Ca\textsuperscript{2+}/H\textsuperscript{+} Countertransport and Electrogenicity in Proteoliposomes Containing Erythrocyte Plasma Membrane Ca-ATPase and Exogenous Lipids*  

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A reconstituted proteoliposomal system was obtained with Ca-ATPase purified from human erythrocyte membrane (plasma membrane, PM ATPase), and liposomes prepared by reverse-phase evaporation. The reconstituted PM ATPase behaved as an electrogenic Ca\textsuperscript{2+}/H\textsuperscript{+} exchanger and, under optimal conditions, utilization of 1 mol of ATP was accompanied by uptake of one Ca\textsuperscript{2+} by the vesicles, and ejection of one H\textsuperscript{+} from the lumen of the vesicles. Ca\textsuperscript{2+} uptake was greatly (5-fold) stimulated by the addition of calmodulin, and by collapsing the H\textsuperscript{+} gradient with the ionophore carbonyl cyanide p-trifluoromethoxyphenylhydrazone. In the presence of calmodulin and p-trifluoromethoxyphenylhydrazone, the reconstituted system sustained transport rates of 1.00 ± 0.12 µmol of Ca\textsuperscript{2+}/mg of protein min\textsuperscript{-1} (30 °C), reaching asymptotic levels of 0.05 ± 0.41 µmol of Ca\textsuperscript{2+}/mg of protein (i.e., 20 nm lumenal Ca\textsuperscript{2+}). The corresponding net charge transfer produced a maximal electrical gradient of 40.5 ± 1.8 mV at steady state. Demonstration of the electrogenic behavior of the PM ATPase, obtained for the first time with these experiments, was critically dependent on the detergent used in the reconstitution procedure. The lumenal pH rise had a much greater rate-limiting effect on the pump, than the electrical potential developed by the pump.

The outward directed Ca\textsuperscript{2+} pump plays an important role in the long-term maintenance of a steep Ca\textsuperscript{2+} gradient across the plasma membrane (PM). Mechanism and regulation of the PM Ca\textsuperscript{2+} pump have been reviewed in detail (Carafoli, 1991, 1992). However, disagreement is still found in the literature regarding countertransport and/or electrogenic properties of the pump. There is considerable evidence to support the existence of Ca\textsuperscript{2+}/H\textsuperscript{+} countertransport. Concerning the H\textsuperscript{+}/Ca\textsuperscript{2+} stoichiometry, however, a ratio of 1 was observed with erythrocyte membrane resealed inside-out vesicles, while a ratio of 2 was obtained with purified ATPase in reconstituted proteoliposomes (Niggli et al., 1982; Smallwood et al., 1983; Gassner et al., 1988).

A H\textsuperscript{+}/Ca\textsuperscript{2+} ratio of 1 or 2 would determine whether the pump is or is not electrogenic. In this regard, experiments with inside-out vesicles (Waisman et al., 1983; Rossi et al., 1982; Smallwood et al., 1983) provided evidence for an electrogenic behavior of the pump, consistent with a H\textsuperscript{+}/Ca\textsuperscript{2+} ratio lower than 2. On the contrary, no development of electrical potential was observed using proteoliposomes reconstituted with purified ATPase (Niggli et al., 1982), suggesting that the PM Ca\textsuperscript{2+} pump operates as an electroneutral H\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, with a 2:1 stoichiometric ratio.

Further disagreement is found in the literature on whether the kinetics of the plasma membrane Ca\textsuperscript{2+} pump are sensitive to H\textsuperscript{+} and/or electrical gradients. Using purified ATPase in reconstituted proteoliposomes, Niggli et al. (1982) obtained evidence for inhibition of the Ca\textsuperscript{2+} pump by H\textsuperscript{+} gradients, but not by electrical potentials generated by Na\textsuperscript{+} or K\textsuperscript{+} gradients. On the one hand, Gassner et al. (1988) reported that Ca\textsuperscript{2+} extrusion from intact red cells is affected by electrical potentials. Furthermore, Smallwood et al. (1983) noted that Ca\textsuperscript{2+} accumulation into inside-out vesicles was enhanced by the diffusion of external anions through the band III protein.

