and ADP. No current signal is observed if caged ATP is replaced by caged ADP. However, the release of ADP from caged ADP generates a pump current in the presence of an ATP generating system such as creatine phosphate and creatine kinase.

Introduction

The Ca^{2+}-ATPase located in the membrane of the sarcoplasmic reticulum is an effective transport system to keep the cytoplasmic Ca^{2+} activity at or below 0.1 μM. Much effort has been devoted to elucidate the molecular mechanisms of its function and elaborate kinetic schemes (Fig. 1) have been suggested to describe its reaction mechanism (for reviews, see Refs. 1–4).

It has been suggested that the Ca^{2+}-ATPase is an electrogenic ion pump (Refs. 5–10, but see Ref. 11 for an opposing view). Up to now direct measurement of the electrical pump current was not possible. Recently a method was described which allows the measurement of electrical pump currents produced by the $(Na^+ + K^+)$-ATPase from kidney membranes [12]. Here we show that the same procedure can be used to study electrical pump currents generated by sarcoplasmic reticu-

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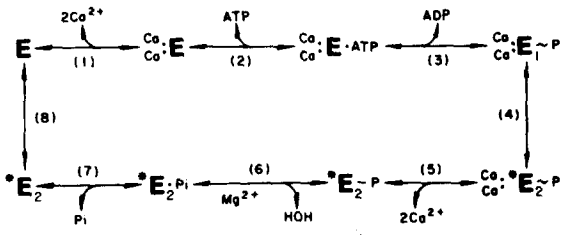

Fig. 1. Reaction cycle of the Ca^{2+} ATPase (according to Ref. 2). Binding of two Ca^{2+} and 1 ATP to the enzyme (step 1 and 2) is followed by the phosphorylation of the enzyme (step 3) a conformational change of the phosphoenzyme (step 4) and the release of Ca^{2+} (step 5) and phosphate (step 7). The cycle is completed by the isomerization of the enzyme to the original state (step 8).

lum vesicles adsorbed to a lipid bilayer membrane. The Ca^{2+}-ATPase of sarcoplasmic reticulum is activated by the photolysis of the protected caged ATP [13] using an ultraviolet light flash which increases the ATP concentration within a few milliseconds. In this report it is demonstrated that the Ca^{2+}-ATPase generates an electrical current and is therefore an electrogenic ion pump. In order to establish this new method as a tool to investigate the molecular mechanisms of the Ca^{2+}-ATPase some elementary properties of the current generated by the Ca^{2+}-ATPase are compared with results obtained before with other methods.

Methods

Sarcoplasmic reticulum vesicles were prepared from rabbit white skeletal muscle as described before [14,15]. The final pellet was suspended in 20 mM histidine buffer (pH 7.2) containing 200 mM sucrose. Small samples of the vesicle suspension were frozen and stored at $-40\,^{\circ}C$ until use. Purified Ca^{2+}-ATPase [16] was reconstituted into asolectin vesicles in a way similar to that described by Shigekawa and Wakabayashi [17]. The synthesis of caged ADP will be described elsewhere (Grell et al., to be published). The modification of the synthesis of caged ATP (P^3-1-(2-nitro)phenylethyladenosine 5′-triphosphate) and other experimental procedures have been described before [12]. In brief: A black lipid membrane (area about 0.01 cm^2) was formed in a hole of a septum separating two compartments of a teflon cell. Lipid membranes were formed from solutions containing diphytanoylphosphatidylcholine (1.5% w/v, Avanti Biochemicals, Birmingham) plus positively charged octadecylamine (0.025% w/v, Riedel-de-Haen, Hannover, F.R.G.) solved in *n*-decane [18]. Both compartments of the cuvette were filled with electrolyte. If not indicated otherwise, the electrolyte standard solution contained 40 mM K_2SO_4, 50 mM Tris-HCl, 5 mM $MgSO_4$, 0.6 mM EGTA, 0.5 mM $CaCl_2$, 1 mM dithiothreitol (pH 6.8). The temperature was 20 to 22 °C. In order to photolyze the caged ATP light pulses (0.125 s) from a 200 W Mercury-Xenon high-pressure lamp (Hannovia 401-B1) or a Xenon high-pressure lamp (Osram XBO 150 W-1) were used. Quartz optics were used throughout.

Vesicles (0.13 to 0.3 mg protein/ml final concentration) and caged ATP (final concentration 100 μM) were added to the *cis*-compartment (Fig. 2A). Stable current signals were obtained about 1 hour after the addition of vesicles. Light pulses of 125 ms duration with an intensity between 0.1 and 4 W/cm^2 were applied in intervals of 5 or 10 min. The current flow across the lipid bilayer is measured with a current-to-voltage converter (model 427, Keithley). The amount of ATP released by a light pulse was estimated by the luciferin-luciferase assay (Boehringer Mannheim, F.R.G.) taking into account the geometry of the optical pathway in the *cis*-compartment of the cuvette. Depending on the intensity of the light source between 1 to 40% of the caged ATP were converted within the light beam. This corresponds to a concentration change by 1 to 40 μM ATP in the vicinity of the membrane. ATP concentrations indicated in the text always refer to the ATP concentration near the membrane after a light flash. This concentration may be approximately constant for several seconds as indicated by the stationary current (Fig. 3B). The illuminated volume is about 1% of the total volume of the *cis*-compartment. Thus 0.2% or less of the total caged ATP is split by a single flash and the consumption of caged ATP is negligible if the time interval between successive flashes is long enough to allow the equilibration of concentration gradients within the cuvette. The light intensity can be decreased by using calibrated neutral density filters for ultraviolet light (Melles and Griot, Irvine, CA). In some experiments

apyrase (3 units/ml, grade 5, Sigma, München, F.R.G.) was added to avoid the accumulation of ADP within the cuvette during an experiment.

