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Calcium transport by sarcoplasmic reticulum Ca-ATPase can be investigated on a solid-supported membrane

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Abstract

Sarcoplasmic reticulum (SR) native vesicles incorporating Ca-ATPase are adsorbed on a solid-supported lipid membrane (SSM). Upon adsorption, the ion pumps are chemically activated by concentration jumps of ATP and the capacitive current transients generated by SR Ca-ATPase are measured under potentiostatic conditions. The Michaelis–Menten constant, K_M, for ATP is evaluated by varying the concentration of ATP in the activating solution. This preliminary result shows that ion transport by SR Ca-ATPase can be suitably investigated by a technique based on concentration jumps on an SSM.

Keywords: Sarcoplasmic reticulum Ca-ATPase; Active transport; Solid-supported membrane; Capacitive current transients

1. Introduction

Contraction and relaxation of muscle fibers is controlled by the cytoplasmic Ca^{2+} concentration, which is kept at or below 0.1 μM, thanks to the pumping activity of the integral membrane protein Ca-ATPase of the sarcoplasmic reticulum (SR), the main storage compartment of Ca^{2+} ions [1,2].

SR Ca-ATPase forms an aspartyl-phosphorylated intermediate during the enzymatic reaction cycle. According to the E_1–E_2 model [3,4], Ca-ATPase in the E_1 conformation binds two calcium ions sequentially and an ATP molecule on its cytoplasmic side. After phosphorylation of the enzyme at the catalytic site (Asp 351) and occlusion of the Ca^{2+} ions, a conformational change from E_1 to E_2 takes place, which causes the translocation of the Ca^{2+} ions and their release to the lumen. This is followed by the hydrolytic cleavage of the phosphorylated intermediate P-E_2 and a return to the E_1 conformation, which can start a new cycle [5].

Time resolved investigation of charge translocation by SR Ca-ATPase from rabbit skeletal muscle was carried out by using an experimental technique that combines the high stability and sensitivity of a solid-supported lipid membrane (SSM) with a method for rapid solution exchange at the surface of the SSM [6]. The SSM consists of an alkanethiol monolayer firmly anchored to the gold surface via the sulphydryl group, with a second phospholipid monolayer on top of it. This method was successfully used to investigate the electrogenic partial reactions in the enzymatic cycle of Na,K-ATPase [6–9] and to study charge transport by melibiose permease [10]. In the present preliminary study, SR vesicles containing Ca-ATPase were adsorbed on the SSM. Upon adsorption, the ion pumps were activated by performing concentration jumps of ATP at the surface of the SSM, and the capacitive current transients generated by SR Ca-ATPase were measured under potentiostatic conditions.

2. Experimental

2.1. Chemicals

Calcium and magnesium chlorides and Tris(hydroxymethyl)-aminomethan (TRIS) are Merck analytical grade reagents. Adenosine-5′-triphosphate disodium salt (ATP, ~97%) and dithiothreitol (DTT, ≥99%) were obtained from Fluka. Octadecyl-mercaptan (98%) from Aldrich was used without further purification. Ethylene glycol-bis[β-aminoethyl ether]-*N*,*N*,*N*′,*N*′ -tetraacetic acid (EGTA) and calcimycin (calcium ionophore A23187) were purchased

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from Sigma. The lipid solution contained diphytanoylphosphatidylcholine (Avanti Polar Lipids) and octadecylamine (puriss., Fluka) [60:1] and was prepared at a concentration of 1.5% (w/v) in *n*-decane (Merck) as described by Bamberg et al. [11]. SR vesicles are a generous gift from Prof. Giuseppe Inesi and were prepared as described by Eletr and Inesi [12].

Free Ca^{2+} concentrations were calculated by the computer program Winmaxc v. 2.40 [13].

