Glycerol Inhibits or Uncouples the Plasma Membrane $(Ca^{2+}+Mg^{2+})$ATPase of Kidney Proximal Tubules Depending on the Ca^{2+} Concentration

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Plasma Membrane $(Ca^{2+}+Mg^{2+})$ATPase Glycerol Uncoupling of Ionic Pump, Polyol, Hydrophobic Interactions

In this report it is shown that glycerol (0.5–10% v/v) stimulate the C_{12}-E_9-solubilized renal $(Ca^{2+}+Mg^{2+})$ATPase in the presence of low concentrations of free Ca^{2+} ($< 10^{-6}$ M). At 4% (v/v), the polyol decreases the $K_{0.5}$ for Ca^{2+} from 1.15 to 0.22 µM at the high-affinity site, and a very-high-affinity Ca^{2+} component appears. This component has a $K_{0.5} \leq 10^{-9}$ M and its maximal velocity is about one-third that of the fully activated enzyme (at 10–20 µM Ca^{2+}), which is not affected by glycerol (21.1 and 20.2 $\text{nmol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$ in the absence and presence of the polyol, respectively). The low-affinity, inhibitory component of the Ca^{2+} curve (50–1000 µM) is also unaffected by glycerol. With 0.07 µM free Ca^{2+} and soluble enzyme, the stimulatory effect of glycerol saturates at ≈10% (v/v). In contrast, with 17 µM free Ca^{2+}, glycerol has little effect up to 10% (v/v), and then progressively inhibits ATPase activity. These data indicate that the effect of the polyol is modulated by the occupancy of the high-affinity Ca^{2+} sites. In native vesicles, the stimulation promoted by low concentrations of glycerol at low concentrations of Ca^{2+} is accompanied by inhibition of active Ca^{2+} transport, indicating that, in these conditions, the polyol uncouples ATPase activity and ATP-driven Ca^{2+} influx.

Introduction

The basolateral membrane $(Ca^{2+}+Mg^{2+})$ATPase plays an important role in the fine-tuned regulation of intracellular calcium in renal proximal tubule cells. ATP hydrolysis by this enzyme is stimulated by Mg^{2+} and micromolar Ca^{2+} concentrations (Gmaj *et al.*, 1982, 1983; Vieyra *et al.*, 1986; Coelho-Sampaio *et al.*, 1991; Guilherme *et al.*, 1991). It belongs to the P-ATPase class (Pedersen and Carafoli, 1987), alternating between two principal conformational states (E_1 and E_2) during the catalytic cycle. Fig. 1 shows the minimal reaction sequence of the enzyme (Guilherme *et al.*, 1991).

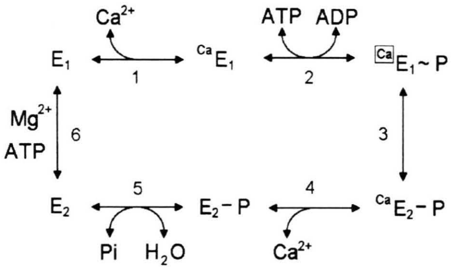

Fig. 1. Reaction sequence of the renal plasma membrane $(Ca^{2+}+Mg^{2+})$ATPase. E_1 represents the enzyme form phosphorylated by ATP, with the Ca^{2+}-binding site in a high-affinity state and facing the cytosolic (extravesicular) compartment. E_2 represents the conformation phosphorylated by Pi during reversal of the pump (Vieyra *et al.* 1991), with the Ca^{2+}-binding site in a low-affinity state and facing the peritubular (intravesicular) aspect of the membrane. ~P and –P indicate high- and low-energy states of the acyl phosphoprotein linkage. The box around Ca in the first phosphorylated intermediate indicates occlusion of the cation, which occurs after ADP release (Pedersen and Carafoli, 1987). Adapted from Guilherme *et al.* (1991).

