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Proton Countertransport by the Reconstituted Erythrocyte Ca^{2+}-Translocating ATPase: Evidence Using Ionophoretic Compounds

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Summary. Human erythrocyte Ca^{2+}-translocating ATPase was solubilized from calmodulin-depleted membranes using the detergent Triton X-100, and subsequently purified by calmodulin-affinity chromatography. The purified enzyme was reconstituted in artificial phospholipid vesicles using a cholate-dialysis method and various phospholipids. The reconstituted enzyme was able to translocate Ca^{2+} inside the vesicles, both in the absence and in the presence of the Ca^{2+}-chelating agent, oxalate, inside the vesicles. The tightness of coupling between ATP hydrolysis and cation translocation was investigated by the use of different ionophoretic compounds. The efficiency of Ca^{2+} translocation was measured by the ability of the ionophores to stimulate ATP hydrolytic activity of the reconstituted enzyme. It was found that the maximum stimulation of the ATP hydrolytic activity was induced by the electroneutral $Ca^{2+}/2H^+$ ionophore A23187 (9 to 10-fold). A Ca^{2+} ionophore unable to translocate H^+, CYCLEX-2E, was less efficient in stimulating the activity of the reconstituted enzyme (two- to threefold). However, the combined addition of CYCLEX-2E plus protonophores further increased the ATP hydrolytic activity (around fourfold), whereas, the protonophores did not further stimulate ATP hydrolysis in the presence of A23187. Furthermore, in the absence of Ca^{2+} ionophore, the electroneutral $K^+(Na^+)/H^+$ ionophoretic exchanger, nigericin, or the electroneutral $Na^+(K^+)/H^+$ ionophoretic exchanger, monensin, stimulated the rate of ATP hydrolysis in the reconstituted enzyme two- or threefold, respectively. These results suggest that the Ca^{2+}-ATPase not only translocates Ca^{2+} but also H^+ in the opposite direction.

Key Words Ca^{2+}-ATPase · proteoliposomes · proton countertransport · ionophores

Introduction

The plasma membrane Ca^{2+}-translocating ATPase is responsible for the extrusion of calcium ion from the red blood cell against a steep electrochemical Ca^{2+} gradient (Schatzmann, 1966). This enzyme is thought to be primarily responsible for the attainment of a low concentration of free Ca^{2+} in the cytoplasm in the order of 10^{-7} to 10^{-8} M. The low concentration of free calcium ion in the cytoplasm of most cells allows efficient use of this ion for the transduction of various extracellular stimuli in the activation/inactivation of given biochemical processes. For efficient modulation of cellular function the cytoplasmic free concentration of Ca^{2+} should oscillate in the range of the affinity constants of Ca^{2+} for its specific target reactions. However, the electrical membrane potential (negative inside) across the plasma membrane, maintained by the electrogenic 3 Na^+/2 K^+-ATPase (Skou, 1965) and selective cation channels, create an energetic barrier preventing sufficient efflux of the doubly positive charged Ca^{2+} cation, that already must surmount transport against an external concentration of this ion in the order of 10^{-3} M. This problem could be partially overcome by countertransport of other positively charged species by the enzyme mechanism cycle. In fact, several recent reports have indicated that H^+ may participate in such a countertransport mode of operation of the enzyme, both in inside-out membrane vesicles (Smallwood et al., 1983) and in proteoliposomes (Niggli et al., 1982; Villalobo & Roufogalis, 1984). The present report examines the effects of different ionophores of known mechanism of action on the ATP hydrolytic activity of the Ca^{2+}-ATPase reconstituted in artificial phospholipid vesicles. The stimulation of Ca^{2+} translocation by ionophoretic agents able to selectively collapse various ion gradients suggests indeed, that ATP hydrolysis is mechanistically coupled to the exchange of Ca^{2+} for H^+. However, the question of electrogenicity versus electroneutrality of the transport mechanism remains to be further clarified.

Materials and Methods

Chemicals

Soybean L-α-phosphatidylcholine (types II-S and IV-S), egg yolk L-α-phosphatidylcholine (type X-E), rabbit muscle lactate dehydrogenase, EC 1.1.1.27 (types II and XI), rabbit muscle pyruvate kinase, EC 2.7.1.40 (types II and III), bovine brain phosphodiesterase 3′,5′-cyclic nucleotide activator-Agarose gel, Triton X-100, cholic acid (sodium salt), dithiothreitol, Hepes[1], ATP (disodium salt and magnesium salt), EGTA, β-NADH, CCCP, FCCP and phospho*enol*pyruvate were purchased from Sigma Chemical Co. (St. Louis, MO). Asolectin was obtained from MCB Manufacturing Chemical Inc. (Cincinnati, OH). Bovine brain calmodulin, monensin, nigericin and A23187 were purchased from Calbiochem Behring Corp. (La Jolla, CA), EDTA from BDH (Toronto) and valinomycin was obtained from Boehringer Mannheim (Dorval, Quebec). Samples of CYCLEX-2E were kindly supplied by Dr. Charles M. Deber from the Research Institute Hospital for Sick Children, Toronto, Ontario. All other chemicals used in this work were of analytical grade. The various phospholipids employed for the reconstitution procedure were used without further treatment or purification.

Preparation of Calmodulin-Depleted Erythrocyte Membranes and Solubilization and Purification of the Ca^{2+}-Translocating ATPase

The methods used in this work for the preparation of calmodulin-depleted human erythrocyte plasma membranes and the solubilization and purification of the enzyme have been described in detail recently by us (Villalobo et al., 1986).