Free Ca^{2+} and Mg^{2+} concentrations are calculated by a program kindly provided by Dr. Burckhardt (Frankfurt). The stability constants of the divalent cations with the chelating agents EGTA and EDTA and ATP are those published by Raaflaub [19] and O'Sullivan and Smithers [20]. The Ca^{2+} ionophore A23187 was supplied by Sigma (München, F.R.G.) and Serva (Heidelberg, F.R.G.), respectively. The ionophore 1799 was kindly provided by Dr. Heytler (DuPont, Wilmington, DE). The protonophore 1799 is very suitable for these experiments because it is insensitive to ultraviolet light at wavelengths above 210 nm (Ref. 21 and Christensen, B., unpublished data).

Non-linear curve fitting is performed by a modified Marquardt algorithm. Current densities given in the figures refer to the area of the black lipid membrane, not to the surface area of the vesicles.

The 2,4-dinitrophenylphosphatase activity of the Ca^{2+}-ATPase is measured at different concentrations of caged ATP with 1 mM lutidinium 2,4-dinitrophenyl phosphate in 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.01 mM EGTA and 25 mM imidazole-HCl containing 5 mM or 40 mM KCl and 60 μg enzyme/ml at pH 6.8 and 37°C.

Results

General properties of ATP-dependent currents

Fig. 2A shows a schematic representation of the cuvette and the optical pathway and Fig. 2B depicts a vesicle adsorbed to the black lipid bilayer with the major transport systems (Ca^{2+}-ATPase and ionophores). The electrical equivalent circuit corresponding to the black lipid membranes and the adsorbed vesicles is depicted in Fig. 2C [12,22–24]. Vesicles containing membrane-bound Ca^{2+}-ATPase are adsorbed on one side of the lipid bilayer membrane. Upon illumination with an ultraviolet light flash (125 ms, 0.1 to 4 W/cm^2) ATP is released from caged ATP resulting in an ATP concentration jump of about 1 to 40 μM depending on the light intensity and the concentration of caged ATP. After the release of ATP the Ca^{2+}-ATPase will start to pump Ca^{2+} ions

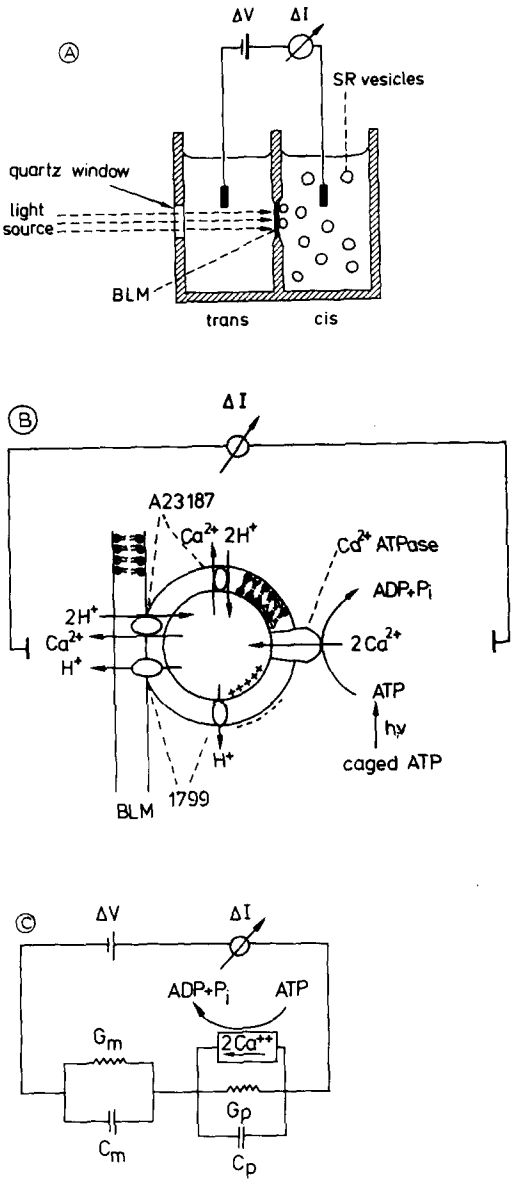

Fig. 2. Schematic representation of the bilayer setup. (A) Teflon chamber with black lipid bilayer (BLM) and adsorbed sarcoplasmic reticulum (SR) vesicles. Note the definition of the *cis* and *trans*-compartments. A battery (ΔV) and a current to voltage converter (ΔI) are connected to the compartment of the cuvette via KCl/Agar bridges and Ag/AgCl electrodes. (B) Proposed experimental situation of SR vesicles adsorbed to a BLM and possible ion fluxes due to pump activity of the Ca^{2+}-ATPase and other ionophores. (C) Electrical equivalent circuit of the BLM and adsorbed SR vesicles. G_m represents the ohmic conductance of the combined parts of the BLM and the SR vesicles. G_p is the conductance of the residual vesicle membrane. C_m and C_p are the capacities of the two membrane systems.