The activity of both detergent-solubilized and membrane-bound $(Ca^{2+}+Mg^{2+})$ATPase preparations of proximal tubule basolateral membranes

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Abbreviations: C_{12}–E_9 or Polydocanol, polyoxyethylene-9-lauryl ether; EGTA, ethylene glycol-bis(β-aminoethyl ether) *N,N,N′N′*-tetraacetic acid; Pi, orthophosphate; Tris, tris(hydroxymethyl)-aminomethane.

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can be stimulated by calmodulin (De Smedt *et al.*, 1981; Coelho-Sampaio *et al.*, 1991). Using the purified (Ca^{2+}+Mg^{2+})ATPase from erythrocyte plasma membranes, Benaim and de Meis (1989, 1990) showed that glycerol and other organic solvents apparently can mimic calmodulin, increasing both the affinity for Ca^{2+} and the turnover number of the enzyme.

More recently, it has been shown that calmodulin induces the appearance of a very-high-affinity Ca^{2+} site in the solubilized renal (Ca^{2+}+Mg^{2+})ATPase (Coelho-Sampaio *et al.*, 1991). In this work, we demonstrate that low concentrations of glycerol can also elicit a very-high-affinity Ca^{2+} site in this enzyme. This activation at very low Ca^{2+} concentrations, however, is not identical to that seen in the presence of calmodulin since it is associated with inhibition of the ATP-dependent Ca^{2+} flux across the membrane.

In recent papers from this laboratory, it has been shown that polyols such as trehalose, sorbitol and mannitol uncouple the renal Ca^{2+} pump since they inhibit the Ca^{2+} flux without modification of (Ca^{2+}+Mg^{2+})ATPase activity (Sola-Penna *et al.*, 1994, 1995). The results shown in the present report indicate that glycerol can affect the pump in different ways, so that depending on the degree of saturation of the enzyme by Ca^{2+}, the pump can be either inhibited or uncoupled.

Materials and Methods

Reagents

ATP, C_{12}-E_9 and ouabain were purchased from Sigma Chemical Co., Percoll was from Pharmacia, and other chemical reagents were of the highest purity available. [^{32}P]Pi was from the Brazilian Institute of Energy and Nuclear Research. $^{45}CaCl_2$ was obtained from New England Nuclear (MA, U.S.A.). Distilled water deionized by the MilliQ system of resins (Millipore Corp., MA, U.S.A.) was used in the preparation of all solutions. [γ-^{32}P]ATP was prepared according to Maia *et al.* (1983). Free Ca^{2+} concentrations in the solutions were calculated using an iterative computer program that was modified (Inesi *et al.*, 1980; Sorenson *et al.*, 1986) from that described by Fabiato and Fabiato (1979), and that took into account the different species involved in the equilibria between EGTA, Ca^{2+}, ATP, Mg^{2+} and H^+, and the influence of monovalent cations on the association constant for CaEGTA. At pH 7.4, K_{app} for CaEGTA was 2.8×10^7.

Preparation of membrane vesicles and solubilization of (Ca^{2+}+Mg^{2+})ATPase

Basolateral membranes from sheep kidney proximal tubules were isolated by the Percoll gradient method (Boumendil-Podevin and Podevin, 1983; Grassl and Aronson, 1986). The final membranous preparation containing inside-out vesicles was resuspended in 250 mM sucrose at a concentration of 10–20 mg protein/ml. The specific activity of the basolateral membrane marker (Na^++K^+)ATPase was enriched by 10–12 fold when compared to the initial homogenate. This membranous preparation consistently contains about 10% of sealed inside-out vesicles (Boumendil-Podevin and Podevin, 1983). Protein concentration was determined by the method of Lowry *et al.* (1951).

Solubilization of the enzyme was carried out by incubating 4 mg/ml membrane protein for 10 min in a solution containing 4 mg/ml C_{12}-E_9 (Polydocanol), 12 mM Tris-HCl buffer (pH 7.4), and 250 mM sucrose. After solubilization, the samples were centrifuged at 106,000 xg_{max} for 30 min. The supernatant was used immediately.