Incorporation of Purified Ca^{2+}-Translocating ATPase in Phospholipid Vesicles

For the reconstitution procedure a cholate-dialysis method derived from the original method by Kagawa and Racker (1971) was employed. A typical procedure was as follows: a 5-ml suspension of 1.5% (wt/vol) phospholipids was prepared by sonication in 100 mM KCl, 20 mM K-Hepes, 5 mM $MgCl_2$, 2 mM DTT and 50 μM $CaCl_2$ at pH 7.4 and in the presence of 1% (wt/vol) sodium cholate. The sonication was carried out at room temperature at 90 to 95 watts of power in a sonicator equipped with a microprobe, with alternate 30-sec periods on and 30-sec periods off. In order to prevent overheating, the suspension of phospholipids was maintained on ice during the intervals between sonication bursts. The procedure was carried out about 20 times until total clarification of the suspension was attained. The sonicated mixture was cooled on ice once more and 1 ml of purified enzyme (30 to 70 μg protein) was added and mixed gently. The enzyme-phospholipid mixture was settled on 1 cm diameter dialysis bags (prehydrated), and dialyzed at 4°C for 26 to 28 hr against 1 liter dialysis buffer of the same composition as described above. The dialysis buffer was changed five times. The above method was modified depending on the required ionic composition of the outer media in the proteoliposomes (*see* legend of Tables and Figures) or the amount of proteoliposomes used. When a different composition was required on the inner space of the proteoliposomes, a second dialysis was performed to change the medium on the outer side of the proteoliposomes.

[1] *Abbreviations:* Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; EGTA, ethyleneglycol *bis* (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; DNP, 2,4-dinitrophenol; CYCLEX-2E, cyclo[Glu(OBz)-Sar-Gly-(N-cyclohexyl)Gly]$_2$; EPR, electron paramagnetic resonance.

Analytical Procedures

The rate of ATP hydrolysis was followed by measuring the amount of inorganic phosphate released to the medium by a colormetric method (Raess & Vincenzi, 1980) or by coupling the rate of ADP production to an ATP-regenerating system (pyruvate kinase/lactate dehydrogenase) and following the rate of NADH oxidation at the wavelength pair of 340 and 360 nm with an SLM/Aminco DW-2C dual-wavelength spectrophotometer. Calcium ion transport was followed with a Ca^{2+}-selective electrode (Radiometer, model F2110 Ca) using as a reference a pH glass electrode (Fisher) in a 3.5 ml thermostated chamber. The electrode outputs were amplified up to 1000-fold through a homemade amplifier and fed into a three-channel recorder (Soltec, model 1234). Known amounts of standard solutions of $CaCl_2$ were added to calibrate the Ca^{2+}-electrode response in each experiment. Protein concentration was determined by the method of Lowry et al. (1951) after the protein was precipitated in a final concentration of 10% (wt/vol) trichloroacetic acid at room temperature. Bovine serum albumin was used as a standard. All stock solutions of ionophores were prepared in ethanol or N,N′-dimethylformamide. The final concentration of each solvent in the assay system was never higher than 1% (vol/vol) and the same concentration of solvents was always added to the controls in the absence of ionophore; they were found not to produce any significant increase in the rate of ATP hydrolysis (less than 1%).

Results

Degree of Coupling and Ca^{2+} Transport by the Reconstituted Ca^{2+}-ATPase

In the first series of experiments we reconstituted the Ca^{2+}-translocating ATPase in phospholipid vesicles of different phospholipid types. In order to establish both the degree to which the enzyme is incorporated into the vesicles and the degree of permeability of the phospholipid membranes we tested the effect of the $Ca^{2+}/2H^+$ electroneutral exchanger A23187 on the ATP hydrolytic activity of the reconstituted enzyme. Table 1 shows the rate of ATP hydrolysis in the absence and in the presence of A23187 in various proteoliposomes, as well as in the nonreconstituted enzyme. In addition, the ATP hydrolysis control ratio (ratio of the rate of ATP hydrolysis in the presence versus the absence of the ionophore) is also included in the same Table. From these data it was possible as well to calculate the degree of coupling (q) between the ATP hydrolysis and the Ca^{2+} translocation. Applying nonequilibrium thermodynamic formalisms (Rottenberg,

Table 1. Degree of coupling of the reconstituted Ca^{2+}-ATPase in different phospholipid vesicles[a]

Phospholipid vesicles	ATPase activity ($nmol \cdot min^{-1} \cdot mg\ prot^{-1}$)		ATP hydrolysis control ratio	Degree of coupling (q)
	−A23187	+A23187		
Nonreconstituted	2331 ± 84(2)	2274 ± 73(2)	1.0 ± 0 (2)	0 (2)
Asolectin (soybean)	245 ± 36(5)	2150 ± 309(2)	8.8 ± 0.4(5)	0.94 ± 0.003(5)
L-α-phosphatidylcholine (soybean, type II-S)	78 ± 6(2)	821 ± 96(2)	10.7 ± 2.0(2)	0.95 ± 0.01 (2)
L-α-phosphatidylcholine (soybean, type IV-S)	37 (1)	345 (1)	9.3 (1)	0.94 (1)
L-α-phosphatidylcholine (egg yolk, type X-E)	39 (1)	227 (1)	5.8 (1)	0.91 (1)

[a] The ATPase activity was assayed at 37°C for 1 hr in a total volume of 1 ml in a reaction mixture as follows: nonreconstituted enzyme (2.2 μg protein), 124 mM KCl, 50 mM K-Hepes pH 7.4, 5 mM $MgCl_2$, 160 μM $CaCl_2$, 160 μM EDTA, 2 $\mu g \cdot ml^{-1}$ calmodulin, 2 mM DTT, 1 $mg \cdot ml^{-1}$ asolectin, 0.5% (wt/vol) Triton X-100, and 2 mM ATP (sodium salt) in the absence and in the presence of 10 $\mu g \cdot ml^{-1}$ A23187; reconstituted enzyme (2.2 to 4.5 mg of the indicated phospholipids plus 1.5 to 2.3 μg protein), 30 or 115 mM KCl, 25 or 75 mM K-Hepes pH 7.4, 3.8 or 5.7 mM $MgCl_2$, 57 or 200 μM $CaCl_2$, 0.6 or 2 $\mu g \cdot ml^{-1}$ calmodulin, 0.3 or 0.6 mM DTT and 2 mM ATP (sodium salt) in the absence or in the presence of 10 or 50 $\mu g \cdot ml^{-1}$ A23187. The change of concentration of the different reagents as indicated above did not modify significantly the degree of coupling of the proteoliposomes. The reaction mixture in all cases contains 0.5 to 1.0% (vol/vol) ethanol or N,N′-dimethylformamide. The proteoliposomes were prepared in: 100 mM KCl, 50 mM K-Hepes pH 7.4, 5 mM $MgCl_2$, 2 mM DTT and 50 μM $CaCl_2$. The inorganic phosphate released to the medium was determined as described in Materials and Methods. The ATPase activity is the mean ± SEM of the number of preparations indicated in parentheses. The ATP hydrolysis control ratio is the ratio of the activities in the presence versus the absence of A23187. The degree of coupling (q) was calculated according to Eq. (1) in the Results section.

