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Electrogenic pump current of sarcoplasmic reticulum Ca^{2+}-ATPase reconstituted at high lipid/protein ratio

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When Ca^{2+}-ATPase from sarcoplasmic reticulum was reconstituted with excess phospholipid (at a 1:800 weight ratio) in a monomeric state and activated by Ca^{2+} and ATP a transmembrane potential developed which could be continuously recorded by the fluorochrome oxonol VI. The results demonstrate the electrogenicity of active Ca^{2+} transport during continuous turnover. The fluorescence signal can be quantified in terms of net current electrical flow through the vesicular membranes and compared to the ATP hydrolysis to give the number of electrostatic charges transferred during Ca^{2+} transport. From such measurements a stoichiometry of 1.8 ± 0.4 Ca^{2+} per ATP hydrolyzed at pH 7.1 can be obtained. The method is also convenient for determination of the kinetics of Ca^{2+}-ATPase activation by ATP and free Ca^{2+}.

Ca^{2+}-ATPase; Sarcoplasmic reticulum; Reconstitution; Proteoliposome; Electrogenic transport; Coupling ratio; Oxonol VI

1. INTRODUCTION

Previous results using reconstituted Ca^{2+}-ATPase have suggested that when the reconstitution is performed at a high phospholipid/protein ratio (ca. 800:1) the protein is inserted into the proteoliposomes in a monomeric state, which apparently retains a high ATP hydrolytic activity as well as Ca^{2+}-transport capacity [1]. With this preparation we here report investigations on the electrogenicity of Ca^{2+} transport in liposomes with reconstituted Ca^{2+}-ATPase from sarcoplasmic reticulum by employing the potential sensitive fluorescent probe oxonol VI as previously described for the reconstituted Na^+,K^+-ATPase [2].

It is generally accepted that the turnover of the Ca^{2+}-ATPase results in the translocation of 2 Ca^{2+} per ATP molecule split [3,4]. However, the invariability of this stoichiometry [5,6] and possible existence of counter-transported ions [7–10] are still under debate. A method for quantitative measurements of electrostatic charges carried with Ca^{2+} transport across the membrane, without interference from other membrane constituents, is therefore needed. Previous results using SR-vesicles or reconstituted vesicles have indicated the electrogenic nature of Ca^{2+} transport [11–16]. This has recently been supported by measurements of transient current responses in planar lipid bilayers after incorporation of detergent solubilized Ca^{2+}-ATPase [17] or of SR-vesicles, following the release of ATP from caged ATP [18]. The latter results suggested that less than 4 positive net charges were translocated per ATP hydrolyzed. Here it is demonstrated using liposomes reconstituted with Ca^{2+}-ATPase that the transport of Ca^{2+} is electrogenic and that during turnover a little less than 4 electrostatic charges are transferred across the membrane, corresponding to a transport of 1.8 ± 0.4 Ca^{2+} per ATP molecule split (mean ± SD, $n = 3$). It is concluded that the method is well suited to continuously measure the electrogenic component of Ca^{2+} transport in order to evaluate transport stoichiometry and kinetic characteristics.

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2. MATERIALS AND METHODS

Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle by differential centrifugation according to de Meis and Hasselbach [19]. Ca^{2+}-ATPase was solubilized by treatment with $C_{12}E_8$ and reconstitution performed by a modification of the previously described cholate dialysis method [1] where dioleoylphosphatidylcholine (Avanti Polar Lipids) was used instead of egg lecithin, and polystyrene beads (Biobeads, Biorad) were included to remove detergent from the dialysate. After dialysis for 44 h and centrifugation on a linear 3–12% sucrose density gradient the upper band containing proteoliposomes with a high lipid/protein ratio (ca. 800:1) was collected. The proteoliposomes were prepared in the presence of 1 mM $MgCl_2$, 1 mM sodium azide and 100 mM sodium phosphate (pH 7.1) which acted as an internal precipitant for Ca^{2+} during a subsequent Ca^{2+}-transport assay to prevent interference with the fluorescence from accumulated Ca^{2+} [20].

