The purified Ca\(^{2+}\) Pump of Human Erythrocyte Membranes Catalyzes an Electroneutral Ca\(^{2+}\)-H\(^{+}\) Exchange in Reconstituted Liposomal Systems

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The purified Ca\(^{2+}\)-pumping ATPase of human erythrocyte membranes (Niggli, V., Adunyah, E. S., Penniston, J. T., and Carafoli, E. (1981) J. Biol. Chem. 256, 395-401) transports Ca\(^{2+}\) after reconstitution in asolectin liposomes, and the ATPase activity is stimulated 9- to 10-fold by A 23187. This indicates tight coupling between ATP hydrolysis and Ca\(^{2+}\) transport. In this system "extra" protons (i.e. in excess of those produced during the hydrolysis of ATP) appear in the medium, with a ratio of about 2 per ATP hydrolyzed. Evidently, the ATP-linked Ca\(^{2+}\) uptake induces the ejection of protons from the vesicles. In the presence of the protonophore carbonyl cyanide-m-chlorophenyl hydrazone and of valinomycin, the proton ejection is decreased by about 50%, indicating that a transmembrane proton gradient is indeed formed during Ca\(^{2+}\) transport. In the presence of the Ca\(^{2+}\) ionophore A 23187, no extra protons are produced during ATP hydrolysis.

In addition, evidence that Ca\(^{2+}\) transport is electroneutral is provided by the fact that no accumulation of tetracyphosphorylboron, a lipophilic anion, takes place during Ca\(^{2+}\) transport. Moreover, valinomycin (in the presence of K\(^{+}\)) has no stimulatory effect on Ca\(^{2+}\) transport rates. In the presence of the anion transport inhibitor 4,4'-diisothiocyanato-2,2'-stilbene disulfonic acid, which should inhibit anion transport through residues of band III possibly contaminating the Ca\(^{2+}\)-ATPase preparation, valinomycin was equally ineffective.

It is therefore proposed that the Ca\(^{2+}\) pump of human erythrocyte membranes operates as an electroneutral Ca\(^{2+}\)-H\(^{+}\) antiporter.

ATP-dependent Ca\(^{2+}\) pumps have now been identified in a variety of cell membranes (1) and two of them have even been purified to near homogeneity (2, 3). The mechanism of these pumps, however, has not yet been clarified. For instance, the problem of whether Ca\(^{2+}\) transport is electrogenic, or obligatorily compensated by co- or countertransport of other ions has not yet been solved. As discussed by Sarkadi (4), there is some evidence, based on experiments with resealed ghosts, that Ca\(^{2+}\) uptake is not dependent upon Na\(^{+}\), K\(^{+}\), and not coupled to the countertransport of any divalent cation. Recently, Waisman et al. have presented some evidence that Ca\(^{2+}\) transport in inside-out resealed ghosts is stimulated by phosphate and other anions, like chloride, acetate, etc., and that these anions accumulate in a Ca\(^{2+}\)-dependent way during Ca\(^{2+}\) transport (5, 6). Since this process is inhibited by NAP-taurine, it probably occurs through band 3. Waisman et al. have postulated that Ca\(^{2+}\) transport is electrogenic, resulting in the creation of a positive membrane potential inside the vesicles, which would be the driving force for anion uptake. However, no direct evidence for the generation of a membrane potential has been provided by the authors. Phosphate transport, however, could also be explained by the formation of an insoluble Ca\(^{2+}\)-phosphate complex inside the vesicles, which would result in the establishment of an apparent phosphate gradient across the membrane.

The ion balance during the transport of Ca\(^{2+}\) has been studied in detail in another membrane system, sarcoplasmic reticulum. The sarcoplasmic reticulum Ca\(^{2+}\) pump differs in several respects from the erythrocyte Ca\(^{2+}\)-ATPase (e.g. molecular weight, interaction with calmodulin, and inhibition by vanadate (7)). Zimniak and Racker (8) have found a stimulatory effect of valinomycin in the presence of K\(^{+}\) on Ca\(^{2+}\) uptake in the reconstituted system. Chiesi and Inesi (9) have observed H\(^{+}\) ejection by sarcoplasmic reticulum vesicles during Ca\(^{2+}\) uptake, using conditions where practically no protons resulted from the ATPase reaction, and they have shown that the initial rates of proton release and Ca\(^{2+}\) uptake occur simultaneously. In the reconstituted system, they have found stimulation of Ca\(^{2+}\) transport by protonophores, but they have not shown direct H\(^{+}\) ejection in this system. Chiesi and Inesi (9) have postulated that 1 proton is exchanged for 1 Ca\(^{2+}\), resulting in electrogenic transport. This would explain both their results and those by Zimniak and Racker (8). Ueno and Sekine (10, 11) have found a stimulation of Ca\(^{2+}\) transport in K\(^{+}\)-loaded sarcoplasmic reticulum vesicles by nigericin. They have also observed a rapid H\(^{+}\) ejection during the initial phase of Ca\(^{2+}\) uptake, which, however, was not abolished by the addition of CCCP and valinomycin, indicating that their vesicles are quite permeable to protons. In addition, they have measured uptake of acetate which would correspond to the generation of a small pH gradient, alkaline inside, during Ca\(^{2+}\) uptake. The main difficulty in the cited experiments on the sarcoplasmic reticulum ATPase is to decide if the proton appearance outside the vesicles is an inherent property of the Ca\(^{2+}\) pump, or a secondary effect of proton displacement by