2.2. The solid-supported membrane (SSM)

The SSM consisted of an alkanethiol monolayer tethered to a gold surface via the sulphydryl group, with a phospholipid monolayer on top of it [14,15] (Fig. 1). The SSM was prepared following Pintschovius and Fendler [6]. Typically, the effective membrane area ranged from 2 to 3 mm^2.

2.3. Setup

For the rapid concentration jumps, a plexiglass cuvette with an inner volume of 20 μl was employed. The SSM and an O-ring, which contained the actual reaction volume, were sandwiched between the upper and the lower part of the cuvette. The SSM acted as the working electrode, while a Ag/AgCl (0.1 M KCl) electrode was used as a counter electrode. The counter electrode was separated from the streaming solution by an agar/agar gel bridge [6]. The cuvette was connected to the outlet of a Teflon block on which two solenoid valves were mounted (Model 225T052, NResearch, West Caldwell NJ, USA). The two valves, which were computer-controlled through a digital-to-analog converter (IOtech DAC 488/2), allowed a fast switching between the activating and the non activating solution. All parts of the setup conducting the electrolyte solutions were enclosed in a Faraday cage. Two different 100-ml glass containers were used for the non activating and the activating solution.

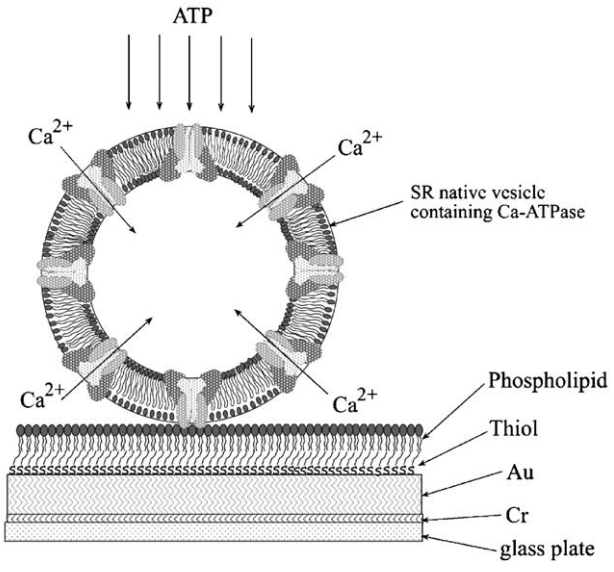

Fig. 1. Schematic representation of an SR native vesicle adsorbed on a solid-supported membrane.

Concentration jump experiments were carried out by switching from the non activating to the activating solution. The flow of the activating solution was kept constant at approximately 60 ml/min by applying a pressure of 0.4 bar to the system and by controlling the pressure with a precision digital manometer. The current generated by the ion pumps upon keeping the applied potential between the SSM and the counter electrode equal to zero, was amplified by a current amplifier (Keithley 428, gain 10^9 V/A), filtered (low-pass, 3 ms), recorded (16-bit analog-to-digital converter, IOtech ADC 488/8SA), visualized (Oscilloscope, Tektronix TDS 340A) and stored (Power PC G3, Macintosh). Operation of the experimental setup and data acquisition were carried out under computer control (GPIB interface, National Instruments board) using a home-made acquisition program written in LabView environment.

2.4. Solution exchange technique

As a rule, 2 h after forming the SSM and filling the cuvette, the capacitance and conductance of the SSM attained time-independent values of the order of 0.2–0.4 μF/cm^2 and 50–100 nS/cm^2, respectively. Control experiments were then performed with the protein-free SSM in order to exclude any artifacts generated by the solution exchange [6]. Subsequently, SR vesicles containing Ca-ATPase were added by injecting 20 μl of their suspension into the cuvette through the outlet opening. The suspension was vigorously mixed using a pipette. The vesicles were adsorbed on the SSM for 30 min upon applying a potential difference of +0.1 V. A schematic picture of an SR vesicle adsorbed on the SSM is shown in Fig. 1. During a concentration jump experiment, the cuvette was first washed with the non activating solution for 1 s; the activating solution was then injected into the cuvette for 1 s; finally, the activating solution was removed from the cuvette with the non activating solution for 1 s.