We describe here the successful reconstitution of purified human erythrocyte PM Ca\textsuperscript{2+}-ATPase with liposomes prepared by reverse-phase evaporation. By the choice of a favorable detergent for the reconstitution procedure, we were then able to obtain parallel measurements of ATP-dependent Ca\textsuperscript{2+} uptake, H\textsuperscript{+} ejection, and development of electrical potential, with the same experimental system. We found that the PM operates as an electrogenic Ca\textsuperscript{2+}/H\textsuperscript{+} exchanger.

MATERIALS AND METHODS

Chemicals—Octaethylene glycol-n-dodecyl ether (C\textsubscript{12}E\textsubscript{10}) was obtained from Nikko Chemical Co. (Tokyo, Japan). Purified egg yolk phosphatidylcholine and phosphatidic acid were obtained from Avanti Polar Lipids Inc. Calmodulin-Sepharose 4B, calmodulin (CaM), valinomycin, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), calmodylin (A23187), and all other chemicals were obtained from Sigma. Preparation of Human Red Cell Membrane—Human red cell membrane ghosts were prepared from outdated human erythrocytes obtained from the blood bank as described by Niggli et al. (1979), except for the use of 10 mM MOPS-K\textsuperscript{+} instead of Tris-HC1 in the last 4 washings. The entire procedure was performed at 4 °C. Purification of Erythrocyte Membranes Ca\textsuperscript{2+}-ATPase—The Ca\textsuperscript{2+}-ATPase was purified from red cell membrane ghosts by calmodulin affinity chromatography, an adaptation of the method described by Niggli et al. (1981) and Koek-Kosicka et al. (1986). Briefly, 300-500 mg of red cell membrane deficient in calmodulin were solubilized with a buffer containing 0.4% C\textsubscript{12}E\textsubscript{10} (w/v), 130 mM KC\textsubscript{l}, 20 mM PIPES-K\textsuperscript{+}, pH 7.2, 100 µM CaCl\textsubscript{2}, 2 mM MgCl\textsubscript{2}, 2 mM diithiothreitol, and 0.3 M sucrose to obtain a final concentration of 6-8 mg of protein/ml, and kept on ice for 10 min. Nonsolubilized material was removed by centrifugation at 40,000 x g for 40 min. Phosphatidylcholine was added to the supernatant to a concentration of 0.5 mg/ml. After sonicating 1 min on ice with a micro-ultrasonic cell disruptor (Kontes, model 1400-4000) at power settings 2-3 and tone 3-4, the supernatant was applied to a calmodulin-Sepharose 4B column (1.5 x 10 cm) pre-equilibrated with 0.05% C\textsubscript{12}E\textsubscript{10}, 150 mM KC\textsubscript{l}, 20 mM PIPES-K\textsuperscript{+}, pH 7.2, 100 µM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 2 mM
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dithiothreitol, 0.3 x sucrose, and 0.05% (w/v) phosphatidylycholine. The column was washed with several bed volumes of the same buffer until the elution medium did not contain anymore protein (monitored by 280 nm absorption). Then the column was further eluted with the same buffer, containing 1 mM EDTA-K\(^+\) instead of CaCl\(_2\). The Ca\(^{2+}\)-ATPase activity peak eluted by EDTA-K\(^+\) was collected, 50 \(\mu\)g CaCl\(_2\) and 20 mM MgCl\(_2\) were added, and the pooled enzyme was stored at -80 °C for use within 1 month. The purification procedure was carried out at 4-5 °C.

**Preparation of Liposomes**—Unilamellar liposomes were prepared by reverse-phase evaporation by the method described by Rigaud et al. (1983) and Yu et al. (1993). For these experiments, the aqueous medium contained 20 mM PIPES-K\(^+\), pH 7.1, and 190 mM KCl.