At low ATP concentrations (below 100 μM) the hydrolytic activity of reconstituted Ca^{2+}-ATPase was assayed with [^{32}P]ATP employing the method of Lindberg and Ernster [21], while at higher ATP concentrations activity was measured spectrophotometrically with an ATP generating system [22]. Ca^{2+} uptake was measured with ^{45}Ca after ion-exchange separation (Biorex, Bio-Rad) of non-transported Ca^{2+}. The phospholipid content was measured spectrofluorometrically with diphenylhexatriene [23] and protein was assayed using the modified Lowry-method of Peterson [24].

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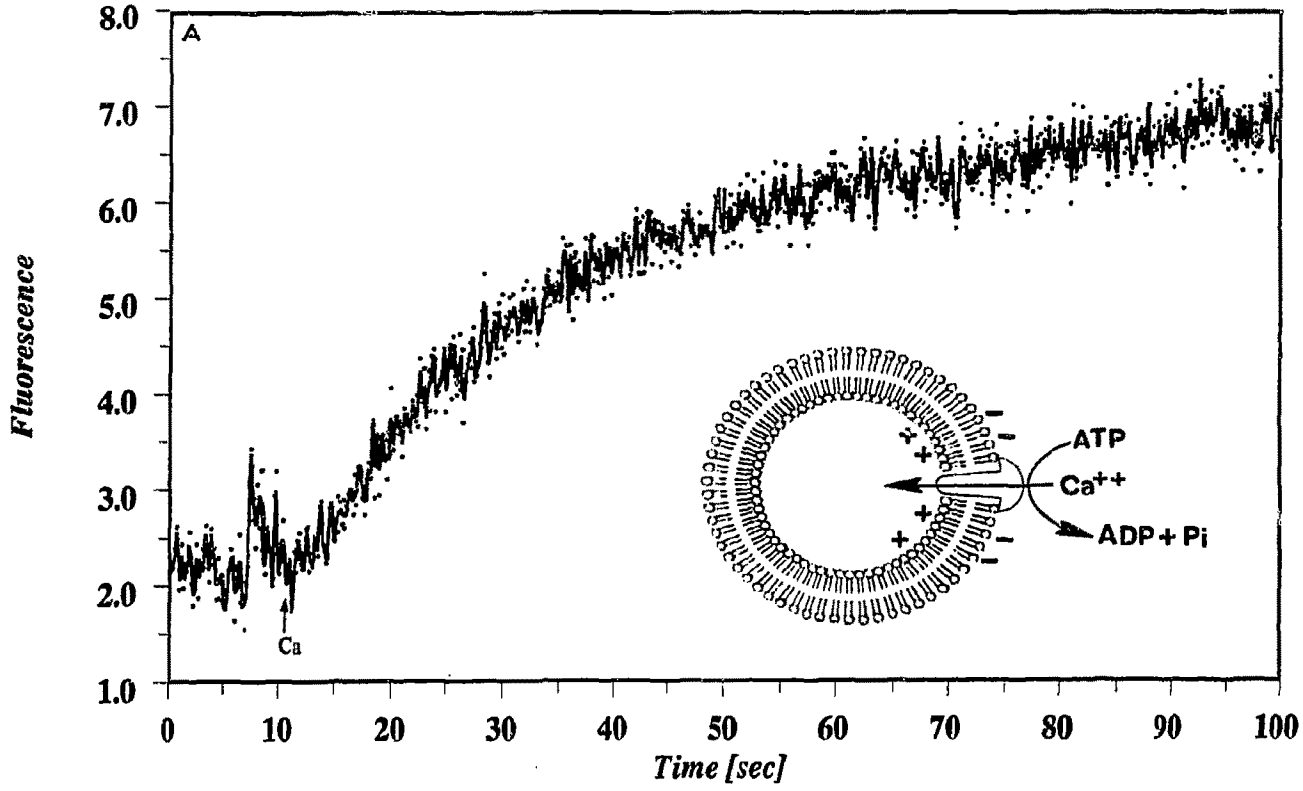