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1 The abbreviations used are: NAP-taurine, N-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate; CCCP, carboxy cyanide-m-chlorophenyl hydrazine; Hepes, N-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis[β-aminoethy]- N,N,N',N'-tetraacetic acid; TPB, tetrathylenethionate; HEDTA, N-(2-hydroxymethyl)-ethylendiamine-N,N',N'-triacetic acid; DIDS, 4,4'-disothiocyanato-2,2'-stilbene disulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid.
calcium inside the vesicles, and subsequent passive H⁺ diffusion due to the pH gradient across the membrane.

So far, H⁺ extrusion by an ATP-driven Ca⁺⁺ pump has not been measured directly in a reconstituted system. In this paper, measurements of this type will be reported. The work will describe the charge and ion transfer during Ca⁺⁺ transport by the purified Ca⁺⁺ pump of erythrocytes (2), reconstituted in asolectin liposomes (12).

**Materials and Methods**

All reagents were of the highest purity available. Asolectin was obtained from Associated Concentrates (Woodside, NY). Cholic acid was treated with activated carbon and recrystallized twice from ethanol, 20% water (v/v). The recrystallized acid was neutralized with KOH.

**Preparation of the Purified Ca⁺⁺-ATPase from Human Red Cell Membranes**

The enzyme was isolated in the presence of phosphatidylcholine by using a protoplasmin affinity column, as described previously (12). Protein was determined as described in Ref. 12.

**Reconstitution of the Purified Ca⁺⁺-ATPase in Asolectin Liposomes by Cholate Dialysis**

A modification of the procedure of Kagawa and Raether (13) has been used (see also Ref. 14). 90 mg of asolectin, dried under nitrogen, was dispersed in 1 ml of a medium containing 80 mM potassium chloride, 100 mM KCl, 20 mM Hepes, pH 7.2 at 4°C, 1 mM MgCl₂, and 50 mM CaCl₂. This suspension was sonicated to clarity at 0°C. 100 p1 of the lipid mixture was added to 350 p1 of the isolated enzyme (0.1 to 0.4 mg of protein/ml). This mixture was briefly mixed on a Vortex mixer and then dialyzed against 1000 volumes of 130 mM KCl, 20 mM Hepes (pH 7.2 at 4°C), 1 mM dithiothreitol, and 0.5 mM MgCl₂. This basic reconstitution procedure was used with the modifications indicated for all experiments described.

**Determination of Ca⁺⁺-ATPase Activity**

The ATPase activity was measured spectrophotometrically, using a coupled enzyme assay. The medium contained 120 mM KCl, 60 mM Hepes, pH 7 at 37°C, 1 mM MgCl₂, 0.5 mM K⁺-ATP, 0.2 mM NADH, 0.5 mM phosphoenolpyruvate, 1 IU of pyruvate kinase/ml assay medium, 1 IU of lactate dehydrogenase/ml of assay medium, 10 mM CaCl₂. The ATPase activity was followed by measuring continuously the difference in absorbance at 366 and 550 nm, using a dual wavelength spectrophotometer. After preincubation of the assay medium (total volume: 1 ml) for 4 min at 37°C, the reaction was started by the addition of the reconstituted ATPase (2 to 5 p1 of protein) to the medium. The activity in the presence of EGTA was practically zero.

**Measurement of TPB⁺ Movements**

The purified Ca⁺⁺-ATPase was reconstituted using the basic procedure described above, except that in all buffers, K⁺ was replaced by Na⁺. TPB⁺ movements were determined with a TPB⁺ electrode (15) in a medium containing the same components as the assay medium described above for the coupled enzyme assay, plus 0.5 mM EGTA and 0.5 mM HEDTA. K⁺ was replaced by Na⁺. The reconstituted enzyme was preincubated 4 min at 37°C with 0.5 mM ATP and 30 to 100 p1 of TPB⁺, and the reaction was started by the addition of 0.6 mM CaCl₂ (free Ca⁺⁺: 10 pM).

**Effect of Valinomycin on Ca⁺⁺ Transport by the Reconstituted Enzyme**

Ca⁺⁺-ATPase Reconstituted in the Presence of K⁺⁺—The purified enzyme was reconstituted using the basic procedure described above. An aliquot of the reconstituted enzyme was dialyzed for a second time for 2 h against a medium containing 130 mM NaCl, 20 mM Hepes, pH 7.2 at 4°C, 1 mM dithiothreitol, 50 pM MgCl₂, and was then immediately assayed in a medium containing Na⁺ instead of K⁺⁺.