1979) the degree of coupling (q) may be calculated using the following expression:

$$q = \left[1 - \frac{(J_{ATP})_{J_{Ca^{2+}}=0}}{(J_{ATP})_{\Delta\tilde{\mu}_{Ca^{2+}}=0}}\right]^{1/2} \tag{1}$$

where $(J_{ATP})_{J_{Ca^{2+}}=0}$ is the rate of ATP hydrolysis in static head conditions [i.e., when the net rate of calcium ion transport ($J_{Ca^{2+}}$) became zero. Its value equals the rate of ATP hydrolysis at equilibrium, in the absence of A23187]. $(J_{ATP})_{\Delta\tilde{\mu}_{Ca^{2+}}=0}$ is the rate of ATP hydrolysis when the electrochemical Ca^{2+} potential difference ($\Delta\tilde{\mu}_{Ca^{2+}}$) across the membrane is zero. Its value should equal the rate of ATP hydrolysis in the presence of A23187, assuming the electrical potential differences across the membrane ($\Delta\psi$) is negligible. This last assumption is warranted because, as we will show in Table 2, the presence in the assay system of $\Delta\psi$-collapsing agents in addition to A23187 does not further modify significantly the rate of ATP hydrolysis in the reconstituted enzyme (*see below*). The degree of coupling (q) expected will oscillate between the value of 0 (no coupling and/or total permeability) and the value of 1 (perfect coupling). Table 1 shows that the degree of coupling (q) between the Ca^{2+} transport and the ATP hydrolysis was very high (0.91 to 0.95) in all the types of vesicles tested, with differences depending on the phospholipids used. Moreover, as expected, the nonreconstituted enzyme yields a value of q equal to zero, since no vectorial Ca^{2+} movement could take place.

Table 1 also shows that the ATP hydrolytic activity of the enzyme, when net Ca^{2+} uptake is prevented (in the presence of A23187), varies greatly depending on the phospholipids used to prepare the vesicles. The highest activity was found with asolectin vesicles (average value 2150 $nmol \cdot min^{-1} \cdot mg\ prot^{-1}$), approaching 95% of the average values of the nonreconstituted enzyme (2274 $nmol \cdot min^{-1} \cdot mg\ prot^{-1}$) (both in the presence of calmodulin). In another series of experiments (*see*, for example, Fig. 4), the relative activity of the reconstituted enzyme in asolectin in the presence of A23187 reached only 65 to 70% of the activity of the nonreconstituted enzyme. Commercial grade soybean L-α-phosphatidylcholine (type II-S) significantly decreased the enzymatic activity in these experimental conditions (821 $nmol \cdot min^{-1} \cdot mg\ prot^{-1}$). The decrease in activity was more pronounced when highly purified soybean L-α-phosphatidylcholine (type IV-S) or egg yolk L-α-phosphatidylcholine (type X-E) was employed (345 and 227 $nmol \cdot min^{-1} \cdot mg\ prot^{-1}$, respectively). Two factors could contribute to these results: inactivation of the enzyme during the reconstitution procedure and/or random incorporation of the enzyme such that only part of the ATP catalytic site(s) is exposed to the outer side of the vesicles. At this point we decided to perform the subsequent experiments only in asolectin or L-α-phosphatidylcholine (Type II-S) vesicles.

In a subsequent series of experiments we studied the time course of ATP hydrolysis of the reconstituted enzyme both in the absence and in the presence of A23187. Figure 1 shows the results of experiments performed using asolectin and soybean