into the adsorbed vesicles making the inside of the vesicles positive. The equivalent circuit (Fig. 2C) shows that charging of the vesicular membrane will drive a current through the underlying membrane. The direction of the current flow should be from the compartment containing vesicles (*cis*-compartment) to the compartment without vesicles (*trans*-compartment, see Fig. 2A) if Ca^{2+} is pumped into the vesicles. Part of the pump current will flow across the vesicular membrane and another part will flow across the combination of the vesicular membrane and the black lipid membrane. Only the latter part can be measured under these conditions. The equivalent circuit also predicts that the current flow across the combined membrane system will be transient. In the beginning most of the membrane current will flow through the membrane capacity C_m, but as soon as C_m is charged the current flow will be mostly across the conductance G_m of the combined membrane. In practice G_m is so low that a stationary current flow is hardly detectable without additional application of ionophores (see below).

Some properties of the pump current generated by vesicles adsorbed to a black lipid bilayer are shown in Fig. 3. Trace A demonstrates the transient behaviour of the observed current after the release of ATP by an ultraviolet light flash. Here, as in all other experiments, the direction of the current suggests that positive charges flow into the vesicles. To demonstrate that the transient current is not only due to the displacement of intrinsic charges of the Ca^{2+}-ATPase during the first cycle of the pump after the concentration jump, it is necessary to demonstrate a continuous current flow during several pump cycles. To achieve this the Ca^{2+}/H^+ exchanging ionophore A23187 and the protonophore 1799 were added to the *cis*-compartment. The protonophore 1799 increases the conductance of the membrane system and the neutral exchanger A23187 is used to prevent the accumulation of Ca^{2+} in the vesicles which may lead to inhibition of the pump activity [25]. Control experiments show that ultraviolet light itself does not affect the conductance of the black lipid membrane in the presence of both ionophores in the absence of caged ATP.

In the presence of A23187 and 1799 the transient current signal is followed by a quasi-stationary phase (Fig. 3B) indicating continuous activity of the pump. The slow decline of the stationary current is probably due to a decrease of the concentration of ATP near the bilayer membrane by enzymatic hydrolysis and by diffusion of ATP in the non-illuminated parts of the cuvette. Fig. 3C also shows that the stationary phase is largely abolished if the decay of the ATP concentration is accelerated by the addition of hexokinase (27 units/ml) and glucose (1 mM).

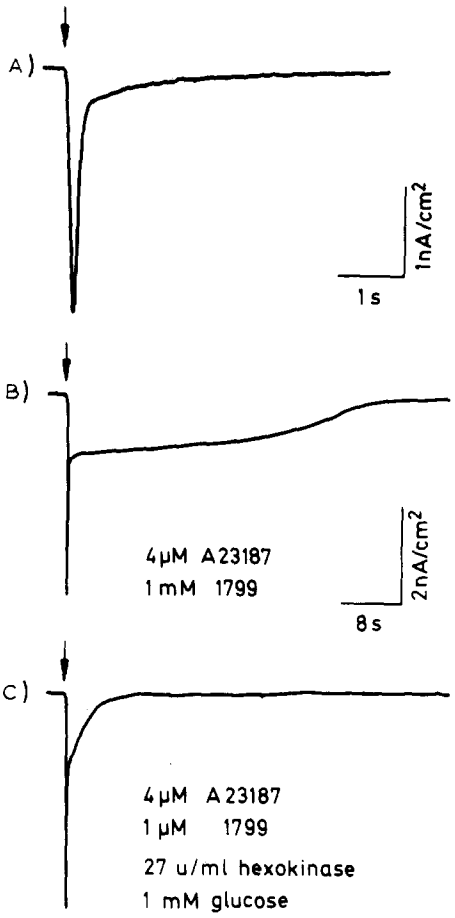

Fig. 3. Short-circuit current across the lipid bilayer system after a concentration jump of ATP. The arrow indicates an ultraviolet light flash of 125 ms, which increases the ATP concentration by about 20 μM. (A) Transient short circuit current in the absence of ionophores. (B) Demonstration of a stationary current after the addition of the ionophores A23187 (4 μM) and 1799 (1 μM). (C) The duration of the stationary current is reduced after the addition of hexokinase and glucose to convert ATP to ADP (Same calibration as in B). Note the different time scales in parts A and B.

Fig. 4. Short-circuit current across the black lipid bilayer in the presence of asolectin vesicles reconstituted with purified Ca^{2+}-ATPase. Both compartments contained 0.1 M K_2SO_4, 10 mM Tris, 0.5 mM $MgSO_4$, 0.5 mM $CaCl_2$, 0.6 mM EGTA at pH 6.8.

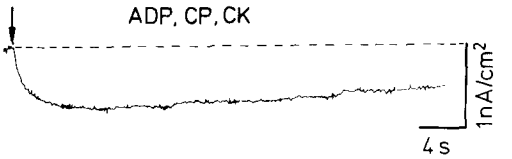

Fig. 5. Short-circuit current after a concentration jump of ADP in the presence of creatine kinase (100 units/ml) and creatine phosphate (1 mM) to convert ADP into ATP. The *cis*-compartment contained 5 μM caged ADP. The yield was 11% per flash.