Enzyme Reconstituted in the Presence of Na⁺⁺—350 p1 of the purified enzyme were added to 100 p1 of an asolectin-cholate mixture as described for the basic procedure. However, K⁺ was replaced by Na⁺. This mixture was then dialyzed for 30 min at 4°C against 100 volumes of a medium containing 130 mM NaCl, 20 mM Hepes, pH 7.2 at 4°C, 50 mM MgCl₂, and 18 mM sodium cholate. In a second dialysis step, the enzyme was then dialyzed overnight against the same buffer not containing cholate.

**Ca⁺⁺ Transport Measurements—Ca⁺⁺ transport was measured with a Ca⁺⁺-selective liquid membrane electrode in an incubation vessel specially devised for the use of these electrodes (Ref. 16), to ensure constant stirring, short mixing time, and small incubation volume. The incubation medium contained 130 mM NaCl, 20 mM Hepes, 1 mM MgCl₂, 10 mM CaCl₂ in a total volume of 1 ml. 50 p1 of proteoliposomes were added to the assay medium and preincubated 15 to 30 s with or without 0.4 pM of valinomycin/ml. The reaction was started by the addition of 0.5 mM K⁺-ATP to the K⁺⁺ medium as described above. The reaction was started by the addition of 0.5 mM K⁺-ATP. Initial rates were determined after about 5 s. The Ca⁺⁺ concentration of the stock solutions of ATP was adjusted to that of the assay medium.

**Effect of CCCP and Valinomycin on the Steady State Rate of the Reconstituted Ca⁺⁺-ATPase**

The enzyme was reconstituted in KCl using the basic procedure, with the following modification: the dialysis buffer contained 50 mM K-MEES, pH 7.2 at 4°C, instead of Hepes. The Ca⁺⁺-ATPase activity was determined as described above, by using a coupled enzyme assay. The assay medium contained the components described above, plus 0.5 mM EGTA and 0.5 mM HEDTA. Reconstituted ATPase (2 to 5 p1 of protein) was added to the medium, and the reaction was started by the addition of 0.6 mM CaCl₂ (free [Ca⁺⁺]: 10 pM). The rate of ATP hydrolysis was followed until linear, and about 2 min after starting the reaction, 2 mM CCCP or 0.4 pM of valinomycin/ml, or both agents together, were added to the medium, and the rate was again recorded for another 2 min. In other experiments, 0.5 pM A 23187 was first added to the liposomes, and afterwards, valinomycin or CCCP or both were added to the medium.

**Measurements of H⁺ Movements during Ca⁺⁺ Transport by the Reconstituted Ca⁺⁺-ATPase**

The purified Ca⁺⁺-ATPase was mixed with a lipid-cholate solution as described for the basic reconstitution procedure. This mixture was first dialyzed 30 to 45 min at 4°C against 100 volumes of a buffer containing 18 mM potassium cholate, 200 mM potassium Hepes, pH 7.2, 25 mM KCl, 40 mM potassium oxalate, 50 mM CaCl₂, 1 mM MgCl₂, 1 mM dithiothreitol. The medium was then dialyzed 16 h against 500 volumes of a buffer containing the same components as the first buffer, but without cholate. Finally, the mixture was dialyzed 4 h against 500 volumes of a buffer containing 260 mM KCl, 50 mM CaCl₂, 1 mM dithiothreitol, and 4 mM Hepes, pH 7.2. The vesicles were used within 6 h. H⁺ movements were measured with a combination pH electrode (Philips CA 14/92), using the same incubation vesicles as described above for Ca⁺⁺ transport (16). The medium (final volume: 1 ml) contained 260 mM KCl, 10 mM CaCl₂, and 1 mM MgCl₂. The pH of the medium was adjusted after addition of the reconstituted enzyme to 6.5, 7.0, or 7.2 at 37°C, as indicated, with KOH or HCl. The electrode was calibrated by adding known amounts of HCl or Titrisol (Merck). The full scale sensitivity was 0.1 pH unit and the 90% response time of the electrode was ≤1 s. All measurements were done at 37°C. 50 to 100 p1 of the reconstituted enzyme were added to the assay medium and the reaction was started by the addition of 500 pM TPB (water solution, the pH adjusted to the pH of the medium, so as minimize the pH jump upon addition to the assay medium). When indicated, 0.4 pM A 23187 or 2 p1 CCCP and 0.4 pM of valinomycin/ml assay medium were added to the vesicles. The K⁺⁺ content of the buffers was adjusted to obtain the same concentration inside and outside the vesicles.