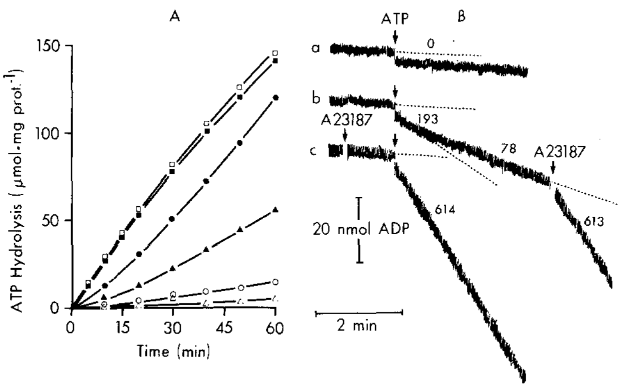

Fig. 1. Time course of ATP hydrolysis in the presence and absence of A23187 by the nonreconstituted and reconstituted enzyme. (*A*) The nonreconstituted Ca^{2+}-ATPase (24.5 μg protein) was assayed at 37°C in a total volume of 10 ml of the following medium: 112 mM KCl, 50 mM Na-Hepes pH 7.4, 5 mM $MgCl_2$, 85 μM $CaCl_2$, 80 μM EDTA, 0.6 μg · ml^{-1} calmodulin, 0.1 mM ouabain (not necessary), 0.05% (wt/vol) Triton X-100, 0.1% (wt/vol) asolectin, 2 mM DTT and 2 mM ATP (sodium salt) in the absence (open squares) or in the presence of 10 μg · ml^{-1} A23187 (filled squares). The reconstituted enzyme (18 mg phospholipids plus 12 μg protein) was assayed at 37°C in a total volume of 8 ml of the following medium: 115 mM KCl, 50 mM Na-Hepes pH 7.4, 5.7 mM $MgCl_2$, 57 μM $CaCl_2$, 0.6 μg · ml^{-1} calmodulin, 0.3 mM DTT and 2 mM ATP (sodium salt) in the absence (open symbols) or in the presence of 10 μg · ml^{-1} A23187 (filled symbols). At indicated times 1 ml of the reaction mixture was withdrawn and assayed for inorganic phosphate, as indicated in Materials and Methods. All the preparations contain 0.5% (vol/vol) ethanol. The proteoliposomes were prepared in 100 mM KCl, 50 mM K-Hepes pH 7.4, 5 mM $MgCl_2$, 2 mM DTT and 50 μM $CaCl_2$: nonreconstituted enzyme (squares), enzyme reconstituted in asolectin (circles) and enzyme reconstituted in L-α-phosphatidylcholine (II-S type) (triangles). (*B*) The reconstituted Ca^{2+}-ATPase (45 mg L-α-phosphatidylcholine type II-S plus 33 μg protein, traces *b* and *c*) was assayed at 37°C in a total volume of 3.1 ml of the following medium: 100 mM KCl, 20 mM K-Hepes pH 7.4, 1 mM $MgCl_2$, 32 μM $CaCl_2$, 1.6 μg · ml^{-1} calmodulin, 2 mM DTT, 3.9 mM phospho*enol*pyruvate, 44 units pyruvate kinase, 112 units lactate dehydrogenase and 0.1 mM NADH. Where indicated, 32 μg · ml^{-1} A23187 and 16 μM MgATP was added. Trace *a* is an experiment performed in the same medium in the absence of proteoliposomes. The proteoliposomes were prepared in (mM): 100 KCl, 20 K-Hepes pH 7.4, 2 DTT and 1 $MgCl_2$. The rate of ATP hydrolysis was determined by following the rate of NADH oxidation at the wavelength pair of 340 and 360 nm. 0.06% (vol/vol) N,N'-dimethylformamide was added with A23187

L-α-phosphatidylcholine (type II-S) and with nonreconstituted enzyme. The rate of inorganic phosphate released to the medium was measured colorimetrically in Fig. 1*A* as described in the Materials and Methods section. Near maximum rate of ATP hydrolysis was obtained with the enzyme reconstituted in asolectin in the presence of A23187, approaching values of the activity of the nonreconstituted enzyme. It was expected, however, that in the absence of A23187, after a brief period of active ATP hydrolysis coinciding with the buildup of a calcium ion concentration gradient, the rate of ATP hydrolysis would slow down to a point where the rate of Ca^{2+} uptake will be compensated by the rate of Ca^{2+} leaking out of the vesicles. The limitation in the colorimetric determination of very small amounts of inorganic phosphate, however, limited the detection of this transient fast initial rate of ATP hydrolysis. The transient initial phase of rapid ATP hydrolysis was expected to be very short, since the small inner volume of the proteoliposomes will result in a very fast buildup of a near maximum Ca^{2+} concentration gradient across the proteoliposome membrane in a short period of time. Consequently, we measured the rate of ATP hydrolysis using an ATP regenerating system (pyruvate kinase/lactate dehydrogenase) and following the initial rate of NADH oxidation spectrophotometrically at the wavelength pair of 340 and 360 nm. Figure 1*B* shows the result of these experiments. It can be seen that in the absence of A23187 (trace *b*) the initial rate of ATP hydrolysis slows down with time, as expected, from 193 to 78 nmol · min^{-1} · mg prot^{-1}. In the same trace *b* (Fig. 1*B*) it is seen that a further addition of A23187 increases the rate of ATP hydrolysis to the same level as that when A23187 was in the assay before the addition of ATP (trace *c*). A control (trace *a*) in the absence of proteoliposomes shows a small transient NADH oxidation induced by addition of ATP, due to a small amount (5% by molarity) of contaminating ADP in the stock solution of ATP. The numbers along the traces indicate the actual rate of NADH oxidation (ATP hydrolysis) expressed in nmol · min^{-1} · mg prot^{-1}.

The capability of the proteoliposomes to actively transport Ca^{2+} across the proteoliposome membranes was directly confirmed by measuring Ca^{2+} uptake with a selective Ca^{2+} electrode to monitor changes in free Ca^{2+} concentration in the outer media. Several typical traces of Ca^{2+} uptake are presented in Fig. 2. Trace *B* shows Ca^{2+} uptake in proteoliposomes in the absence of any Ca^{2+}-chelating agent inside the vesicles, and trace *C* shows Ca^{2+} uptake in oxalate-loaded proteoliposomes. In contrast, experiments performed on proteoliposomes previously treated with A23187 (trace *A*) show that A23187 totally prevents net Ca^{2+} uptake in the vesicles. The number along the traces indicates the initial rate of Ca^{2+} uptake in ng-ions Ca^{2+} · min^{-1} · mg prot^{-1}.

Effects of Different Ionophores on the Activity of the Reconstituted Ca^{2+}-ATPase

As shown previously, the electroneutral $Ca^{2+}/2H^+$ exchanger A23187 induces a strong stimulation of the rate of ATP hydrolysis by the reconstituted enzyme. Since this ionophore not only will collapse a generated Ca^{2+} gradient but a generated proton gra-

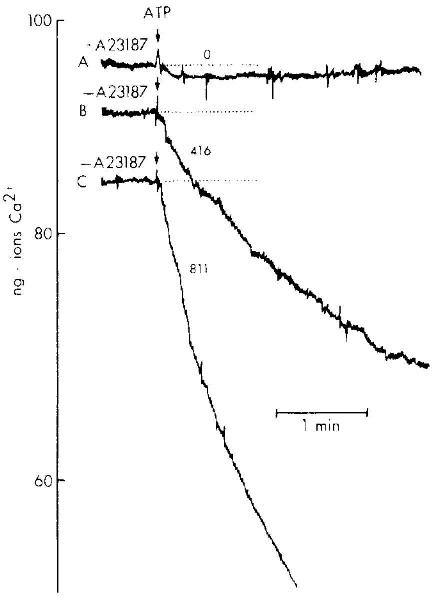

Fig. 2. Time course of Ca^{2+} uptake by the reconstituted Ca^{2+}-ATPase. Ca^{2+} movement was recorded with a Ca^{2+}-selective electrode at 37°C in a total volume of 3.1 ml in the following medium: reconstituted enzyme (45 mg asolectin plus 31 μg protein), 100 mM KCl, 20 mM K-Hepes pH 7.4, 2 mM DTT, 1 mM $MgCl_2$, 32 μM $CaCl_2$, 6 μg · ml^{-1} calmodulin, 3.9 mM phospho*enol*pyruvate, 94 units pyruvate kinase and 7 mM $(NH_4)_2SO_4$. Where indicated, 16 μM MgATP was added. The system in trace *A* contains, in addition, 6.4 μg · ml^{-1} A23187. The proteoliposomes were prepared in 100 mM KCl, 20 mM K-Hepes pH 7.4, 2 mM DTT and 1 mM $MgCl_2$ (traces *A* and *B*) and the same medium plus 100 mM potassium oxalate (trace *C*). The oxalate was removed from the outer medium by a second dialysis against the same medium without oxalate before the experiment was performed. Known amounts of $CaCl_2$ where used as a standard to calculate the response of the Ca^{2+}-selective electrode