(Ca^{2+}+Mg^{2+})ATPase activity

(Ca^{2+}+Mg^{2+})ATPase activity was assayed by measuring the release of Pi from [γ-^{32}P]ATP at 37 °C in a standard assay medium containing 30 mM Tris-HCl buffer (pH 7.4), 10 mM NaN_3, 1 mM ouabain, 10 mM $MgCl_2$, 120 mM KCl, 5 mM [γ-^{32}P]ATP, 0.1 mM EGTA, and enough $CaCl_2$ to obtain the desired free Ca^{2+} concentration. The concentrations of glycerol are indicated in the figures and table legends. The reaction was started by addition of the native membranes or the detergent-solubilized preparation (0.2 mg protein/ml in either case) and quenched after 30 min with 2 vols of a suspension of activated charcoal in 0.1 N HCl (Grubmeyer and Penefsky, 1981). After centrifugation, aliquots of the supernatant containing ^{32}Pi were counted in a liquid scintillation counter. The Ca^{2+}-stimulated activity was calculated by subtracting the activity in the presence of 3.5 mM

EGTA from the activity in the presence of Ca^{2+} measured in tubes run in parallel.

Ca^{2+} uptake

Except when otherwise noted the basic medium for Ca^{2+} uptake contained, in a final volume of 0.5 ml (at 37 °C), 30 mM Tris-HCl buffer (pH 7.4), 5 mM ATP, 5 mM $MgCl_2$, 1 mM ouabain, 10 mM NaN_3, 0.1 mM EGTA and enough [^{45}Ca]$CaCl_2$ to obtain the desired free Ca^{2+} concentrations. [^{45}Ca]Ca^{2+} uptake was started by the addition of membranes (protein concentration 0.2 mg/ml) and stopped by Millipore filtration (Martonosi and Feretos, 1964), using 0.45 μm pore size filters. The filters were washed with a cooled solution containing 2 mM $La(NO_3)_3$, 100 mM KCl and 20 mM MOPS-Tris (pH 7.0). The [^{45}Ca]Ca^{2+} remaining in the vesicles was counted in a liquid scintillation counter.

Results

Effects of glycerol on the Ca^{2+} concentration dependence of (Ca^{2+}+Mg^{2+})ATPase activity using solubilized enzyme

In previous reports, it has been shown that the renal (Ca^{2+}+Mg^{2+})ATPase activity is modulated by Ca^{2+} ions in a biphasic manner. Concentrations of Ca^{2+} up to ≈20 μM stimulate its activity; above 50 μM, Ca^{2+} causes a decrease in the rate of ATP hydrolysis (Vieyra *et al.*, 1989; Coelho-Sampaio *et al.*, 1991; Guilherme *et al.*, 1991). This dual effect of Ca^{2+} is attributed to Ca^{2+} binding at sites in different functional states: one that is stimulatory, with higher affinity ($K_{0.5}$ ≈1 μM), and one that is inhibitory, with lower affinity (K_i ≈600 μM).

The Ca^{2+}-stimulated ATPase of plasma membranes can be further activated in different ways, such as with calmodulin (Gopinath and Vincenzi, 1977; Jarrett and Penniston, 1977), by acidic phospholipids (Niggli *et al.*, 1981a, 1981b), by limited proteolysis (Taverna and Hanahan, 1980; Niggli *et al.*, 1981b) or by organic solvents (Benaim and de Meis, 1989, 1990). All of these agents appear to enhance exposure of hydrophobic domains of the enzyme and/or promote novel interactions of specific domains with the surrounding medium.

Since the (Ca^{2+}+Mg^{2+})ATPase represents a small fraction of the total protein in plasma membranes (Carafoli, 1992), partial solubilization and purification using nonionic detergents has been employed to enhance its activity (De Smedt *et al.*, 1981), especially in experimental conditions in which the activity is low, such as in the presence of low ATP concentrations (Coelho-Sampaio *et al.*, 1991). It should be mentioned that all functional properties of the solubilized renal (Ca^{2+}+Mg^{2+})ATPase such as biphasic activation by ATP, affinities for Ca^{2+}, pH dependence and inhibition by vanadate, are similar to those of the membrane-bound enzyme (Meyer-Fernandes J. R., Sola-Penna M. and Vieyra, A., unpublished observations). In the present work we used both a native vesicular preparation and a membranous preparation treated with C_{12}-E_9. The later has a (Ca^{2+}+Mg^{2+})ATPase activity that is three fold higher than that of native membranes at saturating ATP and Ca^{2+} concentrations.