In all experiments described in this section, where ethanolic solutions of ionophores were used, the control experiments without ionophores were done in the presence of the same amount of ethanol. In the experiments, where a solution of DIDS in dimethyl sulfoxide was used, the control measurements were done in the presence of the same amount of dimethyl sulfoxide (maximally, the concentration of ethanol was 0.6% (v/v), and that of dimethyl sulfoxide, 0.5% (v/v)).
RESULTS

Properties of the Reconstituted ATPase

Fig. 1 shows the Ca\(^{2+}\)-ATPase activity of enzyme purified in the presence of phosphatidylcholine and reconstituted by cholate dialysis in asolectin in the presence of high concentrations of buffer and oxalate. The initial rate of ATPase activity (measured about 10 s after addition of the vesicles) declined progressively with time, to reach a steady rate which corresponded to about one-sixth of the initial rate. After addition of A23187 the steady state rate was stimulated about 10-fold. This experiment shows clearly that the Ca\(^{2+}\)-ATPase activity is tightly coupled to Ca\(^{2+}\) transport.

In analogy with calculations made for the case of the stimulation of reconstituted cytochrome oxidase by uncouplers (17), the amount of ATPase incorporated in a transmembrane functional fashion may be estimated considering only the molecules presenting the ATP-splitting portion to the outside medium (enzyme molecules of opposite polarity are inactive in the present experiments, due to the impermeability of the liposomes to the ATP substrate). A 10-fold stimulation of the steady state rate by A23187 corresponds then to 90% of the active enzyme molecules incorporated in a transmembrane fashion, assuming ideally ion-impermeable liposomes. This actually represents an underestimation, due to the impermeability of liposomes. In all experiments to be described in this work, reconstituted Ca\(^{2+}\)-ATPase with a stimulation factor by A23187 of at least 9-fold, was therefore used. It was found that these high “coupling” ratios were only obtained by reconstituting the enzyme in asolectin by the cholate dialysis method. The Triton X-100-Bio-Beads method (see Ref. 12) yields a reconstituted enzyme which was stimulated only about 2- to 3-fold by A23187. Vesicles reconstituted in phosphatidylcholine, even by the cholate dialysis method, were, on the other hand, invariably poorly coupled (about 2-fold stimulation by A23187).

In addition to ATPase activity, Ca\(^{2+}\) transport has also been measured directly in asolectin liposomes by using a Ca\(^{2+}\)-sensitive electrode (see Fig. 2).

Investigation of Charge Transfer during Ca\(^{2+}\) Transport

Movements of TPB during Ca\(^{2+}\) Accumulation in the Reconstituted System—It has been shown that TPB\(^-\), a lipophilic membrane-permeable anion, is accumulated into sonicated submitochondrial particles in an energy-dependent manner. These particles maintain, upon energization, a positive potential inside (18). Therefore, one should be able to demonstrate uptake of TPB\(^-\) by the isolated reconstituted Ca\(^{2+}\)-ATPase during Ca\(^{2+}\)-transport, if a membrane potential, positive inside, is generated. The movements of TPB\(^-\) were investigated with a TPB\(^-\)-sensitive electrode (15) using purified ATPase reconstituted into asolectin liposomes in the presence of Na\(^+\) instead of K\(^+\). In the uptake medium, Na\(^+\) replaced K\(^+\), since the potassium salt of TPB is extremely insoluble. Under these conditions, the reconstituted vesicles readily accumulated Ca\(^{2+}\), but no change in the free concentration of TPB\(^-\) (total concentration: 30 to 100 \(\mu\)M) could be observed in the medium upon addition of ATP. Controls were done to ensure that TPB\(^-\), at the concentrations used, did not inhibit Ca\(^{2+}\) transport or Ca\(^{2+}\)-ATPase activity.

Effect of Valinomycin on the Initial Rate of Ca\(^{2+}\) Transport of the Reconstituted ATPase, in the Absence and in the Presence of DIDS—1. The ATPase was reconstituted and assayed in a potassium medium in which the permeability of the liposomal membrane to potassium is increased by valinomycin. This is expected to stimulate the transport of Ca\(^{2+}\) if the latter were electrogenic, by decreasing the membrane potential created by it. Initial rates of Ca\(^{2+}\) transport have been measured with a Ca\(^{2+}\)-selective electrode, as described under "Materials and Methods." In the presence and in the absence of valinomycin (see Fig. 2A). The variability of the initial rates of the same preparation did not exceed \(\pm 10\%\), and no significant effect of valinomycin could be observed.

2. Other types of reconstitution were performed, where a preformed K\(^+\) gradient across the membrane was present. This was achieved by reconstituting the Ca\(^{2+}\)-ATPase in K\(^+\) or Na\(^+\) media, and by assaying it in Na\(^+\) or K\(^+\) media. 30 s
before starting the experiment by the addition of ATP, valinomycin was added. This result is in line with the idea of a negative or positive transmembrane electrochemical potential due to the preformed K+ gradient. Again, as shown in Fig. 2B, no significant effect of valinomycin on the initial rate of Ca2+ transport could be observed. This shows that the initial rate of Ca2+ transport was not influenced by a preformed transmembrane electrical potential positive or negative inside the vesicles.