dient (ΔpH) as well, we decided to use, in addition, a series of other ionophores to investigate the possibility of the enzyme being involved in H^+ translocation and to study the alternative electroneutral versus electrogenic mode of operation of the pump. Table 2 shows three typical experiments in which the effect of combining different ionophores on the rate of ATP hydrolysis, as well as the ATP hydrolysis control ratio (as described above) is examined using the reconstituted enzyme. It is seen that the electrophoretic K^+ uniport, valinomycin (in the presence of K^+) produced a significant increase (80 to 90%) in the rate of ATP hydrolysis with respect to the control in the absence of ionophores, probably by collapsing the electrical membrane potential difference across the proteoliposome membrane. The effect of valinomycin was found consistently in all of the preparations tested. Valinomycin stimulated the rate of ATP hydrolysis of the reconstituted enzyme at all concentrations of potassium ion examined from 10 to 100 mM (*results not shown*). A similar effect, although to a lesser extent (20 to

Table 2. Effects of different ionophores on the activity of the reconstituted Ca^{2+}-ATPase[a]

Experiment	Addition	ATPase activity (nmol · min^{-1} · mg $prot^{-1}$)	ATP hydrolysis control ratio
1	None	293	
	Val	539	1.8
	CCCP	365	1.2
	FCCP	395	1.3
	Nig	683	2.3
	Mon	1072	3.7
	A23187	2713	9.3
	A23187 + Val	2672	9.1
	A23187 + CCCP	2779	9.5
	A23187 + FCCP	2539	8.6
	A23187 + Nig	2588	8.8
	A23187 + Mon	2641	9.0
2	None	168	
	CCCP	225	1.3
	FCCP	194	1.2
	DNP	296	1.8
	A23187	1442	8.6
3	None	247	
	Val	461	1.9
	CCCP	291	1.2
	Nig	555	2.2
	Mon	852	3.4
	CCCP + Val	351	1.4
	CCCP + Nig	540	2.2
	CCCP + Mon	842	3.4
	Val + Nig	640	2.6
	Val + Mon	916	3.7
	Nig + Mon	1110	4.5
	A23187	2464	10.0

[a] The ATP activity was assayed at 37°C for 1 hr in a total volume of 1 ml in a reaction mixture as follows: Experiments 1 and 3: proteoliposomes (2.2 mg asolectin plus 1.5 μg protein), 115 mM KCl, 57 mM K-Hepes pH 7.4, 5.7 mM $MgCl_2$, 57 μM $CaCl_2$, 0.6 μg · ml^{-1} calmodulin, 0.3 mM DTT and 2 mM ATP (sodium salt) in the presence of the indicated ionophores: 10 μg · ml^{-1} valinomycin, 25 μM CCCP, 25 μM FCCP, 10 μg · ml^{-1} nigericin, 10 μg · ml^{-1} monensin and 10 μg · ml^{-1} A23187. Experiment 2: proteoliposomes (3.8 mg asolectin plus 3.8 μg protein), 100 mM KCl, 26 mM K-Hepes pH 7.4, 3.5 mM $MgCl_2$, 115 μM $CaCl_2$, 2 μg · ml^{-1} calmodulin, 0.6 mM DTT and 2 mM ATP, in the presence of the indicated ionophores: 25 μM CCCP, 25 μM FCCP, 1.5 mM DNP and 20 μg · ml^{-1} A23187. The reaction mixture in all cases contains 1% (vol/vol) ethanol. The proteoliposomes were prepared in: 100 mM KCl, 50 mM K-Hepes pH 7.4, 5 mM $MgCl_2$, 2 mM DTT and 50 μM $CaCl_2$. The inorganic phosphate released to the medium was determined as described in Materials and Methods. The ATP hydrolysis control ratio is the ratio of the activities in the presence versus the absence of the ionophores.

30%), was observed with the protonophores CCCP and FCCP, although 2,4-dinitrophenol was more effective and stimulated about 80%. The results could be interpreted as indicating the presence of an electrical membrane potential across the membrane fol-

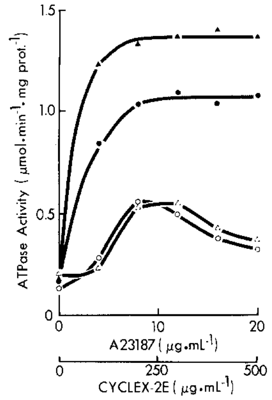

Fig. 3. Effects of different concentrations of A23187 and CYCLEX-2E on the ATPase activity of the reconstituted enzyme. Reconstituted Ca^{2+}-ATPase (4.5 mg asolectin plus 3.7 μg protein) was assayed at 37°C for 1 hr in a total volume of 1 ml in the following medium: 100 mM KCl (triangles) or 100 mM NaCl (circles), 26 mM K-Hepes (triangles) or 26 mM Na-Hepes (circles), 3.5 mM $MgCl_2$, 115 μM $CaCl_2$, 2 μg · ml^{-1} calmodulin, 0.6 mM DTT and 2 mM ATP (sodium salt). The proteoliposomes were prepared in: 100 mM KCl (triangles) or 100 mM NaCl (circles), 20 mM K-Hepes (triangles) or 20 mM Na-Hepes (circles) pH 7.4, 5 mM $MgCl_2$, 50 μM $CaCl_2$ and 2 mM DTT. A23187 (filled symbols) or CYCLEX-2E (open symbols) at the indicated concentration was added to the assay system. 1% (vol/vol) ethanol or N,N'-dimethylformamide were also included in all the tubes. Inorganic phosphate was determined as indicated in Materials and Methods

lowing Ca^{2+} transport. However, it was observed that the combined addition of A23187 plus protonophores did not produce a significant change in the rate of ATP hydrolysis. Consequently, it could be reasonably assumed that the suspected membrane potential, if present, is of a very low magnitude. On the other hand, 1 mM SCN^- or 1 mM NO_3^- did not stimulate the rate of ATP hydrolysis (*results not shown*), possibly due to their low permeability across the artificial proteoliposome membrane, in contrast to the high permeability observed in natural biological membranes.