Fig. 2 shows that glycerol (4%, v/v) stimulates the solubilized renal (Ca^{2+}+Mg^{2+})ATPase, and that this effect is more pronounced at very low Ca^{2+} concentrations. Both in the absence and in the presence of the osmolyte the Ca^{2+} concentration dependence in the range 0.007–27 μM was best fitted according to the equation:

$$v = V_c + V_{max} \cdot [Ca^{2+}]/(K_{0.5} + [Ca^{2+}]) \qquad (1)$$

where V_c is the average rate of ATP hydrolysis observed at free Ca^{2+} concentrations between 0.007 and 0.02 μM after subtraction of the value obtained in parallel with 3.5 mM EGTA, [Ca^{2+}] is the free Ca^{2+} concentration, and V_{max} and $K_{0.5}$ are, respectively, the maximal velocity and the affinity constant for Ca^{2+} in the range 0.02–20 μM of the Ca^{2+} curve indicated in Fig. 2.

In the absence of glycerol (Fig. 2, empty circles) it is clearly seen that at very low Ca^{2+} concentrations (up to 0.02 μM) there is a low but non-zero rate of Ca^{2+}-stimulated ATP hydrolysis (V_c). In the presence of 4% (v/v) glycerol (Fig. 2, filled circles), V_c increases to a value more than three times higher than in the control conditions with no glycerol (Table I), whereas the basal, Mg^{2+}-dependent rate remains unchanged (data not shown). From the Ca^{2+} concentrations at which this component of the Ca^{2+} curve saturates, it may be inferred the $K_{0.5}$ for Ca^{2+} is $\leq 10^{-9}$ M. In addition, in the presence of glycerol, the $K_{0.5}$ for Ca^{2+} in the 0.02–20 μM range of the Ca^{2+} curve decreases from 1.2 to