**Reconstitution of Ca\(^{2+}\)-ATPase with Liposomes**—For this purpose the liposomes stock (16 mg of lipid/ml) was diluted with the aqueous medium used in the preparation of the liposomes, to yield a final lipid concentration (including the enzyme added later) of 4 mg/ml. The liposomes were then solubilized by adding incremental amounts of CH\(_2\)Cl\(_2\), while stirring slowly, until solubilization of lipids resulted in a dramatic decrease of turbidity. At this time, the C\(_{577}/\)lipid molar ratio was approximately 2. If measurements of luminal alkalization were planned, the fluorescent pH indicator pyranine was added at this time, to reach a 200 \(\mu\)M concentration. Purified plasma membrane Ca\(^{2+}\)-ATPase (120-180 \(\mu\)g of protein/ml) was solubilized by the addition of 0.5 mM CH\(_2\)Cl\(_2\), and the liposomes were used to obtain a lipid/protein ratio (w/v) of 150. The detergent/protein/phospholipid mixture was kept for 1 min under gentle stirring, then the detergent was removed by adding 0.6 g/ml prewashed wet Bio-Bead SM-2. The mixture was gently stirred at room temperature for 1.5 h, and then the Bio-Bead was removed by passing the mixture through empty poly-prep columns (Bio-Rad). Finally, the proteoliposomes were passed through an anion exchange chromatography column (AG 1-X8, Bio-Rad) to eliminate the pyranine in the medium outside the proteoliposome. One-ml bed volume per 4 ml of proteoliposome suspension was sufficient for this purpose. Preparation of liposomes and proteoliposomes was carried out at room temperature.

**Functional Measurements of Proteoliposomes**—Ca\(^{2+}\) uptake, electro- potential, and pH changes across the membrane of the proteoliposomes were measured by slight modifications of the methods described by Yu et al. (1993). Ca\(^{2+}\) uptake was followed by monitoring the differential (680 versus 687 nm) absorption changes undergone by Arsenazo I11 (340 nm) at 20 °C and measuring in parallel fluorescence (lumenal pyranine) (Levy et al., 1992). The reaction mixtures contained Ca\(^{2+}\)-ATPase and exogenous lipids yields vesicles which are able to sustain a high Ca\(^{2+}\) uptake activity, as compared to the erythrocyte ghost (Table I).

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**RESULTS**

**Characterization of the Purified ATPase**—The extent of ATPase purification obtained in our experiments is shown in Fig. 1. In the whole membrane electrophoretic pattern the differential (800 versus 687 nm) absorption changes were obtained under Arsenazo III (Scarpia, 1979), which were linearly proportional to changes of the Ca\(^{2+}\) concentration in the external medium. Most cases the reaction mixture contained 130 mM KCl, 20 mM PIPES-K\(^+\), pH 7.2, 5 mM MgCl\(_2\), 5 mM CaM, 50 \(\mu\)M Arsenazo III, and 2.5-3.0 \(\mu\)g of proteoliposomal protein/ml. Addition of Ca\(^{2+}\)-ATPase (5 \(\mu\)l increments) was added after obtaining the absorption base line in order to standardize the absorption changes. Then the ATPase reaction was performed by adding 0.2 mM ATP.

**Reconstitution of Plasma Membrane Ca\(^{2+}\)-ATPase**—Reconstitution of proteoliposomes with purified ATPase and exogenous lipids yields vesicles which are able to sustain a high Ca\(^{2+}\) uptake activity, as compared to measurements in the outside medium of purified erythrocyte Ca\(^{2+}\)-ATPase. SDS electrophoresis was performed according to Laemmli (1970) on a slab gel of 7% polyacrylamide and stained with Coomassie Blue. Lane 1, ghost membrane, 40 \(\mu\)g; Lane 2, purified erythrocyte Ca\(^{2+}\)-ATPase, 1.5 \(\mu\)g.