The addition of either the electroneutral $K^+(Na^+)/H^+$ exchanger, nigericin, or the electroneutral $Na^+(K^+)/H^+$ exchanger monensin, induces a strong increase in the rate of ATP hydrolysis (2.3- and 3.7-fold, respectively) (Table 2). These results were taken as convincing evidence of the transport of H^+ during Ca^{2+} translocation by the reconstituted enzyme. Furthermore, the effect of nigericin or monensin is not additive to the effect of A23187, as expected, further suggesting that the effect of those ionophores results from the collapse of the generated ΔpH. Conditions designed to collapse the ΔpH plus $\Delta\psi$, namely, the combined addition of valinomycin and the electroneutral exchanger nigericin or monensin, resulted in a further increase in the rate of ATP hydrolysis when compared with the addition of each ionophore individually. However, substitution of valinomycin by CCCP did not produce the same results (experiment 3, in Table 2). Table 2 also shows that the combined addition of nigericin plus monensin was more efficient than the addition of each ionophore separately. All the ionophores employed were used at concentrations able to induce maximum rate of stimulation of the reconstituted enzyme, except for the cases of nigericin and monensin. We found that although both nigericin and monensin increased the rate of ATP hydrolysis in a concentration-dependent manner, a plateau was difficult to reach, most probably because of dimerization of the ionophores in the membrane, which could result in the formation of nonspecific channels if excessively high concentrations are used. Moreover, it was shown that the activity of the enzyme before reconstitution was not stimulated by valinomycin, nigericin, monensin, CCCP, FCCP, 2.4-dinitrophenol or A23187. However, valinomycin (10 μg · ml^{-1}) and 2,4-dinitrophenol (1.5 mM) produce a 7 and a 10% inhibition, respectively (*results not shown*).

Use of CYCLEX-2E Plus Protonophores to Further Demonstrate H^+ Translocation by the Reconstituted Ca^{2+}-ATPase

Since A23187 should collapse both a Ca^{2+} and a H^+ gradient in an electroneutral fashion, it was not possible, by the use of this ionophore alone or in combination with other ionophores, to unequivocably decide whether or not H^+ translocation takes place, although as discussed above the stimulatory effects induced by nigericin or monensin made this a strong possibility. However, it could not be excluded that the countertransported species could be K^+ rather than H^+. In order to solve this problem, we chose to use a Ca^{2+} ionophore that does not cotransport protons, but rather exchanges Ca^{2+} for monovalent cations. The synthetic cyclic peptide ionophore, CYCLEX-2E, meets these requirements (Deber, 1980; Deber et al., 1980; Diobries & Deber, 1982). In the experiments of Fig. 3 is shown a comparison between the concentration requirement of A23187 and CYCLEX-2E for stimulation of the rate of ATP hydrolysis in the reconstituted enzyme, both in a KCl- and a NaCl-containing medium. Inhibition by CYCLEX-2E at concentrations higher than 250 μg · ml^{-1} is observed. This inhibition by high concentrations of CYCLEX-2E was also observed on the nonreconstituted enzyme (*results not shown*). Consequently, an appropriate concentration of this ionophore was carefully chosen in the next series of

Table 3. Combined effects of CYCLEX-2E plus proton-conducting ionophores on the activity of the reconstituted Ca^{2+}-ATPase[a]

Proton-conducting ionophore	ATPase activity (nmol · min^{-1} · mg prot^{-1})	
	−CYCLEX-2E	+CYCLEX-2E
None	180	492
CCCP	200	711
DNP	200	786
Nig	296	746
Mon	574	1330
A23187	1537	1663

[a] The ATPase activity was assayed at 37°C for 1 hr in a total volume of 1 ml in a reaction mixture as follows: proteoliposomes (3.6 mg asolectin plus 3 μg protein), 100 mM KCl, 26 mM K-Hepes pH 7.4, 3.5 mM $MgCl_2$, 2 μg · ml^{-1} calmodulin, 0.6 mM DTT and 2 mM ATP (sodium salt) in the presence of the indicated ionophores: 25 μM CCCP, 1.5 mM DNP, 20 μg · ml^{-1} nigericin, 20 μg · ml^{-1} monensin, 20 μg · ml^{-1} A23187, 500 μg · ml^{-1} CYCLEX-2E. The reaction mixture in all cases contains 1% (vol/vol) ethanol plus 0.5% (vol/vol) N,N'-dimethylformamide. The proteoliposomes were prepared in: 100 mM KCl, 20 mM K-Hepes pH 7.4, 5 mM $MgCl_2$, 2 mM DTT and 50 μM $CaCl_2$. The inorganic phosphate released to the medium was determined as described in Materials and Methods.