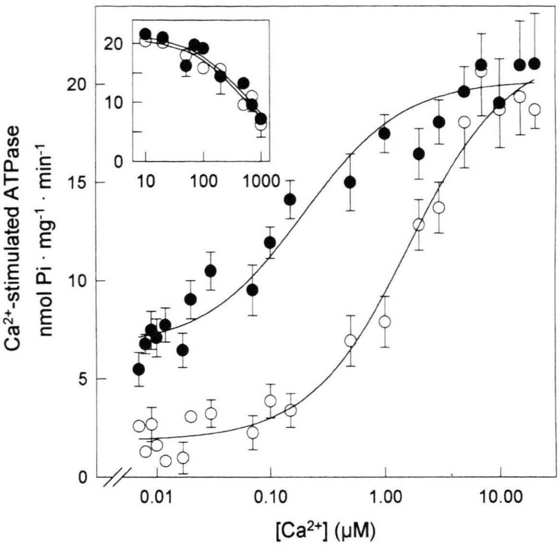

Fig. 2. Effect of 4% glycerol (v/v) on the Ca^{2+} concentration dependence of (Ca^{2+}+Mg^{2+})ATPase activity catalyzed by a C_{12}-E_9-treated membrane preparation. Assay media contained 30 mM Tris-HCl (pH 7.4), 10 mM NaN_3, 1 mM ouabain, 10 mM $MgCl_2$, 120 mM KCl, 5 mM [γ-^{32}P]ATP, 0.1 mM EGTA and enough $CaCl_2$ to obtain the free Ca^{2+} concentrations shown on the abscissa, considering contamination of the solutions with 10 μM calcium. (○): control without glycerol; (●): with 4% (v/v) glycerol. The reaction was started by the addition of C_{12}-E_9-solubilized preparation to a final protein concentration of 0.2 mg/ml and quenched after 30 min at 37 °C as described under Materials and Methods. Control experiments showed a linear relationship between Pi release and time. Bars represent the standard error of the means of 8 experiments. All experimental points were corrected for the activity measured in tubes run in parallel in the absence of added $CaCl_2$ and containing 3.5 mM EGTA. The Ca^{2+} concentration profile in the range below 0.007 μM can not be determined due to the impossibility of buffering Ca^{2+} and to the scatter in the experimental measurements. Eq. (1) (see text) was fitted to the points by nonlinear regression. Goodness of fit was assessed by computing reduced χ^2 of the fits, as described by Motulsky and Ransnas (1987). Inset: Inhibitory low-affinity component of the Ca^{2+} curve (20–1000 μM) measured in the absence (○) or in the presence (●) of 4% (v/v) glycerol.

0.2 μM (Table I). The glycerol-induced stimulation of (Ca^{2+}+Mg^{2+})ATPase activity diminishes with increasing Ca^{2+} concentrations, so that at 10 μM free Ca^{2+} the curves obtained in the absence and in the presence of the polyol coincide at a maximal activation value of about 20–21 nmol · mg^{-1} · min^{-1}, and remain superimposed as Ca^{2+} is increased into the range of the low-affinity, inhibitory component (inset to Fig. 2).

Table I. Kinetic parameters for the Ca^{2+} dependence of C_{12}-E_9-solubilized (Ca^{2+}+Mg^{2+})ATPase in the absence and presence of glycerol. Assay media and experimental procedures were as described in the legend to Fig. 2. Data are the mean values ± standard errors obtained by fitting the individual experiments in Fig. 2, using Eq. (1) (see text). Asterisk (*) indicates statistical difference with respect to the corresponding control without glycerol ($p < 0.001$).

Parameter	Control	Glycerol (4% v/v)
V_c (nmol Pi · mg^{-1} · min^{-1})	2.2 ± 0.2	7.4 ± 0.6*
$K_{0.5}$ (μM)	1.15 ± 0.10	0.22 ± 0.01*
V_{max} (nmol Pi · mg^{-1} · min^{-1})	21.1 ± 0.1	20.2 ± 1.4

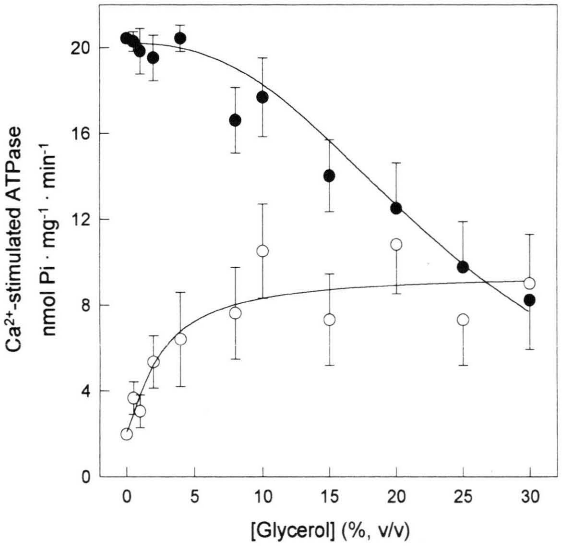

Fig. 3. Stimulation or inhibition of the C_{12}-E_9-solubilized (Ca^{2+}+Mg^{2+})ATPase activity by glycerol. Assay media contained 30 mM Tris-HCl (pH 7.4), 10 mM NaN_3, 1 mM ouabain, 10 mM $MgCl_2$, 120 mM KCl, 5 mM [γ-^{32}P]ATP, 0.1 mM EGTA and enough $CaCl_2$ to obtain either 0.07 μM (○) or 17 μM (●) free Ca^{2+}. The reaction was started by the addition of solubilized enzyme to a final concentration of 0.2 mg/ml and quenched as described under Materials and Methods after 30 min at 37 °C. Control experiments showed that the release of Pi was linear in this interval. Bars represent the standard error of the mean values of 8 experiments. All values were corrected by subtracting the basal activity measured in parallel in the presence of 3.5 mM EGTA without added $CaCl_2$. The smooth curves were drawn by hand.