The same kind of transient and stationary current signals is obtained with asolectin liposomes reconstituted with purified Ca^{2+}-ATPase from sarcoplasmic reticulum (Fig. 4).

If only the Ca^{2+} ionophore A23187 is added no stationary current is observed but the amplitude of the transient current is increased indicating that there is significant accumulation of Ca^{2+} inside the vesicles within 150 ms which may cause 'back-inhibition' [25].

The transient and stationary current signals are blocked by the addition of vanadate which is known to inhibit the activity of the Ca^{2+}-ATPase [26,27]. A 50% inhibition is obtained at about 40 μM vanadate. Further evidence that the current signal is specific to the Ca^{2+}-ATPase is provided by experiments in which caged ATP is replaced by caged ADP. A light-induced release of ADP (up to 50 μM) does not generate a measurable current signal. If, however, an ATP generating system like creatine phosphate and creatine kinase is added, then the photolysis of caged ADP leads to the generation of a stationary current (Fig. 5) in the presence of ionophores. A transient current is not observed in this experiment possibly because the formation of ATP from ADP is slow at the low concentration of ADP used. The addition of ATP of [γ-S]ATP prior to a light-flash effectively depresses the signal produced by the release of ATP from caged ATP, possibly because the relative amplitude of the concentration change is reduced.

Oxalate (5 mM) which increases the capacity of vesicles to take up Ca^{2+} [14, 28] does not affect transient and stationary current signals obtained in the presence of 0.1 μM valinomycin. In the absence of ionophores a slow increase of the transient current by a factor 1.5 to 2 is observed within 20 to 30 min after the addition of oxalate.

Similar transient and stationary current signals are obtained if K^+ is replaced by Na^+ and if SO_4^{2-} is replaced by Cl^- or cyclamate. A quantitative comparison is, however, not performed.

In the following sections the Ca^{2+}, Mg^{2+}, ATP and ADP dependence of the transient current will be described. Qualitatively the stationary current depends on the Ca^{2+}, Mg^{2+} and ATP concentration in a similar manner as the transient current. The effect of ADP on the stationary current was not investigated.

Ca^{2+} dependence of the transient current

The Ca^{2+} dependence of the transient current signal is determined by the addition of increasing amounts of calcium dicyclamate or $CaCl_2$ to the electrolyte solution in both compartments containing a fixed amount of EGTA. The concentration of free Ca^{2+} is then calculated as described in methods. Changes of pH are less than 0.01 pH units if the total Ca^{2+} concentration is increased from 0.2 to 2 mM. Fig. 6 shows that the amplitude of the transient current signal increases with increasing free Ca^{2+} concentration, saturates at a free Ca^{2+} concentration of about 10 μM and decreases again at concentrations greater than 0.1 mM. In the range of low free Ca^{2+} concentrations the amplitude is half-maximal at about 1 μM (range 0.5 to 1.2 μM). The experiment shown in Fig. 6 is performed in the presence of 4 μM A23187, a Ca^{2+} ionophore which establishes equal Ca^{2+} concentrations on both sides of the vesicle membrane. A very similar concentration dependence is observed if the experiment is performed in the absence of the Ca^{2+} ionophore provided

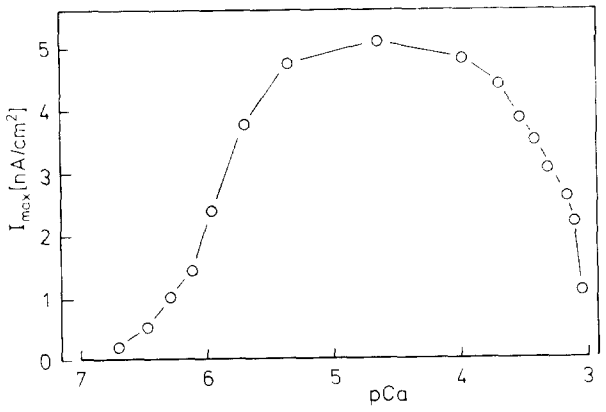

Fig. 6. Ca^{2+} dependence of the peak short-circuit current. Both compartments contained 80 mM KCl, 5 mM $MgCl_2$, 50 mM Tris-Hepes, 1 mM EGTA at pH 6.8. The initial total Ca^{2+} concentration was 0.2 mM. The *cis*-compartment contained 4 μM A23187. The ultraviolet light flash increased the ATP concentration by 6 μM.

there is enough time for Ca^{2+} to equilibrate across the membrane. The ATP concentration is either 1 or 10 μM in different experiments. There is no significant effect of the ATP concentration on the Ca^{2+} dependence. No qualitative difference is seen between experiments using KCl or sodium cyclamate as the major electrolyte.

Mg^{2+} dependence of the transient current

The effect of Mg^{2+} on the transient current signal is determined by the variation of the total Mg^{2+} concentration while the amount of ATP released is kept constant (20 μM). Binding of Mg^{2+} by EGTA does not change the Mg^{2+} concentration significantly but the free Ca^{2+} concentration increases by about 30% at 10 mM Mg^{2+}. Fig. 7 shows the Mg^{2+} dependence of the transient current signal. The transient current signal is maximal at about 1 mM total Mg^{2+} and at higher concentrations the signal declines by about 30% of the maximum (see inset Fig. 7). For Mg^{2+} concentrations less than 1 mM the Mg^{2+} dependence can be fitted by the Michaelis-Menten formalism with $K_m = 53 \pm 4.2$ μM ($\bar{x} \pm$ S.E., $n = 4$).