Finally, the effect of valinomycin on the initial rates of Ca2+ transport was also investigated in the presence of the anion transport inhibitors DIDS and NAP-taurine. It could have been argued that the Ca2+-ATPase preparation is contaminated by very small amounts (≤5%) of the anion transport protein band 3 (see 2). A potential generated by the Ca2+ uptake could thus be dissipated by net anion uptake through this protein, which would render a presumed electrogenic transport of Ca2+ electroneutral. Inhibition of the anion transporter by the inhibitors DIDS or NAP-taurine (19) should therefore eliminate this mechanism of charge compensation and reveal an electrogenic transport, if present. During the investigation of the effect of these agents on Ca2+ transport, it was unexpectedly found that low concentrations of DIDS had a pronounced inhibitory effect on the reconstituted Ca2+-ATPase activity. The effect was seen in the absence and in the presence of A 23187, and was observed also with the detergent solubilized enzyme. The inhibition of the Ca2+-ATPase activity was paralleled by the inhibition of the Ca2+ uptake and was observed, although less effectively, also with NAP-taurine. In the presence of 0.2 mg of phospholipid/ml of assay medium, 1 to 2 μM DIDS inhibited the Ca2+-ATPase activity by 50%, whereas 50 to 100 μM NAP-taurine was required to produce an equivalent inhibition. Clearly, these inhibitors have a direct effect on the ATPase which is unrelated to the charge-neutralization mechanism. Therefore, they were used at concentrations that produced only 30 to 40% inhibition of the ATPase activity. Under these conditions, valinomycin did not reverse the inhibition of the Ca2+ uptake by DIDS (Fig. 3) and by NAP-taurine.

The results presented above indicate that Ca2+ transport by the reconstituted Ca2+-ATPase is not an electrogenic process. Therefore, charges must be compensated by an obligatory anion/Ca2+ cotransport or a Ca2+ /cation antipor if not solubilized enzyme. Since evidence has been provided, that the Ca2+-ATPase of sarco-plasmic reticulum functions as a Ca2+-H+ antiporter (9, 11), the possibility of H+ fluxes compensating charges also in the Ca2+-ATPase of erythrocytes was investigated.

**Fig. 3. Effect of valinomycin on initial rates of Ca2+ uptake by the reconstituted Ca2+-ATPase in the presence of DIDS.**

When indicated, 2 μM CCCP, 0.4 μg of valinomycin/ml, or 0.4 μM A 23187 were added to the medium as ethanolic solutions (for details see "Materials and Methods"). Relative rates of ATP hydrolysis are given (for comparison, the absolute rate of ATP hydrolysis in the control experiment was 2.4 nmol of ATP hydrolyzed/min/20 μl of enzyme). The small stimulation (5 to 15%) resulting from the addition of ethanol alone was subtracted from the actual rates.

**Table I**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Relative rate of ATP hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.0</td>
</tr>
<tr>
<td>CCCP</td>
<td>1.45</td>
</tr>
<tr>
<td>Valinomycin</td>
<td>1.40</td>
</tr>
<tr>
<td>Valinomycin/CCCP</td>
<td>1.50</td>
</tr>
<tr>
<td>A 23187</td>
<td>9.8</td>
</tr>
<tr>
<td>A 23187/CCCP</td>
<td>9.5</td>
</tr>
<tr>
<td>A 23187/CCCP/valinomycin</td>
<td>9.4</td>
</tr>
</tbody>
</table>

40%. When the two ionophores were added together, a 50% stimulation was observed. When CCCP and valinomycin were added to the reconstituted enzyme after A 23187, no stimulatory effect could be observed. These data will be discussed below.

**H+ Fluxes during Ca2+ Transport of the Isolated and Reconstituted Ca2+-ATPase**

H+ translocation during Ca2+ transport was measured directly with a pH electrode. In this case, the ATPase was reconstituted in the presence of high concentrations of Hepes and oxalate. This was done to minimize, at least during the first turnovers, changes in the intraliposomal pH and free Ca2+ concentration that would inhibit the enzyme. In a subsequent dialysis step, Hepes and oxalate were then removed from the outside of the liposomes, and proton translocation was measured in a medium with low pH-buffering capacity, and having the same concentration of KCl as that of the liposomes. The results of experiments of this type are shown in Fig. 4.

Clearly, a fast initial rate of H+ extrusion by the reconstituted enzyme could be observed upon addition of ATP which

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corresponded to 93 nmol of H⁺/min/50 µl of enzyme (initial rates corresponded to rates measured 2 to 3 s after addition of ATP). This rate slowed down gradually, to a rate of about 10 nmol of H⁺/min/50 µl after 30 s. By the addition of A 23187 at this point, the H⁺ production was stimulated, to a linear rate of 23 nmol H⁺/min/50 µl of enzyme. When A 23187 was added before ATP (see Fig. 4A), the initial phase of rapid H⁺ extrusion described above was not observed upon addition of ATP to the enzyme and only a linear rate of H⁺ production could be measured, which corresponded very well to the rate of H⁺ production measured in the same preparation, when A 23187 was added after ATP. In addition, the rate of H⁺ production in the presence of A 23187 was well comparable to the rate of ATP hydrolysis measured using the coupled enzyme assay system with the same enzyme preparation in the presence of A 23187 (30 nmol of ATP hydrolyzed/min/50 µl) (see Fig. 1), taking into consideration that at pH 7, only 0.7 nmol of H⁺ is released/nmol of ATP hydrolyzed (20).

