

# Voltage-dependent pump currents of the sarcoplasmic reticulum $\text{Ca}^{2+}$ -ATPase in planar lipid membranes

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Sarcoplasmic reticulum vesicles containing largely  $\text{Ca}^{2+}$ -ATPase were incorporated into planar lipid membranes. The ATPase was activated by a UV flash-induced concentration jump of ATP from a photolabile caged ATP. Under these conditions stationary pump currents were observed. The dependence of these pump currents on applied voltages was investigated. The current-voltage curve of the  $\text{Ca}^{2+}$ -ATPase shows monotonously increasing pump currents with increasing positive potentials of the ATP containing compartment. This indicates the existence of electrogenic steps in the direction of the transported  $\text{Ca}^{2+}$  ions. From the extrapolated reversal potentials of the curve is concluded that less than four positive net charges are transported per hydrolyzed ATP.

$\text{Ca}^{2+}$ -ATPase; Sarcoplasmic reticulum; Reconstitution; Planar lipid membrane; Voltage dependence; Caged ATP

## 1. INTRODUCTION

The contraction of skeletal muscle is initiated by calcium release from the sarcoplasmic reticulum (SR). During muscle relaxation the calcium must be pumped back to the SR. The enzyme responsible for this process is the  $\text{Ca}^{2+}$ -ATPase. It is generally accepted that during its action in vivo 2 calcium ions are pumped per molecule of ATP hydrolyzed [1]. The knowledge about the stoichiometry, however, is still incomplete and under debate [2,3].

Indirect evidence for an electrogenic action of the  $\text{Ca}^{2+}$ -ATPase has already been presented by the demonstration that the calcium pump activity can be influenced by  $\text{K}^+$ /valinomycin-induced diffusion potentials [4]. More recently, SR-vesicles have been attached to preformed planar bilayer membranes and transient pump currents observed in response to an ATP concentration jump [5]. Increasing the membrane conductance of the underlying bilayer by addition of the  $\text{Ca}^{2+}/\text{H}^+$  exchanger A23187 and the protonophore 1799 induced stationary current responses, thus providing strong evidence for the electrogenicity of the  $\text{Ca}^{2+}$ -ATPase [5]. In both approaches ionophores were used for the demonstration of the electrogenic properties of the enzyme, which could lead to an ambiguous interpretation of electrogenicity demonstrated by indirect means. In addition, nonelectrogenic ion pumps and the anion exchanging band III protein from erythrocytes reconstituted into proteoliposomes and

oocytes have also been shown to respond to applied potentials [6–8].

The most straightforward way to investigate the electrogenicity of an ion pump is, to measure directly the pump currents through a bilayer which contains only the pump. In contrast to the  $\text{Na}^+/\text{K}^+$ -ATPase, which is present in the plasma membrane of several cell types, the  $\text{Ca}^{2+}$ -ATPase is located in an intracellular organelle that is not readily accessible by electrophysiological methods. The same technical limitations exist with regard to vesicular preparations of biological membranes. Therefore, the SR- $\text{Ca}^{2+}$ -ATPase was incorporated into a planar lipid membrane separating two bulk electrolyte phases following a protocol used for the reconstitution of the light-driven proton pump bacteriorhodopsin [9]. This technique is substantially different from that shown in [5]. The main advantage of this method is that the incorporated ion pumps are readily accessible from both sides of the membrane by different electrolytes and by various applied membrane potentials which is impossible in the experiments where a composed membrane system was used [5].

Recently, Nishie et al. [10] reported evidence for an electrogenic activity of the  $\text{Ca}^{2+}$ -ATPase after fusion of liposomes containing the enzyme into planar lipid membranes. Here we demonstrate a substantially improved functional reconstitution of the SR  $\text{Ca}^{2+}$ -ATPase into planar lipid membranes by an alternative method. The use of the caged ATP led to about 10-fold increased pump currents and a much better signal-to-noise ratio. The electrogenicity and voltage dependence of the  $\text{Ca}^{2+}$ -ATPase are unambiguously demonstrated. In addition, the results indicate that

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under these conditions less than four positive net charges are transported per ATP hydrolyzed.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Sarcoplasmic reticulum vesicles (of which about 90% of the membrane protein is  $\text{Ca}^{2+}$ -ATPase) were prepared from rabbit skeletal muscle according to procedures described elsewhere [11,12].