**Fig. 1. Electrophoretic analysis of erythrocyte PM proteins and purified Ca\(^{2+}\)-ATPase.** SDS electrophoresis was performed according to Laemmli (1970) on a slab gel of 7% polyacrylamide and stained with Coomassie Blue. Lane 1, ghost membrane, 40 \(\mu\)g; Lane 2, purified erythrocyte Ca\(^{2+}\)-ATPase, 1.5 \(\mu\)g.
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**TABLE I**

| Activity                        | Ghost membrane | Purified Ca-ATPase | Proteoliposomes | +FCCP | +CaM |
|--------------------------------|----------------|-------------------|----------------|-------|------|
| µmol/mg/min                    |                |                   |                |       |      |
| ATP hydrolysis -CaM            | 0.0025 ± 0.0004| 2.46 ± 0.25       | 0.16 ± 0.02    | 0.12 ± 0.01 | 1.13 ± 0.12 |
| ATP hydrolysis +CaM            | 0.0281 ± 0.0003| 3.78 ± 0.21       | 0.80 ± 0.04    | 1.31 ± 0.16 | 2.19 ± 0.18 |
| Ca\textsuperscript{2+} uptake -CaM |                |                   | 0.18 ± 0.02    | 0.13 ± 0.01 |     |
| Ca\textsuperscript{2+} uptake +CaM |                |                   | 0.89 ± 0.15    | 1.05 ± 0.15 |     |

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calmodulin. If we perform our measurements at 12 °C, H\textsuperscript{+} ejection and Ca\textsuperscript{2+} uptake proceed initially with a stoichiometric ratio of approximately 1, which then declines after 3–4 min (Fig. 4A). On the other hand, if we measure the two activities at 30 °C, the Ca\textsuperscript{2+}/H\textsuperscript{+} stoichiometric ratio is lower than 1 (Fig. 4B). Since we found in parallel experiments that the passive permeability of the proteoliposomes to H\textsuperscript{+} is quite low in the absence of ATP, the variable H\textsuperscript{+}/Ca\textsuperscript{2+} countertransport ratio suggests a temperature-dependent slippage of H\textsuperscript{+} during the operation of the pump. Under comparable conditions, slippage of the pump appears to effect H\textsuperscript{+} more than Ca\textsuperscript{2+} (compare Figs. 3 and 4).

Notwithstanding the variable Ca\textsuperscript{2+}/H\textsuperscript{+} ratio, a maximal (net) H\textsuperscript{+} ejection of approximately 2 µmol/mg protein was obtained at steady state (Fig. 4B). Based on a lumenal volume of 400 µl/mg protein, this corresponds to a 5 ms drop of luminal concentration, including free H\textsuperscript{+} and H\textsuperscript{+} released by buffers. The actual pH rise is 0.35 units, as determined by measurements with a pH electrode during the standardization procedures (see "Materials and Methods").

It is noteworthy that H\textsuperscript{+} ejection is not affected significantly by vanadomycin in the presence of K\textsuperscript{+}, but is totally reversed by FCCP (Fig. 5). Therefore, H\textsuperscript{+} ejection is not driven by any electrical gradient, but is a primary event linked to ATP-dependent Ca\textsuperscript{2+} uptake in the form of countertransport.

Establishment of Electrical Potential—It can be shown by the use of the indicator oxonol VI that ATP-dependent Ca\textsuperscript{2+} uptake generates an electrical potential across the proteoliposomal membrane, reaching steady state levels of 13–15 mV (Fig. 6). Establishment of the electrical potential is favored by the addition of FCCP. In this case the steady state potential increase up to 30–40 mV (Fig. 6), likely due to collapse of the H\textsuperscript{+} gradient and removal of its contribution to charge neutralization, and also due to stimulation of Ca\textsuperscript{2+} transport activity (Fig. 2). In the absence of FCCP and CaM, the initial rates of electrical potential formation are 1.8 ± 0.1 and 5.1 ± 0.3 mV min\textsuperscript{-1} at 12 °C and 30 °C, respectively (Fig. 7). In the presence of FCCP and CaM the initial rate rises to 22.1 ± 1.6 mV min\textsuperscript{-1} at 30 °C (Figs. 6 and 7).