In the presence of CCCP and valinomycin, the initial rate of H⁺ extrusion was markedly and reproducibly decreased (see Fig. 4B), to a value of 49 nmol of H⁺/min/50 µl. Appropriate controls showed that CCCP and valinomycin, in the presence of ATP, did not change the H⁺-buffering capacity of the medium. The rate in the presence of CCCP and valinomycin, measured in five different enzyme preparations, was reproducibly 40 to 50% lower than the initial rate in the absence of these agents. Experiments of the type shown in Fig. 4 have been carried out at pH 6.8, 7.0, and 7.2, and the results are summarized in Table II. On the basis of these data, we have calculated, for each pH, the amount of "extra" protons extruded in the initial phase. The calculations are based on the assumption that after addition of ATP, the Ca⁺⁺-ATPase in the initial phase is fully uncoupled. We have therefore subtracted from the initial rate of proton extrusion the rate of H⁺ production measured in the same experiment after the addition of A 23187. In addition, we have calculated the ratio of the amount of extra protons extruded in the initial phase per ATP hydrolyzed in the same time (see Table II). Well comparable values have been obtained at the three pH values ranging from 1.7 to 2.4 H⁺/ATP. If one assumes (based on the data described under "Investigation of Charge Transfer during Ca⁺⁺ Transport"), that the Ca⁺⁺ transport is electroneutral (2 H⁺ extruded/Ca⁺⁺ accumulated), then the data fit best the assumption of 1 Ca⁺⁺ transported/ATP hydrolyzed. The amount of "extra" protons extruded in the initial phase in the presence of CCCP and valinomycin have also been calculated and a figure of 0.7 H⁺/ATP has been obtained at pH 7.0 and at pH 7.2.

**DISCUSSION**

The investigation of a membrane potential or of ion gradients generated during Ca⁺⁺ transport requires a system with tight coupling between ATP hydrolysis and Ca⁺⁺ transport. We have now achieved to improve the properties of our reconstituted Ca⁺⁺-ATPase by using the cholate dialysis method (see Ref. 14). The initial rate of ATP hydrolysis was about 6-fold higher than the steady state rate and the latter was stimulated about 10-fold by A 23187. This corresponds (see "Results") to the incorporation of at least 90% of the active enzyme in the membrane of essentially ion-impermeable vesicles. As mentioned, tight coupling between ATP hy-

**TABLE II**

Proton production by the isolated and reconstituted Ca⁺⁺-ATPase upon addition of ATP, in the absence, and in the presence of A 23187, measured at three different pH values

<table>
<thead>
<tr>
<th>pH</th>
<th>Initial rate of H⁺ production (nmol/min/50 µl enzyme)</th>
<th>H⁺ production in the presence of A 23187 (pmol/min/50 µl)</th>
<th>H⁺ produced/ATP hydrolyzed (Ref. 20)</th>
<th>Rate of ATP hydrolysis (nmol/min/50 µl)</th>
<th>Corrected rate of initial H⁺ extrusion (H⁺ due to ATP hydrolysis subtracted)</th>
<th>H⁺ extruded/ATP hydrolyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.8</td>
<td>65 ± 5 <em>(n = 4)</em></td>
<td>13 ± 2 <em>(n = 5)</em></td>
<td>0.6</td>
<td>22 ± 3</td>
<td>52 ± 7</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td>7.0</td>
<td>93 ± 1 <em>(n = 3)</em></td>
<td>24 ± 3 <em>(n = 6)</em></td>
<td>0.7</td>
<td>34 ± 4</td>
<td>69 ± 8</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>7.2</td>
<td>98 ± 6 <em>(n = 3)</em></td>
<td>31 ± 3 <em>(n = 7)</em></td>
<td>0.8</td>
<td>39 ± 4</td>
<td>67 ± 6</td>
<td>1.7 ± 0.2</td>
</tr>
</tbody>
</table>
drosis and Ca\textsuperscript{2+} transport was only observed when the enzyme was reconstituted in asolectin by the cholate dialysis method, a system in which the ATPase was already in the high affinity state in the absence of calmodulin due to the presence of acidic phospholipids in asolectin (see Refs. 11 and 13). The following findings made here are taken as evidence that the operation of the Ca\textsuperscript{2+} pump does not create a membrane potential. First (Fig. 1), the initial rate of ATP hydrol-
ysis, which is 60% of the rate in the presence of A 23187 at the time of the first determination (10 s), can be extrapolated to the same value as in the presence of A 23187 at time zero.