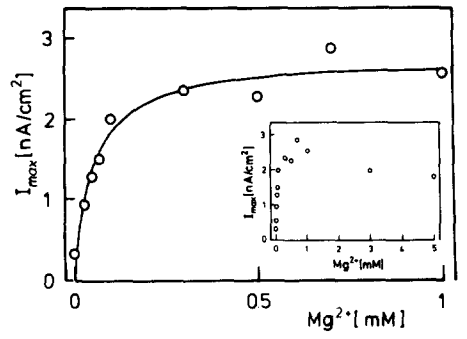

Fig. 7. Mg^{2+} dependence of the peak short-circuit current. For Mg^{2+} concentrations ≤ 1 mM the Mg^{2+} dependence can be described by the Michaelis-Menten formalism with an apparent $K_m = 66$ μM. The inset shows that the amplitude of the peak current decreases for Mg^{2+} concentrations > 1 mM. The dots represent the average of two successive measurements. The light-induced ATP concentration was 20 μM.

ATP dependence of the transient current

The ATP dependence of the amplitude of the transient current is determined in the presence of 5 mM Mg^{2+} which assures that more than 95% of ATP is in the form of MgATP. The ATP dependence is determined using two different methods. In the first case the ATP concentration is increased by adding increasing amounts of caged ATP to the *cis*-compartment and applying identical light flashes to photolyze caged ATP. In the other case a constant high concentration of caged ATP is added and the concentration of ATP is manipulated by adjusting the intensity of the light flash with calibrated filters (see Methods). Both methods can be used in the same experiment by first increasing the concentration of caged ATP to the final concentration and then decreasing the light intensity. An experiment of this kind is shown in Fig. 8A. The concentration of caged ATP is increased up to 0.2 mM and the maximal concentration of ATP is 22 μM. Afterwards the light intensity is decreased in the same experiment as described above. Both dose-response curves are identical within experimental error and can be described by the Michaelis-Menten formalism. The apparent K_m values are 9.7 μM (addition of caged ATP) and 7.4 μM (variation by light intensity). The extrapolated maximal currents are 176 and 180 pA. The curve fit is less good for the relation obtained by the variation of caged ATP. The stepwise addition of caged ATP lasts for 2.5 h and there is probably a slight drift of the current amplitude superimposed. On the average the apparent K_m for the ATP dependence is 10 ± 2 μM ($x \pm$ S.E., $n = 4$).

The addition of the protonophore 1799 which reduces the potential difference across the vesicular membrane created by the pump current increases the amplitude of the transient current but does not modify the apparent K_m. Fig. 8B shows the dose-response curve in the presence of 4 μM A23187 and in the presence of additional 4 μM 1799. Curve fitting according to the Michaelis-Menten formalism shows that the maximal current increases by 53% but the apparent K_m remains nearly constant (8.7 vs. 8.8 μM).

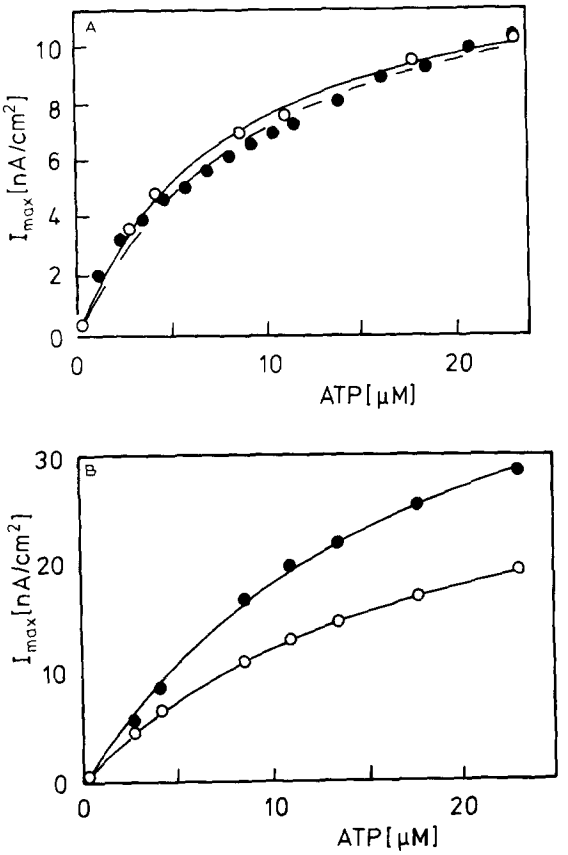

Fig. 8. ATP dependence of the peak short-circuit current. (A) The ATP dependence was determined first by increasing the concentration of caged ATP in the *cis*-compartment in steps to 200 μM at a fixed yield of 11% per light flash (●). Then the release of ATP per light flash was decreased by changing the intensity of the light flash with neutral density filters (○). The ATP dependency obtained by the variation of the concentration of caged ATP could be fitted by the Michaelis-Menten formalism with $K_m = 9.7$ μM (broken line) and for the ATP dependency obtained by the variation of the light intensity a K_m of 7.4 μM was obtained. (B) Effect of the protonophore 1799 on the ATP dependence of the peak short-circuit current. The amount of ATP released per flash was changed by modifying the intensity of the light at a constant concentration of caged ATP (200 μM), the maximal yield was 11% per flash. The plot shows the ATP dependence in the presence of 4 μM A 23187 (○) and in the presence of 4 μM A23187 plus 1 μM 1799 (●, same experiment as before). The continuous lines indicate Michaelis-Menten kinetics with K_m values of 8.7 μM (A23187) and 8.8 μM (A23187+1799).