Regulation of Transport and ATPase Activities—It is shown in Fig. 8 that a marked stimulation of Ca\textsuperscript{2+} uptake, ATPase activity, and electrical potential development is obtained by the addition of CaM. FCCP was added in these experiments to prevent inhibition by luminal alkalinization, thereby allowing occurrence of the CaM effect without interference. Comparable stimulation of Ca\textsuperscript{2+} uptake, ATPase activity, H\textsuperscript{+} ejection, and charge transfer demonstrates that these four parameters are subject to the same regulation and, therefore, linked by the same mechanism. It is noteworthy that stimulation by CaM, which is quite evident with native erythrocyte membrane (Schatzmann, 1993; Gopinath and Vincenzi, 1977; Jarrett and Penniston, 1977, 1987), is much more modest in the purified ATPase preparation (Kosk-Kosicka and Bzdega, 1988). On the other hand, we find that following its reconstitution in proteoliposomes, the enzyme regains its property of being greatly (5-fold) stimulated by CaM (Table I).
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In the presence of CaM, collapse of the electrical gradient by valinomycin does not affect the rate of Ca\(^{2+}\) uptake (Fig. 9A). On the contrary, collapse of the H\(^{+}\) gradient by FCCP causes a pronounced stimulation of Ca\(^{2+}\) uptake (Fig. 9B). The stimulation is greater if FCCP is added during the steady state when the H\(^{+}\) gradient is maximal (Fig. 9B), as compared to the beginning of the reaction when the H\(^{+}\) gradient is minimal (Table I).

These findings indicate that while a rise of luminal pH inhibits Ca\(^{2+}\) uptake (see also Fig. 2), the electrical potential developed under our condition produces only a minor inhibition if any. This latter conclusion is further substantiated by experiments in which 65, 130, or 260 mM KCl was added to proteoliposomes formed in the presence of 65 mM KCl in the luminal medium. It is shown in Fig. 10 that in the absence of valinomycin the KCl additions had no effect. In the presence of valinomycin, addition of 130 or 260 mM KCl is expected to generate a 18- or 36-mV diffusion potential, which is in the range of the potential produced by the pump. We found only a minor inhibition of the rate of transport by the 36 mV diffusion potential (curve f in Fig. 10), while the 18-mV potential had no significant effect.

It is noteworthy that the ATPase activity is subject to a regulatory pattern which is consistent with its linkage to Ca\(^{2+}\) transport. In addition to the effect of CaM (Table I), the consistent pattern of regulation includes ionophores and inhibitors. It is shown in Fig. 11 that collapse of the electrical potential by valinomycin does not change the ATPase rate, while...
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FIG. 6. Development of transmembrane electrical potential as a consequence of Ca\(^{2+}\) uptake by proteoliposomal vesicles. The reaction mixture contained 130 mM KCl, 20 mM PIPES-K\(^{+}\), pH 7.2, 20 mM CaCl\(_2\), 5 μg of CaM, 5 mM MgCl\(_2\), 1 μM oxonol VI, and 2.75 μg of protein (lipid:protein: 145:1:1). Four μM FCCP or 10 nM valinomycin were added when indicated. Calibration of oxonol absorption with membrane potential changes is shown in the inset. For this purpose, 130 mM KCl increments were added (indicated by arrows) to a reaction mixture as described above, containing proteoliposomes and valinomycin, but no ATP or FCCP. The resulting diffusion potentials were calculated according to the Nernst equation (Apell and Bersch, 1987), and plotted as a function of the concomitant absorption changes.

FIG. 7. Initial rates of electrical potential development at different temperatures. The reaction mixture was the same as that in Fig. 6, without valinomycin and FCCP. The measurement was carried out at 30 °C (a) and 12 °C (b), respectively.

collapse of the H\(^{+}\) gradient by FCCP increases the ATPase activity. A further increase is produced by collapsing the Ca\(^{2+}\) gradient with A23187, thereby relieving the inhibition by high luminal Ca\(^{2+}\) and promoting uncoupled ATPase turnover. Finally, it is shown in Fig. 11 that the PM ATPase is inhibited by vanadate which inhibits also Ca\(^{2+}\) uptake (Fig. 2).