The inhibition of the rate of ATP hydrolysis develops gradually over a period of about 2 min. However, if Ca\textsuperscript{2+} transport were electrogenic, a membrane potential positive inside would immediately develop after the first few turnovers of the enzyme, and markedly inhibit both the uptake of Ca\textsuperscript{2+} and ATP hydrolysis already in the first few seconds of the reaction. Second, valinomycin would be expected to stimulate the electrogenic Ca\textsuperscript{2+} transport by increasing the passive permeability of the membrane for K\textsuperscript{+}, resulting in charge equilibration across the membrane. However (Fig. 2), it had no effect on the measured initial Ca\textsuperscript{2+} transport rates. By comparison, one may quote the findings by Zimmnik and Racker (8), who have reported a 50% stimulation of the Ca\textsuperscript{2+} uptake rate by the isolated and reconstituted sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase by valinomycin in the presence of K\textsuperscript{+}. In addition (Fig. 2), a preformed transmembrane potential positive or negative inside the liposomes had no effect on the initial rate of Ca\textsuperscript{2+} transport.

As mentioned, Waisman \textit{et al.} (5, 6) have found inhibition of Ca\textsuperscript{2+} and phosphate accumulation into inside-out resealed erythrocyte vesicles by DIDS. If the Ca\textsuperscript{2+}-ATPase used here were contaminated with even small remnants of the anion-conducting band III, inhibitors of this carrier should have been expected to block anion fluxes through it, and thereby reveal the true nature of the Ca\textsuperscript{2+} pump in terms of electro
genicity. DIDS and NAP-taurine did in fact inhibit the ATPase activity and the Ca\textsuperscript{2+} transport associated with it, but the effects were unrelated to their action on the anion channel in band III. Valinomycin does not reverse the 40% inhibition of the initial rate of Ca\textsuperscript{2+} uptake by DIDS.

A third line of evidence for the electroneutrality of the Ca\textsuperscript{2+} pump is the finding that no uptake of the lipophilic anion tetraphenylboron could be measured under conditions where Ca\textsuperscript{2+} was transported into the liposomes. Based on all these data, it is thus permissible to assume that the Ca\textsuperscript{2+} pump is electrically silent, and operates as a Ca\textsuperscript{2+} ion symporter or antiporter.

A rapid initial rate of proton extrusion upon addition of ATP to the reconstituted Ca\textsuperscript{2+} pump was found (Fig. 4). The initial rate of proton extrusion, measured 2 to 3 s after the addition of ATP, was at least 3-fold higher than the rate of protons produced in the presence of A 23187. If we assume that the initial rate of ATP hydrolysis upon addition of ATP to the reconstituted system corresponds to the rate of ATP hydrolysis measured in the presence of A 23187, then about 2 extra protons appeared in the external medium per ATP hydrolyzed. If the system was made permeable to protons by the addition of CCCP and valinomycin, the initial rate of proton production was about 50% lower than in the absence of these agents, showing that, indeed, a pH gradient had developed during Ca\textsuperscript{2+} transport. The source of the 0.7 extra proton appearing per ATP hydrolyzed in the proton-permeable system is unclear. Probably, the accumulated Ca\textsuperscript{2+} binds to anionic sites inside the liposomes, thereby releasing the protons which appear in the outside medium, as the membrane is very permeable in the presence of valinomycin and CCCP.

In the proton-impermeable system, protons released from internal binding sites would be expected to appear after a lag phase, due to the high intraliposomal pH buffering chosen in our experiments. Moreover, during the operation of the pur
tative Ca\textsuperscript{2+}-H\textsuperscript{+} exchanger, the liposomes should become more alkaline inside, and any protons released from internal binding sites would thus not be expected to appear in the outside medium. Therefore, the 2 extra protons extruded per ATP hydrolyzed in the proton-impermeable system can be safely attributed to the Ca\textsuperscript{2+}-H\textsuperscript{+} exchanger. Based on the finding that Ca\textsuperscript{2+} uptake and ATP hydrolysis were well coupled in the liposomes used here, and on the above-mentioned conclu
dition that the Ca\textsuperscript{2+} transport is electroneutral (2 H\textsuperscript{+} extruded/ 1 Ca\textsuperscript{2+} transported), one can derive the number of Ca\textsuperscript{2+} transported per ATP hydrolyzed from the number of protons extruded per ATP hydrolyzed (see Table II). The experimental data agree well with the ratio of 1 Ca\textsuperscript{2+} transported/ATP hydrolyzed. According to the data presented, a proton gradient, as well as a Ca\textsuperscript{2+} gradient, are developed during Ca\textsuperscript{2+} transport. These gradients are presumably responsible for the observed inhibition of the steady state rate of the Ca\textsuperscript{2+}-ATPase activity (see Fig. 1). An increase in the proton permeability of the membrane is therefore expected to stimulate the steady state rate of the Ca\textsuperscript{2+}-ATPase.