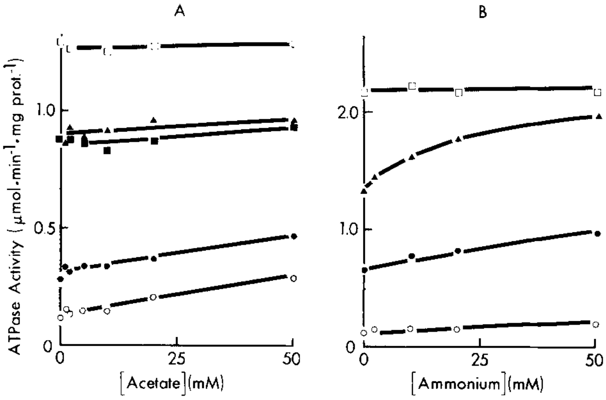

Fig. 4. Effects of acetate and ammonium on the ATPase activity of the nonreconstituted and reconstituted Ca^{2+}-ATPase in the absence and presence of Ca^{2+} ionophores. (*A*) The ATPase activity was determined at 37°C for 1 hr in a 1-ml volume. The nonreconstituted enzyme (3.8 μg protein) (open squares) was assayed as follows: 124 mM KCl, 25 mM K-Hepes pH 7.4, 3.5 mM $MgCl_2$, 160 μM $CaCl_2$, 160 μM EDTA, 2 μg · ml^{-1} calmodulin, 0.05% (wt/vol) Triton X-100, 0.1% (wt/vol) asolectin, 2 mM DTT, 2 mM ATP (sodium salt), and the indicated concentrations of potassium acetate. The reconstituted enzyme (4.4 mg asolectin plus 2.3 μg protein) was assayed as follows: 100 mM KCl, 26 mM K-Hepes pH 7.4, 3.5 mM $MgCl_2$, 115 μM $CaCl_2$, 2 μg · ml^{-1} calmodulin, 0.6 mM DTT, 2 mM ATP (sodium salt), and the indicated concentration of potassium acetate: (open circles) no Ca^{2+} ionophores, (filled circles) 250 μg · ml^{-1} CYCLEX-2E, (filled triangles) 20 μg · ml^{-1} A23187 and (filled squares) 250 μg · ml^{-1} CYCLEX-2E plus 20 μg · ml^{-1} A23187. All the tubes contained 1% (vol/vol) ethanol plus 0.5% (vol/vol) N,N'-dimethylformamide. The proteoliposomes were prepared in: 100 mM KCl, 20 mM K-Hepes, pH 7.4, 5 mM $MgCl_2$ and 2 mM DTT. Inorganic phosphate was determined as indicated in Materials and Methods. (*B*) The nonreconstituted Ca^{2+}-ATPase (3.6 μg protein) (open squares) was assayed as follows: 100 mM NaCl, 24 mM KCl, 25 mM K-Hepes pH 7.4, 3.5 mM $MgCl_2$, 160 μM $CaCl_2$, 160 μM EDTA, 2 μg · ml^{-1} calmodulin, 0.05% (wt/vol) Triton X-100, 0.1% (wt/vol) asolectin, 2 mM DTT, 2 mM ATP (sodium salt) and the indicated concentration of ammonium chloride. The reconstituted enzyme (3.9 mg asolectin plus 1.9 μg protein) was assayed as follows: 100 mM NaCl, 26 mM K-Hepes pH 7.4, 3.5 mM $MgCl_2$, 115 μM $CaCl_2$, 2 μg · ml^{-1} calmodulin, 0.6 mM DTT, 2 mM ATP (sodium salt) and the indicated concentration of ammonium chloride: (open circles) no Ca^{2+} ionophores; (filled circles) 250 μg · ml^{-1} CYCLEX-2E; (filled triangles) 20 μg · ml^{-1} A23187. All the tubes contained 1% (vol/vol) ethanol plus 0.5% (vol/vol) N,N'-dimethylformamide. The proteoliposomes were prepared in: 100 mM NaCl, 20 mM K-Hepes pH 7.4, 5 mM $MgCl_2$, 50 μM $CaCl_2$ and 2 mM DTT. Inorganic phosphate was determined as indicated in Materials and Methods

experiments. The experiment in Table 3 was designed to test the combined action of the Ca^{2+}-ionophore CYCLEX-2E and different agents able to permeabilize the membrane to H^+. In all the cases studied, the combined addition of CYCLEX-2E plus one of the following compounds: CCCP, 2,4-dinitrophenol, nigericin or monensin, dramatically increased the rate of ATP hydrolysis when compared to the addition of each ionophore independently. Controls with A23187, and CYCLEX-2E plus A23187 are also shown for comparison. It becomes clear that in order to attain maximum stimulation of the activity of the reconstituted Ca^{2+}-translocating ATPase, it is necessary to prevent the buildup not only of a Ca^{2+} concentration gradient but of a ΔpH as well.

Effects of Acetate and Ammonium on the Activity of the Reconstituted Ca^{2+}-ATPase

In Fig. 4*A* are shown the results of the progressive addition of potassium acetate on the activity of the reconstituted enzyme, in the absence and presence of Ca^{2+}-ionophores, and on the nonreconstituted enzyme. In the absence of any ionophore or in the presence of CYCLEX-2E, conditions that may result in the generation of a ΔpH (alkaline inside) during ATP hydrolysis, the presence of increasing concentrations of acetate increases progressively the rate of ATP hydrolysis, since buffering of the inner compartment of the proteoliposome is obtained by the continuous transport of the protonated form of acetate, inside the vesicles, as it is permeable through the membrane. However, the same concentration of acetate produces little or no effect in the reconstituted enzyme in the presence of the $Ca^{2+}/2H^+$ electroneutral exchanger, A23187 or A23187 plus CYCLEX-2E, or with the nonreconstituted enzyme, conditions in which no ΔpH is expected to develop, even if the enzyme carries out an electroneutral Ca^{2+} to H^+ exchange. A similar experiment using NH_4Cl instead of potassium acetate is presented in Fig. 4*B*. Intravesicular NH_4^+ is expected to dissociate to NH_3 plus H^+, during ATP hydrolysis so facilitating the extrusion of H^+ by the enzyme. However, in this case the stimulatory effect of NH_4Cl could be observed in the reconstituted enzyme both in the absence and in the presence of either CYCLEX-2E or A23187. However, no effect was observed in the nonreconstituted enzyme. The pronounced effect of NH_4Cl in the presence of A23187 could be understood if it is assumed that the number of H^+ translocated out of the vesicles by the reconstituted enzyme is lower than the number of H^+ entering the vesicles via A23187 by exchange with Ca^{2+}. If that were the case, the reversed ΔpH generated in this condition (acid inside) could be neutralized by the entry of ammonia (NH_3) and the formation of NH_4^+. This result agrees with the indication that perhaps the enzyme is able to translocate Ca^{2+} against one single H^+ in an electrogenic fashion, confirming the effects observed with the ionophores able to collapse an electrical gradient (*see* Table 2).