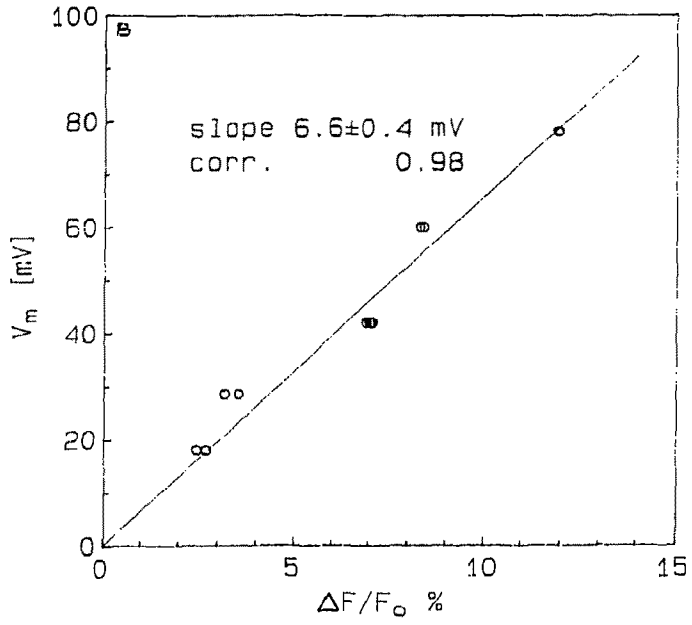

Fig. 1. A. Transmembrane potential generated by reconstituted Ca^{2+}-ATPase (inset) during Ca^{2+} transport. A small volume (50 μl) of proteoliposomes was added to the cuvette containing 530 nM oxonol VI and buffer with 100 mM phosphate, 1 mM $MgCl_2$, 5 mM ATP, 0.3 mM EGTA and 30 mM imidazole, pH 7.1. By addition of 0.33 mM Ca^{2+} (8 μM free concentration) an increase in fluorescence is initiated indicating the production of an inside positive potential. Fluorescence is given in arbitrary units with the steady-state potential corresponding to 90 mV calculated from the calibration curve shown below, which gives the relation of relative increase in fluorescence ($\Delta F/F$) versus K^+ diffusion potential. B. The fluorescent signal was calibrated by addition of increasing K^+ concentrations to proteoliposomes in the presence of valinomycin (1 μM). Calibrations were consistently performed during identical conditions of Mg^{2+} and Ca^{2+} (in the present case 1 mM and 8 μM, respectively) as performed for measurement of transmembrane potential accompanied by Ca^{2+}-ATPase activity, in order to correct for a slight suppression of the fluorescence signal by increasing Ca^{2+}.

The transmembrane potential was measured using oxonol VI as previously described [2,25]. Essentially, 50 μl proteoliposomes were added to a 3-ml cuvette containing buffer with 100 μM ATP and 530 nM oxonol VI and the increase in fluorescence emitted at 660 nm after excitation at 580 nm was detected upon addition of Ca^{2+}. The fluorescence signal was calibrated using known gradients of K^+ in the presence of valinomycin, as described by Apell and Berch [25] for vesicles of the same size as the ones used here [1].

3. RESULTS AND DISCUSSION

When proteoliposomes with reconstituted Ca^{2+}-ATPase are activated by Ca^{2+} in the presence of 100 μM ATP a steady-state transmembrane potential of about 90 mV develops within the first 2 min (Fig. 1A). This response clearly demonstrates the electrogenicity of the reconstituted Ca^{2+} pump. After 30–40 s the initial rapid increase in fluorescence is followed by a slower phase. The first phase probably represents the initial accumulation of internal Ca^{2+}, until a steady-state concentration level is attained due to the precipitation of calciumphosphate by the high concentration of internal phosphate, and the establishment of the transmembrane potential which in itself opposes the influx of Ca^{2+}. Upon addition of vanadate (2 mM) the steady-state transmembrane potential dissipates slowly, due to cessation of Ca^{2+} transport and slow equilibration of the ions (probably Cl^-) across the membrane.