Glycerol concentration dependence of (Ca^{2+}+Mg^{2+})ATPase activity at different free Ca^{2+} concentrations using solubilized enzyme

When a broad range of glycerol concentrations is used, the rate of ATP hydrolysis is modulated differently by the polyol depending on Ca^{2+} concentration (Fig. 3). At 17 μM free Ca^{2+}, when ATP hydrolysis in the absence of glycerol is maximal (Fig. 2, empty circles), concentrations up to 10% (v/v) glycerol have no significant effect on ATP hydrolysis, while higher concentrations are inhibitory (Fig. 3, filled circles). At 500 μM Ca^{2+}, similar effects are observed (data not shown). At 0.07 μM free Ca^{2+}, a concentration normally found in proximal tubule cells (Poggioli *et al.*, 1992), and one that stimulates ATP hydrolysis very little in the absence of glycerol (Fig. 2, empty circles), the rate of ATP hydrolysis is progressively activated by glycerol concentrations up to 10% (Fig. 3, empty circles).

Glycerol concentration dependence of Ca^{2+} uptake by inside-out vesicles at different Ca^{2+} concentrations

Both of the free Ca^{2+} concentrations used with solubilized enzyme in the experiments of Fig. 3 (0.07 and 17 μM) support Ca^{2+} transport into native inside-out vesicles (Fig. 4). At the higher Ca^{2+} concentration, the effect of glycerol on Ca^{2+} uptake is similar to its effect on the (Ca^{2+}+Mg^{2+})ATPase activity of the solubilized enzyme – that is, little or no effect up to 5% (v/v) glycerol, followed by a significant inhibition at higher glycerol concentrations (Fig. 4, filled circles). A similar effect is observed when the effect of glycerol on transport is studied in a medium containing 0.07 μM Ca^{2+} (Fig. 4, empty circles). This profile of progressive inhibition contrasts with the activation observed for (Ca^{2+}+Mg^{2+})ATPase activity (Fig. 3, empty circles). Inhibition of uptake is not due to an increase in the membrane permeability to Ca^{2+}, since the EGTA-induced Ca^{2+} efflux from preloaded vesicles is the same in the absence or presence of glycerol (Fig. 4, inset).

Comparison of the effects of glycerol on (Ca^{2+}+Mg^{2+})ATPase activity and Ca^{2+} uptake using membrane-bound enzyme

As mentioned above, all functional properties of the renal plasma membrane (Ca^{2+}+Mg^{2+})ATPase that have been tested are similar in the detergent-treated and in the native enzyme, including the response to ligands and inhibitors. Similar data have been reported for the (Ca^{2+}+Mg^{2+})ATPase from red cells (Gopinath and Vincenzi, 1977; Muallem and Karlish, 1979; Niggli *et al.*, 1981a; Stieger and Luterbacher, 1981). However, since glycerol could influence the (Ca^{2+}+Mg^{2+})ATPase by affecting its interactions with the phospholipid environment and/or the detergent (Martins and de Meis, 1985), additional experiments were carried out using native vesicles (Fig. 5). Since at very low Ca^{2+} concentrations ATP hydrolysis was increased 2.5 fold (Fig. 5A, left bars) whereas Ca^{2+} uptake