3. Results and discussion

Native vesicles containing SR Ca-ATPase from rabbit skeletal muscle were adsorbed on the SSM. The calcium pump was then activated by performing ATP concentration jumps in the presence of Ca^{2+} and Mg^{2+} ions, and the resulting current transients were recorded.

A typical capacitive current transient following an ATP concentration jump at a constant Ca^{2+} concentration is shown in the inset of Fig. 2. The sign of the current peak is negative and corresponds to the transport of positive charge from the aqueous solution toward the SSM [16]. The sign of the capacitive current indicates that the flux of calcium ions is directed toward the inside of the SR vesicles,

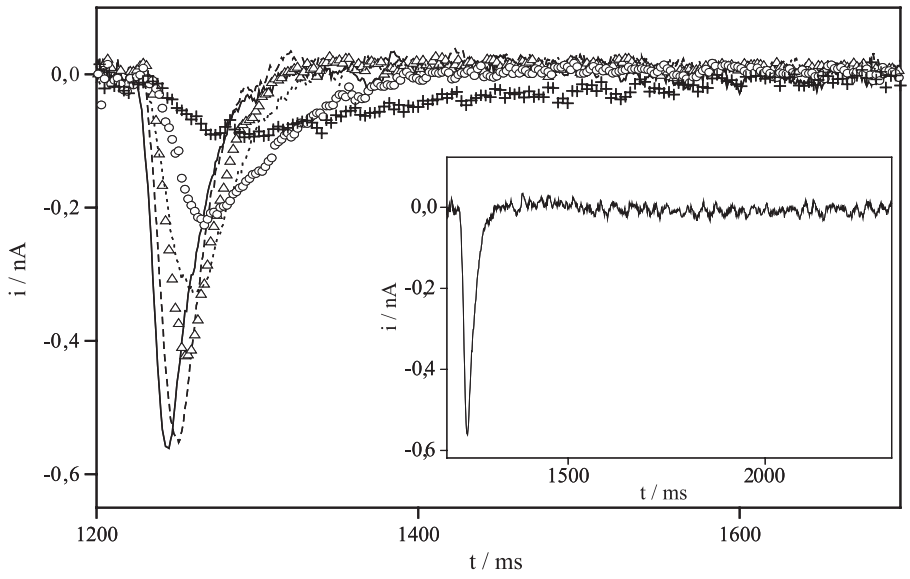

Fig. 2. Current transients generated by SR Ca-ATPase following ATP concentration jumps at different ATP concentrations: 100 (solid), 50 (dash), 25 (△), 10 (dot), 5 (○), 1.5 μM (cross). The non activating solution contained 1 mM $MgCl_2$, 1 mM $CaCl_2$, 25 mM TRIS, 1 mM EGTA, 0.2 mM DTT, pH 7.0. The activating solution had the same composition as the non activating solution plus a variable concentration of ATP. The inset shows a capacitive current transient following a 100 μM ATP concentration jump.

with the vesicles contributing to the electrical signal being adsorbed with the cytoplasmic side facing the aqueous solution. This result clearly shows that Ca^{2+} translocation following the conformational transition $E_1P \rightarrow E_2P$ is an electrogenic event, in agreement with results obtained by different techniques [17,18].

The current signal was fitted with a multiexponential function. The first relaxation time constant, $\tau_1 = 11$ ms, accounts for the rise in the negative current, while the second time constant, $\tau_2 = 21$ ms, accounts for its decay. A third time constant, $\tau_3 = 200$ ms, has a positive amplitude and accounts for a modest current overshoot. Integration of the current peak in the inset of Fig. 2 gives the value of the translocated charge, which amounts to 20 pC. However, as distinct from the above relaxation time constants, the charge under the current peak depends upon the amount of vesicles adsorbed on the gold-supported alkanethiol/lipid bilayer, and hence, to some extent, on the preparation of the SSM.