Glycerolmonooleate (GMO) was purchased from Avanti Polar Lipids (Birmingham, AL).

Caged ATP was synthesized and purified according to the method of Kaplan et al. [13].

The luciferin/luciferase assay kit was from Boehringer Ingelheim (FRG). All other reagents and salts were purchased in analytical grade from Merck, Fluka, Sigma or Serva.

### 2.2. Methods

All experiments were carried out at room temperature in home made teflon cuvettes. The two halves were separated by a teflon septum containing a hole of about 150–200  $\mu\text{m}$  diameter.

Membranes were formed by the application of lipid layers preformed from 2% glycerolmonooleate (w/v) in hexadecane and SR vesicles according to a modified Montal and Mueller method [14]. Electrical measurements were performed by use of Ag/AgCl electrodes separated from the light beam by coloured agar bridges. Currents were  $10^{10}$ -fold amplified (rise time 100 ms), converted to voltage signals (Ithaco, model 1211, Ithaca, NY) and monitored on a storage oscilloscope (Nicolet 3091, Offenbach, FRG).

Membrane capacitances were about  $0.2 \mu\text{F}/\text{cm}^2$ , this is an underestimated value because the real bilayer region is smaller than the hole area ( $2$  to  $3 \times 10^{-4} \text{ cm}^2$ ) due to a torus region consisting of bulk organic solvent; the existence of bilayers was checked by application of Gramicidine A, which is known to form ion channels only in true bilayer membranes (not shown). Moreover, from the mean open time of the Gramicidine A channels it is possible to estimate the membrane thickness and, thus, the real bilayer area of the membrane [15]. Since the mean open time is more than 2 s the membrane should have a thickness of about 3.0 nm. From the observed membrane capacitance together with the total hole area results that approximately one-third of the hole area is covered by a bilayer membrane. Membrane conductances were in the range of  $10^{-7} \text{ S}/\text{cm}^2$ .

ATP release from caged ATP was performed by 125 ms UV-light flashes from a mercury high pressure lamp (Osram HBO 200W). The amount of released ATP was measured quantitatively in control experiments by the luciferin/luciferase assay.

In most experiments we used a standard electrolyte consisting of: 40 mM  $\text{K}_2\text{SO}_4$ , 5 mM  $\text{MgSO}_4$ , 50 mM Tris- $\text{SO}_4$  pH 6.8; 0.6 mM EGTA, 0.5 mM  $\text{CaCl}_2$  ( $5 \mu\text{M}$  free  $\text{Ca}^{2+}$ ), 1 mM DTT (all in both compartments); 15  $\mu\text{M}$  free ATP per UV flash (caged ATP only in one compartment).

50  $\mu\text{g}$  SR protein were added to both chambers of the cuvette prior to membrane formation.

## 3. RESULTS AND DISCUSSION

After forming the planar film in the presence of SR vesicles the pump was activated by a UV flash-induced concentration jump of ATP from a photolabile caged ATP [13]. The simultaneous activation of a large number of pump molecules by means of caged ATP was the prerequisite for resolving a satisfying current signal from the electrical background noise.

In the absence of any ionophores capacitive peak currents were observed followed by stationary pump current components (Fig. 1). These currents were both