The investigation of the effect of the protonophore CCCP, with or without valinomycin, on the steady state rate of the Ca\textsuperscript{2+}-ATPase (see Table I) has shown that the steady state rate of the Ca\textsuperscript{2+}-ATPase was stimulated to a similar extent by either CCCP or by valinomycin added alone, or by the combined addition of the two (40 to 50% stimulation). The unex
gected stimulation by valinomycin alone may be explained by the findings by Biegel and Gould (21), that valinomycin stimulates the passive H\textsuperscript{+} diffusion across asolectin liposomal membranes about 300-fold, thereby contributing to the dissipation of the pH gradient across the vesicle membrane. Fi
nally, it must be stressed that all stimulatory effects of CCCP, of valinomycin, or of both are small (1.5-fold activation) as compared to the stimulation by A 23187 (9-fold activation). This may be explained by the relatively high permeability of the asolectin vesicles to protons (21), that prevents the estab
lishment of a high pH across the membrane. Since A 23187 probably catalyzes an electroneutral Ca\textsuperscript{2+}/H\textsuperscript{+} exchange (22), it would reverse the Ca\textsuperscript{2+} pump and H\textsuperscript{+} movement created by the action of the Ca\textsuperscript{2+} pump proposed here. The finding that valinomycin and CCCP had no stimulatory effect on the ATPase when the latter is stimulated by A 23187 (see Table I) adds further evidence to the mechanism proposed.

In conclusion, it is proposed that the Ca\textsuperscript{2+} pump operates as an obligatory electroneutral Ca\textsuperscript{2+}/H\textsuperscript{+} exchanger. The experimental data presented do not permit to discriminate be
tween an OH\textsuperscript{-}/Ca\textsuperscript{2+} cotransport and a Ca\textsuperscript{2+}/H\textsuperscript{+} antiport, but the second mechanism is considered more likely. The Ca\textsuperscript{2+}-pumping ATPase of the plasma membrane thus apparently contains molecular devices for transferring both Ca\textsuperscript{2+} and the charge-compensating H\textsuperscript{+}. At the present stage, nothing is known on the organization of the enzyme in the membrane. The mode of operation of the enzyme molecule is therefore completely obscure.

Based on our model, the formation of a H\textsuperscript{+} gradient is the primary consequence of Ca\textsuperscript{2+} extrusion from intact red blood cells. An important question at this point is that of the subsequent fate of this pH gradient in vivo. In red blood cells, OH\textsuperscript{-} ions are equilibrated mainly via the anion exchange system of band III, which involves a chloride-bicarbonate exchange (see Ref. 23). CO\textsubscript{2} forms bicarbonate ion by reacting with OH\textsuperscript{-}, exchanges via band III with chloride, and disso
ciates again to OH\textsuperscript{-} and CO\textsubscript{2}. This process results in an OH\textsuperscript{-}
anion exchange across the membrane, and is inhibited by the band III inhibitors DIDS and NAP-taurine. As shown in Fig. 5A, which depicts the polarity of the system in intact cells, a proton gradient resulting from the Ca\(^{2+}\) pump, will be dissipated in vivo by the anion-bicarbonate exchange through band III. Ca\(^{2+}\) transport will therefore result in a band III-dependent anion transport in the same direction as Ca\(^{2+}\) transport. Fig. 5B shows the polarity of the membrane in resealed inside-out erythrocyte vesicles, which corresponds to the situation in the isolated, reconstituted enzyme. In the isolated enzyme, however, the anion exchanger is absent.

Waisman et al. (5,6) have suggested that ion accumulation in human erythrocyte inside-out vesicles proceeds by a primary electrogenic Ca\(^{2+}\) pump, which drives a secondary electrogenic accumulation of anions presumably via band III. However, the net uptake of anions proposed by these authors seems rather unlikely, in view of the fact that band III catalyzes mainly an obligatory exchange of anions. The rate of net anion flux through band III is only about 1/10,000 of the rate of anion exchange (19). A possible alternative explanation for the data of Waisman et al. (5,6) is suggested in the scheme of Fig. 5B. The primary pH gradient resulting from the Ca\(^{2+}\)-H\(^{+}\) exchange catalyzed by the Ca\(^{2+}\) pump as proposed here, is dissipated through band III either by a bicarbonate-anion exchange (resulting in a secondary anion uptake) or, in the absence of bicarbonate, by the H\(^{+}\) anion symport proposed by Jennings (24). However, one should bear in mind that in vivo the Ca\(^{2+}\) leak across the erythrocyte membrane, and therefore, the Ca\(^{2+}\) pump activity are very low under non-pathological conditions.

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Fig. 5. Schematic representation of Ca\(^{2+}\), H\(^{+}\), and anion fluxes via the Ca\(^{2+}\) pump and band III in the intact red blood cell (A) and in resealed, inside-out red blood vesicles (B).
The purified Ca\textsuperscript{2+} pump of human erythrocyte membranes catalyzes an
electroneutral Ca\textsuperscript{2+}-H\textsuperscript{+} exchange in reconstituted liposomal systems.
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