Discussion

The first successful reconstitution of a purified erythrocyte plasma membrane Ca^{2+}-translocating ATPase was performed by a freeze-thaw sonication procedure using an enzyme isolated from pig erythrocytes (Haaker & Racker, 1979). In this preparation, however, the ionophore A23187 stimulated the rate of ATP hydrolysis only by about threefold. Using a purified human erythrocyte enzyme preparation and a cholate-dialysis method to perform the reconstitution, the same type of experiments yielded higher stimulation of the enzymatic activity by A23187, in the order of nine- to 10-fold (Niggli et al., 1982; Villalobo & Roufogalis, 1984). Using the same cholate-dialysis method we also have obtained highly coupled proteoliposomes exhibiting similar ATP hydrolysis control ratios induced by A23187 (*see* Table 1). The obvious advantage in the use of proteoliposomes over inside-out membrane vesicles for the study of the ionic species directly translocated by the enzyme is that we avoid secondary translocation of other ions, which undoubtedly complicates the analysis of the data.

In first generation swelling-type experiments in inside-out membrane vesicles, Rossi and Schatzmann (1982) concluded that the enzyme is electrogenic, rather than supporting the alternative electroneutral Ca^{2+} for $2H^+$ exchange mode of operation. In apparent agreement with the electrogenic nature of the Ca^{2+} transport, earlier experiments on the stimulatory effect induced by different anions on the Ca^{2+} uptake were interpreted as evidence that the anion channel (band III) in the erythrocyte membrane translocates the anions in an electrophoretic mode, so compensating for the electrical charge imbalance (Waisman et al. 1981*a*,*b*). However, these experiments could equally be interpreted by assuming that the Ca^{2+}-translocating ATPase operates as a Ca^{2+}/H^+ exchanger and that the anion channel cotransports H^+ (or exchanges with OH^-) for the anions, resulting in a net accumulation of Ca^{2+} plus the anions and recycling of H^+. Direct measurement of electrical potential difference across the membrane ($\Delta\psi$) in inside-out membrane vesicles has been reported using different $\Delta\psi$-sensitive probes (Gimble et al., 1981, 1982). However, the time-course of the fluorescent signal generation of both $\Delta\psi$-sensitive probes used, 1-anilino-8-naphthalenesulfonate (ANS) and 3,3′-dipropylthiodicarbocyanine (Di-S-C_3(5)) was very slow, taking as much as 20 min to attain equilibrium. This time frame appears to be unusually high for generation of a $\Delta\psi$, since it was shown that the translocation of the probes themselves was not a limiting factor in these experiments. In the same work, the time course for the development of the assumed $\Delta\psi$ when using the $\Delta\psi$-sensitive paramagnetic probe, a nitroxide derivative of triphenyl phosphonium, was not presented (Gimble et al., 1982). Moreover, the sensitivity of the $\Delta\psi$-sensitive probes used to changes in pH on the inside of the vesicles was not tested. Consequently, the possibility remains that the observed signals were due to protonation/deprotonation of the probe. However, the same authors (Gimble et al., 1982) reported that ambiguous results were obtained when different ΔpH-sensitive probes were employed, although distribution of [^{14}C]methylamine and a ΔpH-sensitive EPR probe, indicated indeed increased alkalinization inside the vesicles during ATP hydrolysis.

Our results suggest that a $\Delta\psi$, perhaps of low magnitude, develops in the proteoliposomes during ATP hydrolysis, since valinomycin (in the presence of K^+) stimulated significantly the rate of ATP hydrolysis. Although other authors have observed similar results, the observed effect was considered insignificant (Niggli et al., 1982). In contrast, we have found that this effect is statistically significant and consistently reproducible between experiments. When the Ca^{2+} ionophore A23187 was present, valinomycin did not stimulate further the rate of ATP hydrolysis (*see* Table 2), indicating that the collapse of the chemical Ca^{2+} gradient accounts for a maximum stimulation of the reconstituted enzyme. In addition, valinomycin at these concentrations of 10 $\mu g \cdot ml^{-1}$ slightly inhibits the solubilized enzyme (7%), preventing, perhaps, observation of the expected additional stimulation in the presence of A23187. Moreover, collapsing a $\Delta\psi$ using CCCP, FCCP or DNP instead of potassium ion (+valinomycin) also stimulated the rate of ATP hydrolysis (*see* Table 2), although CCCP and FCCP were less effective, perhaps because these compounds have been shown to interact directly with -SH groups in proteins (Kaback et al., 1974), thereby producing some inhibtion. In our experiments we have shown that high concentrations of CCCP or FCCP (75 μM) do indeed inhibit the reconstituted enzyme.

Our results strongly suggest that the Ca^{2+}-ATPase is involved in H^+ translocation, since the maximum stimulation of activity in the reconstituted enzyme was always obtained by the combination of agents able not only to collapse chemical Ca^{2+} gradients, but ΔpH as well, in agreement with previous results by others in inside-out membrane vesicles (Smallwood et al., 1983) and proteoliposomes (Niggli et al., 1982). However, the ability of the enzyme to exchange 1 Ca^{2+} for 1 H^+ in an electrogenic fashion, or to efficiently translocate Ca^{2+} alone, in some conditions of the cell activation cycle cannot be disregarded at present. This electrogenicity could well represent an alternative mode of operation of the Ca^{2+} pump when the electrical membrane potential across the cell membrane is low. The alternative mode of operation could be attained by changing the stoichiometry of translocated H^+ per each ATP hydrolyzed. In support of this view other transport ATPases (Lee & Blostein, 1980; Forgac & Chin, 1981; Gafni & Boyer, 1985; Cox & Helman 1986*a*,*b*) have been shown able to change the stoichiometric cation/ATP ratio, so perhaps making them more adaptable and capable of coping with demanding changes in physiological cellular metabolism.

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