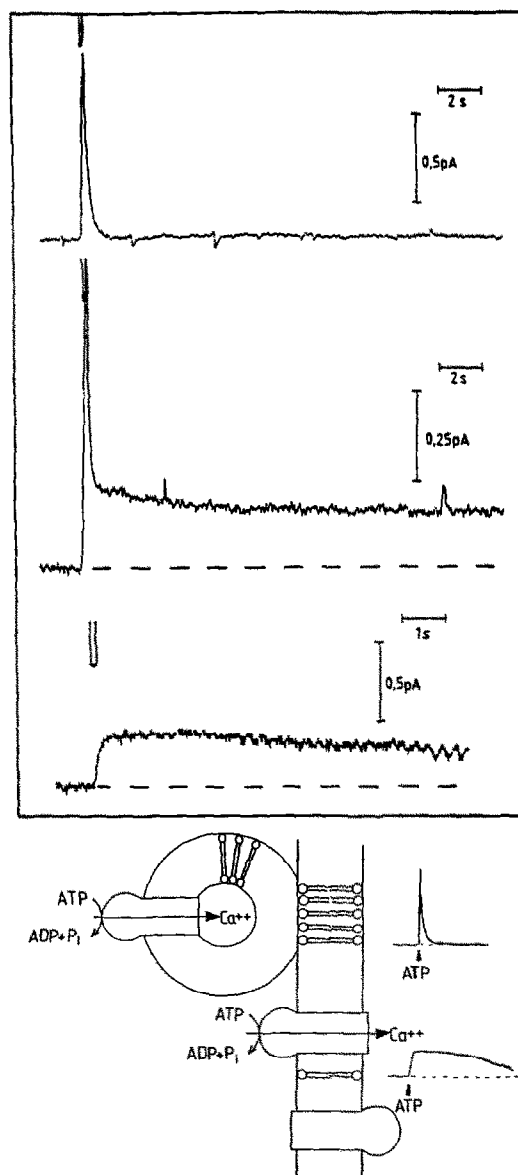


Fig. 1. (A) Types of current responses. The most abundant current pattern after 125 ms UV-light flashes was a capacitive peak current followed by a quasi-stationary current component (middle trace). This indicates the existence of incorporated  $\text{Ca}^{2+}$ -ATPases as well as attached SR vesicles. In some cases no incorporation occurred and thus only the capacitive current of the attached pumps appeared (upper trace) and sometimes the number of incorporated pumps exceeded the attached ones; this led to the appearance of only stationary currents after a rapid onset (lower trace). (B) Scheme of the planar bilayer membrane containing integrated pump molecules as well as attached SR vesicles. ATPase molecules of only one orientation are activated by ATP added to one side of the membrane. The other population remains silent. The attached ATPases (SR vesicles) are expected to produce solely capacitive peak currents [26] as indicated in the upper right part of the scheme. The incorporated pumps are expected to produce stationary pump currents which decline slowly due to ATP consumption and diffusion of the ATP out of the illuminated volume of the cuvette (lower right).

dependent on the simultaneous presence of calcium ions, ATP SR-protein and magnesium ions. The current responses were abolished upon addition of 200  $\mu\text{M}$

sodium vanadate to the ATP containing compartment (Fig. 2) and after removal of calcium ions by EGTA. In accordance with earlier reports,  $\text{Sr}^{2+}$  and  $\text{Mn}^{2+}$  ions are able to substitute for calcium as activating cations (not shown) [3,16]. Only  $\text{Ca}^{2+}$ ,  $\text{Sr}^{2+}$  and  $\text{Mn}^{2+}$  ions stimulate pump currents, other electrogenic ATPases than  $\text{Ca}^{2+}$ -ATPase are not detectable in SR.

Release of ADP from a caged ADP did not lead to pump currents unless the ATP-generating system creatine-kinase/phosphocreatine was also present.

Fig. 1A shows in addition two species of current patterns which occur occasionally. In the upper trace no incorporation of ion pumps took place and therefore only pumps attached to the planar membrane produce a transient, capacitive current [5]. The lower trace shows only stationary currents indicating that the number of incorporated pumps exceeds that of the attached ones. Fig. 1B summarizes schematically both extreme situations of only attached (upper part) or only incorporated pumps (lower part). The most abundant current pattern is therefore the result of the combined action of pumps incorporated in and attached to the planar lipid membrane. This is confirmed by the observation that, after application of the ionophores A23187 and 1799, which allows also the attached pumps to contribute to the transmembrane current, the stationary current increases (not shown). The stationary currents had amplitudes up to 0.5 pA. The sign of the current indicates that positive charges are pumped from the ATP containing compartment across the membrane to the opposite chamber. Assuming a turnover rate of about  $7 \text{ s}^{-1}$  [17] and 4 net charges transported per cycle about  $10^5$  pumps are active at the same time. Since the pump molecules are expected to be randomly oriented in the membrane, another  $10^5$  ATPases are present in the membrane which were not activated by ATP. The total density of incorporated proteins is therefore about  $3 \times 10^9$  pumps/cm<sup>2</sup> (the corrected value of the bilayer area was used).