From the initial rate of development of the membrane potential (dV/dt), calculated after the calibration of the fluorescence response (Fig. 1B) the net current flow can be calculated according to $I = C_m A_m (dV/dt)/e$ in which C_m and A_m are the specific membrane capacitance (1 μF/cm^2, as measured in lipid bilayers and most cell membranes, cf. [26]) and the bilayer surface area, respectively, and e is the elementary charge ($1.6 \cdot 10^{-19}$ C). A_m is calculated from the phospholipid content and a phospholipid surface area of 0.7 nm^2/molecule corresponding to a bilayer thickness of 36 Å and consistent with the average value measured in small vesicles [27]. In a parallel sample the hydrolytic activity is measured and from such data a stoichiometry of 3–4 net charges translocated per ATP molecule hydrolyzed can be calculated. This is in accordance with an electrogenic transport of close to 2 Ca^{2+} per ATP molecule split (1.8 ± 0.4, mean $\pm$ SD), corresponding to coupling ratios that we obtained from steady-state measurements of ^{45}Ca uptake at high ATP concentrations (data not shown). The deviation of the coupling ratio from the 2:1 value is considered insignificant but might signify the presence of some Ca^{2+}-ATPase which is not properly inserted into the liposome membrane, giving rise to hydrolysis unaccompanied by vectorial transport, co-transport of anions, or countertransport of cations. A substantial Ca^{2+}/H^+ countertransport has recently been suggested by Levy et al. [10]. The coupling ratio for electrogenic Ca^{2+} transport as calculated in the present report does not indicate this to be the case in our preparation, although it is subject to some uncertainty in the potential measurement and assignment of values for specific membrane capacitance as well as of surface area of phospholipids.

Fig. 2 shows measurements of the initial transmembrane potential as a function of the ATP concentration. A biphasic curve is obtained which can be resolved according to a second degree rate equation [28] equivalent to the sum of two Michaelis-Menten type equations with apparent ATP affinities of 0.22 μM and 0.3 mM, respectively. The biphasic substrate curve is in accordance with results obtained on Ca^{2+}-ATPase membranes prepared from the SR without reconstitution ([22] and references therein).

If the initial rate of transmembrane potential development is measured as a function of the free Ca^{2+} concentration in the medium a co-operative activation of the potential response is observed with half maximum response at 3.5 μM (Fig. 3). This is slightly higher than usually found for Ca^{2+} activation, but is in agreement with data obtained on intact vesicles and leaky Ca^{2+}-ATPase membranes (not shown). Ca^{2+} concentrations higher than 10^{-5} M cause depression of the fluorescence response; a similar effect of Ca^{2+} is found on the hydrolytic activity of the proteoliposomes indicating that the coupling ratio of Ca^{2+} transport to hydrolysis is retained during suboptimum Ca^{2+} conditions.

The electrogenic transport of Ca^{2+} by the sarcoplasmic reticulum Ca^{2+}-ATPase is clearly demonstrated by the ability of the reconstituted preparations to initiate

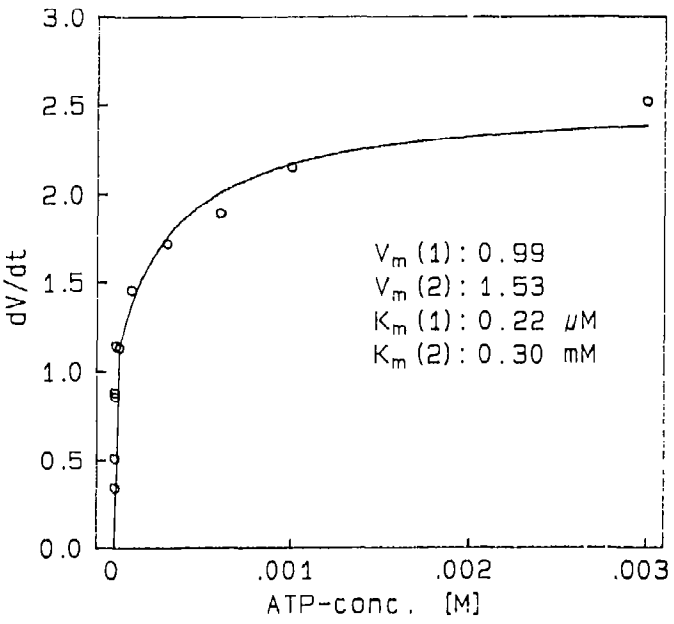

Fig. 2. The ATP activation curve for reconstituted Ca^{2+}-ATPase, measured by initial oxonol response, given in arbitrary units. The curve is a calculated fit according to a second order rate equation [28]. The two calculated fractions (V_m) with corresponding apparent ATP affinities (K_m) are indicated.