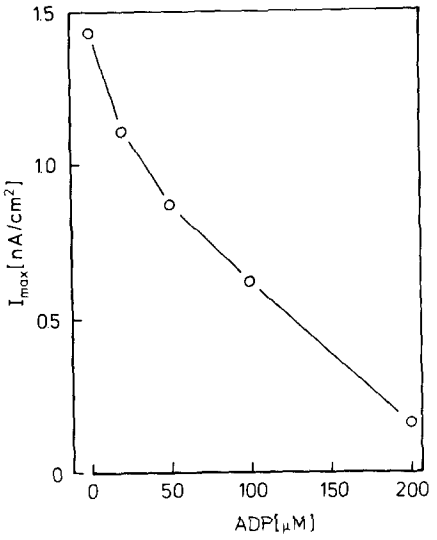

Fig. 9. Inhibition of the peak short-circuit current by ADP. The ATP concentration was 1 μM after an ultraviolet light flash. The electrolyte was 40 mM sodium cyclamate, 5 mM $MgSO_4$, 30 mM Tris, 0.4 mM EGTA, 0.4 mM calcium dicyclamate at pH 6.8.

ADP dependence of the transient current

The effect of ADP on the amplitude of the transient current is investigated by adding increasing amounts of ADP to the *cis*-compartment. The ATP concentration after the light flash is about 1 μM and the free Ca^{2+} concentration is 0.6 μM. Fig. 9 shows the inhibitory effect of ADP on the maximum of the transient current. The amplitude is reduced by 50% at 76 ± 24 μM ADP ($\bar{x} \pm$ S.E., $n = 6$). The inhibitory effect of ADP is reversed if apyrase (3 units/ml) which splits ADP is added to the *cis*-compartment.

Discussion

General properties of the system

In the foregoing sections data are presented

which demonstrate the generation of an electrical current flow across a lipid bilayer doped with adsorbed vesicles after a fast increment of the concentration of ATP. This current signal shows many properties which can be anticipated for an electrical current generated by the Ca^{2+}-ATPase. The direction of the current flow is compatible with the flow of Ca^{2+} into the lumen of the vesicles thus generating a positive potential between the inside of the vesicles and the *trans*-compartment of the cuvette (Fig. 2). The observation of stationary pump currents and their dependence on ATP provides evidence that the observed currents are generated by the continuously running ATP dependent pump and not by a mere displacement of electrical charges within the membrane.

The amplitude of the current signal depends on the ATP, Ca^{2+}, Mg^{2+} and ADP concentration in a way expected for the Ca^{2+}-ATPase from sarcoplasmic reticulum (see below). Furthermore, the release of ADP from caged ADP does not produce a current signal. A stationary current is, however, generated if caged ADP is photolyzed in the presence of creatine kinase and creatine phosphate which convert ADP into ATP. The transient current is inhibited by free ATP or [γ-S]ATP which reduce the relative amplitude of the concentration jump. Vanadate which inhibits the Ca^{2+}-ATPase [26,27] also reduces the transient and stationary current. In view of this evidence it seems justified to consider the observed current as a signal related to the pump current generated by the Ca^{2+}-ATPase. Therefore the measured current will be called 'pump current' in the following sections although it should be clear that it is distorted by the equivalent circuit shown in Fig. 2C.

Under the present conditions (20 °C, 1 to 20 μm ATP) the maximum of the transient current is reached about 150 ms after the beginning of the light-flash (125 ms duration). As the cycle time of the Ca^{2+}-ATPase under comparable conditions is about 150 to 200 ms [29] it is suggested that most of the enzyme molecules have not yet completed the first cycle at the maximum of the transient current. Thus effects of Mg^{2+} or ATP on the reaction steps following the generation of current should have no effect on the maximum of the transient current.

Electrogenicity of the Ca^{2+}-ATPase

Indirect evidence for the electrogenic function of the Ca^{2+}-ATPase has been previously provided by different approaches. In vesicles as well as in liposomes reconstituted with Ca^{2+}-ATPase the generation of a potential difference across the vesicle membrane (inside positive) is reported [5–8,10]. Furthermore it has been shown that the Ca^{2+}-pump activity accelerates the dissipation of a pre-established membrane potential [9]. A pre-established membrane potential with the inside potential being negative appears to increase the Ca^{2+}-uptake rate while positive polarity will reduce it [5,7,10]. On the other hand the electrogenicity of the Ca^{2+}-ATPase has been questioned by Chiu and Haynes [11].

The observation of current flow across the lipid bilayer membrane after activation of the Ca^{2+}-ATPase is possible only if the pump creates an electrical potential difference across the visible membrane (see Fig. 2C). The direction of the current flow across the lipid bilayer indicates that the inside of the vesicles is positively charged by the active pump in agreement with the assumption that Ca^{2+} is the charge carrier. Thus the results of these experiments provide direct evidence that the Ca^{2+}-ATPase acts as an electrogenic ion pump. The number of net charges translocated per cycle cannot be deducted from these experiments. It may be less than 4, e.g. 1 Ca^{2+} may be exchanged for 1 H^+.