Recently, Nishie et al. [10] reported the incorporation of the  $\text{Ca}^{2+}$ -ATPase into planar lipid membranes by fusion of vesicles containing  $\text{Ca}^{2+}$ -ATPase to preformed planar lipid membranes. This method seems to be less effective since the reported currents were extremely small (max. 0.035 pA) and the currents develop within minutes after addition of ATP. The signal-to-noise ratio is poor compared with the direct incorporation of the pumps by the method presented here, which led to 3–15-fold larger current amplitudes. The use of the SR vesicles, with their high density of  $\text{Ca}^{2+}$ -ATPase, may have favoured the improved incorporation 'yield' in our experiments. The ATP concentration jump method by use of the caged ATP enabled us to activate the ATPase within a few milliseconds. This allowed a better time resolution in the range of some hundred milliseconds. The ATP release by an UV-flash permits an activation of the ATPase without

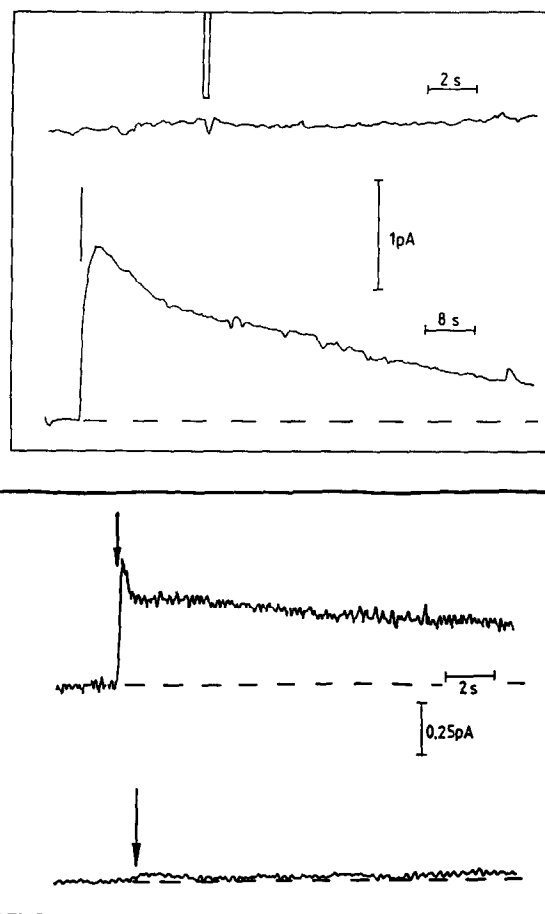


Fig. 2. (A) Activation of pump currents by ATP (caged ATP); electrolyte as in Fig. 1. (Upper trace) In the absence of caged ATP no signal is detected. (Lower trace) After addition of  $100 \mu\text{M}$  caged ATP to one compartment (called cis) a 125 ms UV flash, which releases  $15 \mu\text{M}$  free ATP in the illuminated volume of the cuvette, induces a pump current response. The stationary current component shows a slight decline because of ATP consumption and diffusion of the ATP out of the illuminated volume (7% of cuvette volume). Note the larger time scale of the lower trace. (B) Inhibition of pump currents by vanadate. Electrolyte as in Fig. 1.  $15 \mu\text{M}$  free ATP/flash. Before vanadate application normal pump responses were observed (upper trace); 10 min after addition of  $200 \mu\text{M}$   $\text{Na}_3\text{VO}_4$  to the cis chamber nearly all of the current disappeared.

mechanical disturbance like stirring and perfusion procedures. Therefore, drift effects of the baseline were eliminated and a satisfying signal to noise ratio was obtained.

The finding that  $15 \mu\text{M}$  free ATP are sufficient to stimulate considerable pump currents of the  $\text{Ca}^{2+}$ -ATPase agrees well with the known high affinity activation in the range of  $10^{-6} \text{ M}$  ATP [2,5,19–22]. However, this result contradicts the finding of Nishie et al. [10] who reported a Michaelis constant of ATP activation of  $0.69 \text{ mM}$  ATP without detecting the high affinity activation component. In addition, the report demonstrates only the appearance of currents after the addition of ATP and lacks essential controls of