DISCUSSION

As previously observed with the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (Levy et al., 1990; Yu et al., 1993), reconstitution of proteoliposomes with low protein to lipid ratios has the advantage of providing a large lumenal volume per enzyme unit. This allows transport of large amounts of Ca\(^{2+}\) through repeated enzyme cycles over a relatively long time of experimental observation. Considering the 1:150 (w:w) protein to lipid ratio used in our experiments with the PM Ca\(^{2+}\)-ATPase, we estimate that no more than 2 or 3 molecules of enzyme were reconstituted in each proteoliposome. This estimate was confirmed by electron microscopic observations on frozen and fractured proteoliposomes.

With respect to the transport properties of the proteoliposomes, we obtained our best results when we used a 10% phosphatidylcholine and phosphatidic acid (10:1) mixture for the liposomes, and C\(_{12}E_{8}\) as the detergent for the reconstitution procedure. The transport rates of 1.00 ± 0.12 μmol of Ca\(^{2+}\)/mg of protein min\(^{-1}\) observed in our experiments with reconstituted PM ATPase are in the same range as those obtained by Yu et al. (1993) with reconstituted SR ATPase, and by Niggli et al. (1981) with PM ATPase.

The Ca\(^{2+}\)/ATP stoichiometric ratio of 1 observed in our experiments is consistent with that reported by Niggli et al.
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Fig. 8. Effect of calmodulin on Ca\textsuperscript{2+} uptake, ATP hydrolysis, electrical potential development, and H\textsuperscript{+} ejection. The reaction mixtures for Ca\textsuperscript{2+} uptake (A), ATP hydrolysis (B), membrane potential (C), and H\textsuperscript{+} ejection (D), with (a) or without CaM (b), were the same as those described in the legends to Figs. 2, 3, and 7, respectively. All four measurements were carried out in the presence of 4 \mu M FCCP.

Fig. 9. Effects of valinomycin and FCCP on Ca\textsuperscript{2+} uptake by proteoliposomes. The reaction mixture contained 130 mM KCl, 20 mM PIPES-F\textsuperscript{+}, pH 7.2, 20 mM CaCl\textsubscript{2}, 5 mg of CaM, 5 mM MgCl\textsubscript{2}, 50 \mu M Arsenazo III, and 2.75 mg of protein (lipid/protein: 145)/ml. In the absence of valinomycin and FCCP, the reaction was started by addition of 0.2 mM ATP. Ten \mu M valinomycin or 4 \mu M FCCP were the added when indicated.

(1981) for the PM ATPase, but is lower than the ratio of 2 sustained by the SR Ca\textsuperscript{2+}-ATPase under optimal conditions. In fact, the Ca\textsuperscript{2+}/ATP ratio tends to decrease after a few minutes of reaction, possibly due to “slippage” of the pump when the vesicular Ca\textsuperscript{2+} rises, as also observed with the SR ATPase (Inesi and de Meis, 1989). It is difficult to demonstrate unambiguously whether a ratio lower than 2 is due to detergent exposure during purification, or is rather related to intrinsic features of the PM ATPase which may differ from those of the SR ATPase.

It should be pointed out that sequence alignment of the PM Ca\textsuperscript{2+}-ATPase and of a number of SR Ca\textsuperscript{2+}-ATPase isoforms (SERCA ATPases) reveals extensive homology within the group of SERCA isoforms, and within the group of PM isoforms. However, very little homology is found between the SERCA (MacLennan et al., 1985) and the PM ATPases (Verma et al., 1988), except in the region neighboring the phosphorylation site. In particular, the 6 residues involved in Ca\textsuperscript{2+} binding (Clarke et al., 1989) within the SERCA ATPase transmembrane region...
becomes lower following a few minutes of reaction with ATP, H+ countertransport, net transfer of positive charge into the lumen of the vesicles is sustained by the Ca2+ pump. Accord-