Because of the high conductance of the membrane of the sarcoplasmic reticulum [3] the voltage generated by the pump current may be low and not of physiological relevance but nevertheless the electrogenicity of the Ca^{2+}-ATPase opens new possibilities to investigate the molecular mechanism of this important transport protein.

As mentioned above the measured current represents only a fraction of the total pump current. A rough estimate indicates, that a relatively low number of vesicles may produce pump currents of the order of several nanoamperes. Assuming a vesicle diameter of 100 nm, about 10^4 Ca^{2+}-ATPase molecules per μm^2 [1], 5 cycles per second [29] and 2 Ca^{2+} ions transported per cycle, the pump current generated by one vesicle is about 10^{-15} A and 10^7 vesicles will generate a current of 10 nA. This number of vesicles covers 5% or less

of the total area of the black lipid membrane if it is assumed that 50% or less of the surface area of the vesicles are in contact with the underlying membrane. If all this current (10 nA) were to flow across the underlying membrane this would correspond to a current density of 1 $\mu A/cm^2$, which is much higher than the highest current density observed so far.

Ca^{2+} and Mg^{2+} dependence of the pump current

The pump current is half-maximal activated at free Ca^{2+} concentrations of about 1 μM and at concentrations larger than 0.1 mM it is inhibited. This agrees reasonably with the concentration dependence of the rate of ATP hydrolysis which is half-maximal at about 0.1 to 0.2 μM Ca^{2+} and is inhibited at concentrations larger than 10 to 100 μM [14,30,21,32]. It also agrees with the Ca^{2+} dependence of the stationary concentration of phosphoenzyme, the rate of ATP, ADP + P exchange and the rate of Ca^{2+} uptake which are half-maximal at about 0.1 to 0.3 μM Ca^{2+} [30,33,34]. Ca^{2+} binding studies in the absence of ATP have revealed a high-affinity binding site with a dissociation constant between 0.3 to 0.4 μM and a low-affinity site with a dissociation constant between 1 to 2 mM [16,35,36].

There are at least two explanations for the depressing effect of high Ca^{2+} concentrations on the pump current related to the first cycle of the pump after an ATP concentration jump which are not mutually exclusive. The reduction of the pump current may be due to a shift of the initial equilibrium between the E_1 and E_2 forms of the Ca^{2+}-ATPase (Fig. 1) in such a way that less enzyme is in the form E_1. The other possibility is that in the presence of high Ca^{2+} concentrations inside the vesicle the probability increases that the pump does not complete the cycle but will return to the start position [1], i.e. E_1Ca_2 and this may decrease the net pump current.

At 20 μM ATP the transient current is half-maximal at about 50 μM total Mg^{2+}. Assuming an apparent dissociation constant of about 60 μM [20] about 50% of the ATP forms a MgATP complex at a total Mg^{2+} concentration of 50 μM and at 1 mM Mg^{2+} 95% of the ATP are complexed with Mg^{2+}. Thus the most likely explanation for the stimulating effect of Mg^{2+} on the transient current is that MgATP is the substrate for the electrogenic activity of the Ca^{2+}-ATPase as it is for other forms of its activity (Ref. 37, for more references see Ref. 2). It should be noted, however, that Mg^{2+} in low concentrations may have additional effects on the kinetics of the Ca^{2+}-ATPase by increasing the rate of the transformation from E_1P to E_2P [38].

A decrease of the transient current is commonly observed at Mg^{2+} concentrations above 1 mM. This inhibitory effect of Mg^{2+} may be related to the depressing effect of Mg^{2+} on the rate of phosphoenzyme formation and phosphate release observed in kinetic studies of the early reactions of the enzyme cycle [39]. An inhibitory effect of Mg^{2+} on the steady-state hydrolysis of ATP has been reported by Makinose [33]. The inhibitory effect of Mg^{2+} may be due to a shift of the equilibrium between the E_1 and E_2 state to the E_2 state [40] or to a competition between Ca^{2+} and Mg^{2+} for the high-affinity Ca^{2+}-binding site [41].

ATP dependence of the pump current

In the range of ATP concentrations investigated (40 μM) the ATP concentration dependence of the transient current can be described by the Michaelis-Menten formalism with an apparent K_m of about 10 μM. The ATP dependence is identical if the ATP concentration is adjusted by changing the concentration of caged ATP at constant light intensity or by changing the intensity of the light flash while keeping the concentration of caged ATP constant. This observation strongly suggests that caged ATP does not bind to the Ca^{2+}-ATPase at a site relevant for the catalytic activity of the molecule [42]. If caged ATP bound to the catalytic site of the Ca^{2+}-ATPase or inhibited its activity, then different dose-response curves would be expected because the ratio between ATP and caged ATP (i.e. substrate and inhibitor) is different in both cases at identical concentrations of ATP. In the case of the $(Na^+ + K^+)$-ATPase it has been shown that both methods to change the ATP concentration yield different dose-response curves [55] in agreement with the report that caged ATP binds to the $(Na^+ + K^+)$-ATPase from pig kidney [43]. The suggestion that caged ATP is no inhibitor of the Ca^{2+}-ATPase is also consistent with the result of enzymatic activity studies with 2,4-di-

nitrophenylphosphate which do not show substantial inhibition of hydrolytic activity of the Ca^{2+}-ATPase in the presence of caged ATP (Grell et al., unpublished data).