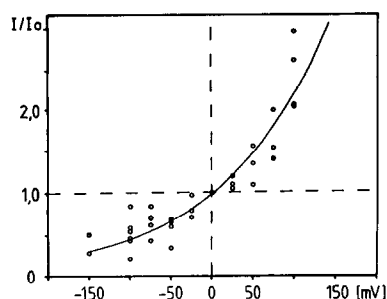


Fig. 3. Current-voltage curve of the incorporated  $\text{Ca}^{2+}$ -ATPase. Because of varying stationary current amplitudes between different experiments pump currents are shown as relative current amplitudes  $I/I_0$ . Values were taken from the quasi-stationary phase 10 s after the light flash. Data with applied voltage were compared with temporally neighbouring data at zero voltage. Voltage was applied prior to UV-illumination in order to allow relaxation to a new baseline. The guideline is the result of a computer fit with a single exponential. Electrolyte: 10 mM  $\text{K}_2\text{SO}_4$ , 5 mM  $\text{MgSO}_4$ , 10 mM Tris- $\text{SO}_4$ , pH 6.8; 0.6 mM EGTA, 0.7 mM  $\text{CaCl}_2$  (106  $\mu\text{M}$  free  $\text{Ca}^{2+}$ ), 1 mM DTT (all in both compartments), 15  $\mu\text{M}$  free ATP per flash (caged ATP only in one compartment).

$\text{Ca}^{2+}$ -ATPase activity like  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  dependence and vanadate sensitivity. In fact, the data could also be the effect of a nonspecific pseudo ion pump described by Hyman [18], which is the result of ion associations at the membrane surface. This is clearly not the case in the present paper, since the specific ion and substrate requirements of the  $\text{Ca}^{2+}$ -ATPase could be demonstrated. Furthermore, the release of ADP from caged ADP, which produces nearly the same charged products as in the photolysis of caged ATP, has no electrical effect.

The presence of stationary currents in the absence of any ionophore clearly demonstrates the direct and unbalanced transfer of cations from one compartment of the cuvette to the other. The electrolytes on both sides of the membrane provide large ion reservoirs and prevent the establishment of diffusion potentials. The results show unambiguously that the  $\text{Ca}^{2+}$ -ATPase from skeletal muscle sarcoplasmic reticulum is electrogenic under conditions where it is cycling continuously.

The activity of an electrogenic ion pump should be modulated by an applied voltage. We tested this by the measurement of stationary pump current amplitudes after application of different membrane potentials. The resulting current-voltage curve (Fig. 3) shows increasing pump currents with increasing positive potentials of the ATP-containing compartment. This indicates the existence of electrogenic steps in the direction of the transported  $\text{Ca}^{2+}$  ions. The current increase is  $e$ -fold for every 125 mV in the observed range. The rate of an enzymatic reaction can be modulated both by changing the rate constants or by changing the equilibrium concentrations of the starting intermediate [23]. Since the slope of the  $I$ - $V$  curve at positive potentials is rather

flat, it is possible – as shown for the  $\text{Na}^+/\text{K}^+$ -ATPase in heart muscle [23] – that the electrogenic step (here assumed to be the major conformational change from  $\text{E}_1\text{P}(\text{Ca}_2)$  to  $\text{E}_2\text{P}(\text{Ca}_2)$  which is possibly connected to the translocation of the calcium ions across the membrane) is not rate limiting [24]. But it influences the turnover of the pump by modulating the equilibrium concentration of the enzyme intermediate preceding a following nonelectrogenic slow step (possibly the dephosphorylation of the  $\text{E}_2\text{P}$  form). In the case of a slow, rate limiting electrogenic step the voltage is expected to influence the turnover rate directly and more drastically.

An important parameter that can be obtained from an  $I$ - $V$  curve is the reversal potential or electromotive force (EMF) for the ion pump. It is defined as the difference of the driving forces (ATP hydrolysis) and the osmotic work which has to be performed by the pump, divided through the transported net charge [25]:

$$\text{EMF} = (G_{\text{ATP}} - A_{\text{osm}})/n \text{ with } G_{\text{ATP}} = G^0 \cdot \ln[\text{ATP}]/[\text{ADP}][\text{P}_i]$$

The currents obtained at negative potentials seem to approach asymptotically a reversal potential which is in the region of high negative values. The theoretical reversal potential of the pump under these conditions, calculated from the ATP, ADP and  $\text{P}_i$  concentrations, in absence of concentration gradients ( $A_{\text{osm}} = 0$ ) and assuming four positive charges transported per ATP, should be about  $-175$  mV. Since the reversal potential is far in excess of this value our results suggest that less than four positive net charges are transported per ATP molecule. This is in accordance with previous observations [2,24] which suggest a reduced coupling ratio at micromolar ATP concentrations. The alternative explanation whether other cations (most probably  $\text{H}^+$  [3]) are countertransported was tested, but in the presence of pH gradients larger than one no pump currents were observed.

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