ingly, development of an electrical potential can be demonstrated during the early time of reaction following the addition of ATP, reaching steady state levels of 13–15 mV. Formation of the electrical gradient is favored (up to 30–40 mV) by collapse of the H+ gradient with FCCP. The effect of FCCP is due to additional positive charge associated with H+ flux into the lumen of the vesicles, and also due to reversal of Ca2+ pump inhibition by high luminal pH. On the other hand, collapse of the electrical potential by the addition of valinomycin does not produce significant stimulation of Ca2+ transport. Furthermore, in agreement with Niggli et al. (1982) we find that imposition of a diffusion potential of 38 mV produces very little inhibition of the rate of Ca2+ transport. Our experiments suggest that the electrogenic step has only a minor rate-limiting influence on the turnover of the pump, in the presence of the voltage range developed under our conditions.

Countertransport and electrogenic features of the PM ATPase are also observed with the SR ATPase reconstituted in a very similar proteoliposomal system (Yu et al., 1993). The reconstituted PM ATPase, however, appears to be electrogenically less efficient than the SR ATPase. In fact, the initial rate of voltage development by the SR ATPase corresponds quite closely to the rate of charge transfer calculated from the initial rates of cation transport (Table II). On the contrary, the initial rate of voltage development by the PM ATPase, under comparable conditions (10 °C, FCCP and calmodulin), is lower than the rate of charge transfer calculated from the initial rate of cation transport. This suggests significant slippage of compensating electrolytes, and may explain previous difficulties in detecting the electrical potential generated by the PM ATPase (Niggli et al., 1982) when less favorable detergents may have been used.

Our experience suggests that various detergents have different influences on the catalytic and transport functions of various enzymes, as well as on the apparent electrogenic properties of the reconstituted systems. For instance, in the reconstitution of the SR ATPase we obtained the best results by solubilizing separately the native SR vesicles with C8E8 and the liposomes with octylglucoside, and then mixing the two solutions together before removal of the detergents. On the other hand, when we used C8E8 for solubilization of both SR vesicles and liposomes, the resulting proteoliposomes displayed the same transport properties, but unfavorable electrogenic properties. As for the PM ATPase reconstitution, we found that the use of octylglucoside during the reconstitution procedure caused impairment
of catalytic and transport activity. On the other hand, the use of Triton or cholate permitted retention of good enzyme activity, but interfered with the development of electrical potential. For this reason we chose C₄E₄, resulting in production of proteoliposomes displaying very good transport activity, but less than ideal electrogenic properties. As for the composition of the reaction media for the functional assays, we found that the use of Cl⁻ as the main anion allows higher PM ATPase transport rates than SO₄²⁻. This is in contrast with our experiments with the SR ATPase, in which we found that minus SO₄²⁻ allows higher activity than Cl⁻ (Yu and Inesi, 1993).

With regard to regulation of the PM ATPase, it is noteworthy that the CaM effect is largely lost in the purified enzyme, but is regained following reconstitution of the proteoliposomal system (Table I and Fig. 2). This apparent loss of the CaM effect has been attributed to catalytic enhancement by enzyme oligomerization following purification (Kosk-Kosicka and Bzdega, 1988). Our finding that the CaM regulatory mechanism is regained following removal of detergent and reconstitution indicates that dilution of the enzyme in the proteoliposomal membrane promotes its native state and conformation.

In conclusion, our experiments indicate that the reconstituted erythrocyte PM Ca²⁺-ATPase sustains a (1:1) H⁺ and Ca²⁺ countertransport, which is electrogenic due to the excess charge associated with Ca²⁺ transport. Rise of the luminal pH by H⁺ ejection inhibits the pump turnover, while formation of electrical potential up to 30–40 mV produces very little inhibition. Demonstration of electrogenicity places a limit (i.e. less than 2) to the H⁺/Ca²⁺ stoichiometric ratio which is very difficult to determine accurately by direct measurements.

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