As shown in Fig. 2, ATP concentration jumps in the presence of a constant concentration of free Ca^{2+} ion of 100 μM induce current transients of increasing height, as the ATP concentration in the buffer solution is increased. The experimental points are satisfactorily fitted by the Michaelis–Menten equation:

$$i_N = i_{N,max} \frac{[ATP]}{[ATP] + K_M} \quad (1)$$

The best fit yields a K_M value for ATP of 2.9 ± 0.3 μM (Fig. 3). This K_M value is in good agreement with the value of 4.6 μM determined by Hartung et al. [18].

It must be mentioned that in this experiment, a correction for the concentration values was required [6]. In fact, at the time t_{peak} of the peak, as measured from the instant at which the valve driver is triggered, the ATP concentration, c_{peak}, at the surface of the SSM is less than its full value, c_0, in the glass container of the activating solution. Therefore, the concentrations were corrected according to the equation [6]:

$$c_{peak} = c_0 \left(1 - \frac{\tau_{app}}{t_{peak}}\right) \quad (2)$$

The empirical parameter τ_{app} was determined as described in Ref. [6]. In the present case, τ_{app} was found to be equal to 92 ms, for the K_M value of 2.9 μM; the confidence interval for τ_{app} was between 85 and 97 ms.

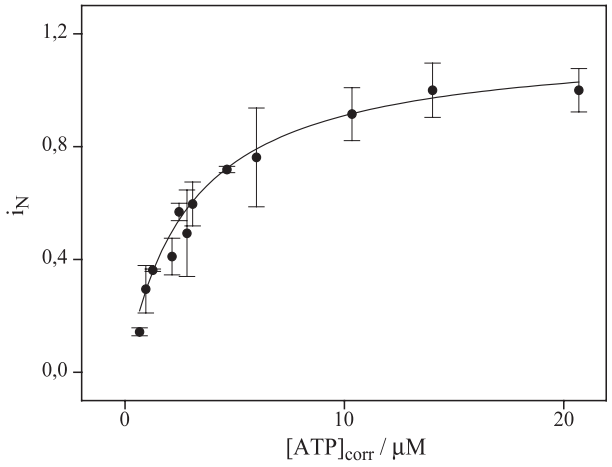

Fig. 3. ATP dependence of the normalized peak currents after an ATP concentration jump experiment. The experimental points were fitted with the Michaelis–Menten equation, yielding a $K_M = 2.9 \pm 0.3$ μM. The ATP concentrations are corrected as explained in the text.

The dependence of the two relaxation time constants τ_2 and τ_3 upon the ATP concentration was obtained by fitting the current transients in Fig. 2 with a multiexponential function. While τ_2 increases with the ATP concentration, the time constant τ_3 of positive amplitude, which accounts for the current overshoot, is independent of it. It is therefore reasonable to ascribe τ_2 to the binding of ATP to Ca-ATPase. As regards the origin of the relaxation time τ_3, it should be noted that current transients with a typical overshoot were reported in charge translocations of Na/K-ATPase, induced either by the photochemical release of ATP [19] or by a Na^+ concentration jump [6]. The overshoot and the corresponding time constant were ascribed to a backflow of charge through the adsorbed membrane fragments incorporating the sodium pump and were considered irrelevant from a kinetic viewpoint. By analogous arguments, we may ascribe the overshoot to the response of the system consisting of the supporting mixed bilayer and of the adsorbed vesicles. In other words, it should depend upon the resistance and capacitance of the vesicles, but not on the parameters expressing the time dependence of the pump current (see also Ref. [16]).

4. Conclusions

These preliminary results show that the ion transport mechanism of SR Ca-ATPase can be conveniently investigated by a technique based on concentration jumps on a solid-supported membrane. Further experiments are currently underway with the aim of unraveling some important aspects of calcium binding and transport by this ion pump.

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