The apparent K_m determined for the ATP dependence of the transient current (10 μM) agrees with the K_m of 20 μM obtained for the rate of phosphoenzyme formation [29] and for the initial rate of Ca^{2+} uptake [44]. The formation of phosphoenzyme saturates at about 50 μM [44], which agrees with the concentration at which the current amplitude saturates. The K_m value obtained here is somewhat higher than the high-affinity K_m (2–3.5 μM) obtained from studies of the steady-state hydrolysis [30,31,37,45–47]. It is also higher than the dissociation constant of the high-affinity binding-site (2 to 3 μM [45,46,48]), which is generally assumed to be the catalytic binding site [2]. It should be noted, however, that other estimates of the dissociation constant of the catalytic binding site are between 8 μM and 35 μM [47,49,50]. The range of the low-affinity binding site (K_d 0.5 mM) which is assumed to be a regulatory site [2] is not covered in these experiments. It is suggested, however, that this regulatory low-affinity site should have negligible effect on the peak of the transient current because it is assumed that binding to this site accelerates the conformational change from the E_2 to the E_1 form of the enzyme [2] which is not expected to occur within the time to peak of the transient current (see above).

ADP dependence of the pump current

In the presence of Ca^{2+} the Ca^{2+}-ATPase is phosphorylated by ATP and ADP is released from the enzyme [31,51]. The first step of the translocation of Ca^{2+} seems to be the occlusion of Ca^{2+} by the phosphorylated enzyme [52,53]. Since it has been shown that the phosphate group may be transferred from the enzyme to ADP [54] and since Ca^{2+} may be released from the occluded state by the addition of ADP [52] it is possible that the addition of ADP reduces the pump current across the vesicular membrane by reducing the probability that the ATPase performs the conformation change from E_1 to E_2 (Fig. 1). The reduction of the pump current agrees with kinetic experiments which show that the release of phosphate is reduced if ADP and ATP are added simultaneously to the enzyme [39].

As has been shown here the transient current is reduced by 50% at 90 μM ADP in the presence of 1 μM ATP. In the absence of ATP the affinity of the phosphorylated enzyme for ADP is about 3 μM [1]. In the presence of millimolar concentrations of ATP the apparent affinity decreases to about 0.1 to 0.7 mM [4,47].

Another possibility to explain the reduction of the pump current by ADP consists in a competition with ATP for the catalytic ATP binding site. Estimates of the dissociation constant for the binding of ADP to the catalytic ATP binding site range from 3 μM to 90 μM [47,48,50]. A binding site for ADP with a dissociation constant of 18 μM is reported by Møller et al. [46].

Concluding remarks

The experiments show that in the presence of vesicles a rapid increment of the ATP concentration generates a current flow across the black lipid membrane which behaves in many ways as it is expected for an electrogenic Ca^{2+}-ATPase. It should be pointed out, however, that, although the procedure allows the measurement of electrical activity of the Ca^{2+}-ATPase the pump current generated by the Ca^{2+}-ATPase is distorted by the network shown in Fig. 2C. Another drawback of the method is that the voltage across the vesicular membrane generated by the pump activity is not measurable and cannot be affected. A similar problem is raised by changes of ion concentrations inside the vesicles during the pump activity. The comparison of the properties of the transient current with other forms of the activity of the Ca^{2+}-ATPase have shown far-reaching agreement between these different experimental approaches indicating that the transient current is a reliable measure of the properties of the Ca^{2+}-ATPase.

Furthermore the distortion of the pump activity by the vesicular membrane potential may be negligible because the membrane of the sarcoplasmic reticulum is so leaky for monovalent cations and anions that the membrane potential generated by pump currents should not exceed 10 mV [3] and problems due to concentration changes inside the vesicles may be overcome by the use of ionophores.

Also in the presence of ionophores the initial transient current is usually larger than the stationary current. The ratio between the transient and the stationary current may become much smaller than in Fig. 3B if the conductance is further increased by ionophores. Therefore it seems that most of this overshoot is due to the capacitative coupling between the vesicles and the underlying membrane. On the other hand it seems possible, that also the true pump current shows an initial overshoot before it settles to the steady-state value. Such an overshoot may be expected from the kinetics of the phosphoenzyme formation [39]. Under the present conditions, however, this problem is difficult to solve because of the incomplete knowledge of the properties of the membrane system.

Finally it seems appropriate to consider the relation between the actual pump current and the reaction cycle of the Ca^{2+}-ATPase. For simplification it is assumed that the Ca^{2+} translocation succeeds in one step, i.e. there is only one current generating step. This could be step 4 in Fig. 1. If the amount of enzyme in the state preceeding the current generating state is denoted by (A) and that in the following state by (B) then the amplitude of the pump current will be proportional to the difference $k'(A) - k''(B)$; k' and k'' are the rate constants for the transition $\leftrightarrow A \leftrightarrow B \leftrightarrow$. In the forward running mode far from equilibrium i.e. briefly after the concentration jump of the ATP or under conditions which inhibit the accumulation of Ca^{2+} in the vesicle, the backward reaction may be negligible and the current amplitude may be proportional to the concentrations of A and the time-course of the current amplitude will reflect the formation of A. Within the framework of the reaction cycle shown in Fig. 1, state A may correspond to the ADP-sensitive phosphoenzyme $E_1 \sim P$.

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