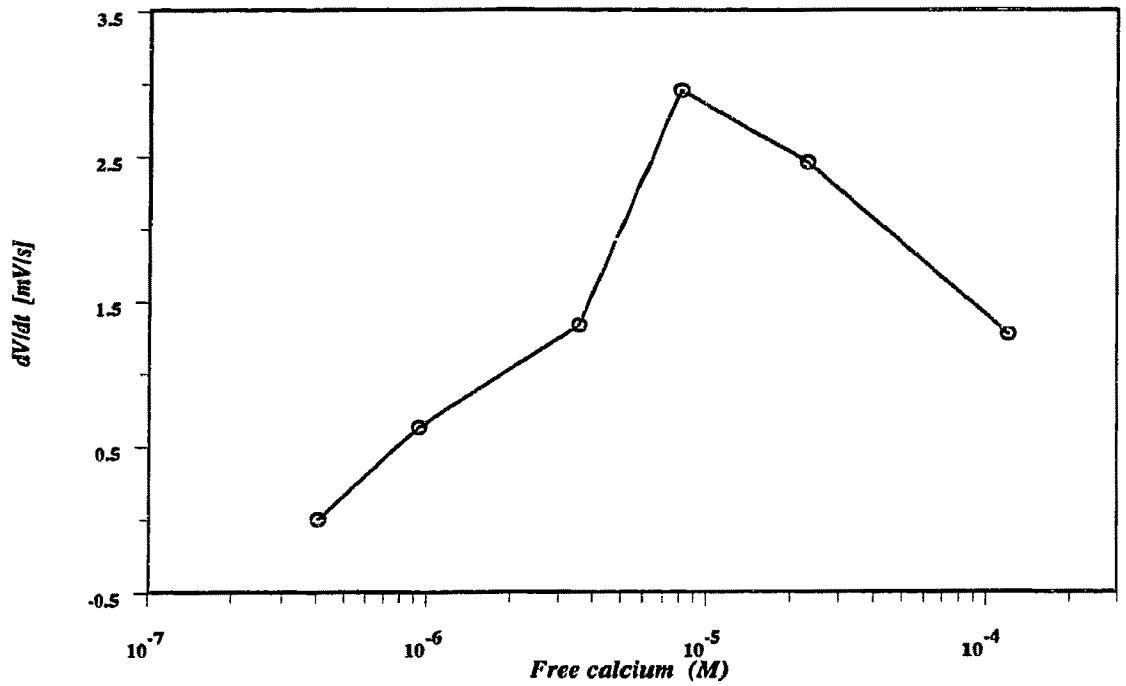

Fig. 3. The activation of transmembrane potential by external free Ca^{2+}. The concentration of ATP is 5 mM and the free conc. of Ca^{2+} calculated according to Cornelius [29]. At $1.2 \cdot 10^{-4}$ M Ca^{2+} dV/dt is decreased to 45% of maximum which is comparable to an observed decrease in hydrolytic activity of 48% by increasing Ca^{2+} from 10^{-5} M to $1.2 \cdot 10^{-5}$ M (not shown).

and sustain transmembrane potential gradients across the bilayer of liposomes reconstituted with the Ca^{2+} pump. By means of the fluorochrome oxonol VI it is possible to continuously measure the electrogenic component of this transport. The dye is also useful in the enzyme-kinetic characterization of the reconstituted preparations which are not so easy to perform in these preparations due to their low protein content. In the present experiments at pH 7.1 we have so far observed no difference between Ca^{2+} transport and electrical current flow through the membranes. However, the method opens upon the possibility for detection of transport stoichiometries under a wide variety of conditions, including pH [3,4] and imposition of different ionic composition of the media on the two sides of the pump proteins to test for 'slippage' in the Ca^{2+}-translocation mechanism or different transport modes under special conditions.

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