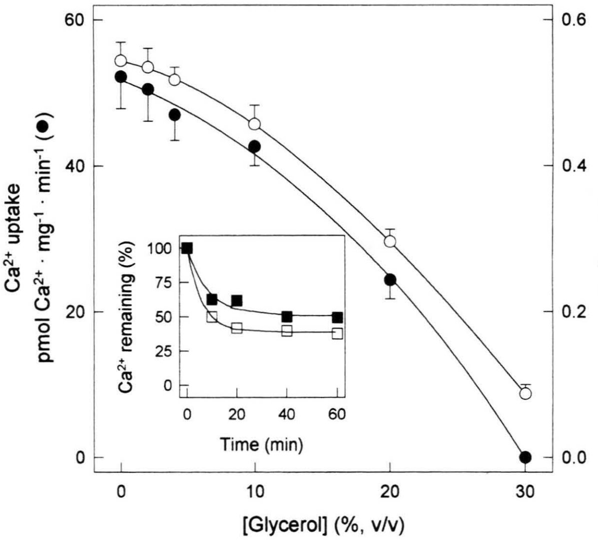

Fig. 4. Effect of glycerol on Ca^{2+} transport measured in inside-out vesicles derived from basolateral membranes. The native vesicular preparation (0.2 mg/ml) was incubated for 30 min at 37 °C in the assay media described under Materials and Methods with 0.07 μM (○) or 17 μM (●) free Ca^{2+}, in the presence of the concentrations of glycerol shown on the abscissa. Control experiments showed that Ca^{2+} accumulation was linear during this time interval. Inset: Ca^{2+} efflux from preloaded vesicles in the absence and presence of glycerol. Vesicles were preloaded for 5 h in the same experimental conditions (17 μM free Ca^{2+}), except that the protein concentration was 5 mg/ml. The Ca^{2+} remaining in the vesicles (indicated as percent values on the ordinate) was measured at the times shown on the abscissa after addition of 2 mM EGTA (□) or 2 mM EGTA plus 15% glycerol (v/v) (■). Curves in both main panel and inset were drawn by hand.

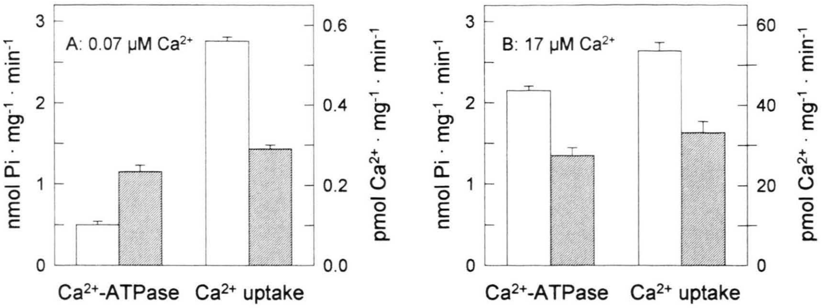

Fig. 5. Comparison of the effects of glycerol on Ca^{2+} transport and (Ca^{2+}+Mg^{2+})ATPase activity measured in parallel at 0.07 µM (A) or 17 µM (B) free Ca^{2+} using a native vesicular preparation. Assays were performed as indicated in the legends to Figs. 3 and 4 except that a native vesicular preparation was used instead of solubilized (Ca^{2+}+Mg^{2+})ATPase. Left bars (A and B): (Ca^{2+}+Mg^{2+})ATPase. Right bars (A and B): Ca^{2+} uptake. Empty bars: Controls without glycerol. Hatched bars: Assays containing 15% (v/v) glycerol.

was inhibited by 50% (Fig. 5A, right bars), we conclude that glycerol uncouples the Ca^{2+} pump. In contrast, in the presence of 17 µM free Ca^{2+} (enough to saturate the stimulatory components shown in Fig. 2), glycerol (15%, v/v) inhibited both Ca^{2+} accumulation and Ca^{2+}-stimulated ATP hydrolysis in parallel by about 40% (compare left and right bars in Fig. 5B). It should be mentioned that the effect of glycerol on (Ca^{2+}+Mg^{2+})ATPase activity was not modified by the addition of 20 nM calmodulin (data not shown).

Effect of glycerol on the steady-state Ca^{2+} influx

To further investigate the mechanism by which glycerol impairs Ca^{2+} accumulation, vesicles preloaded to a steady level with two different concentrations of non-radioactive Ca^{2+} (0.07 and 17 µM) in the absence of glycerol were used to measure the Ca^{2+} influx component of the $^{45}Ca^{2+} \leftrightarrow Ca^{2+}$ exchange in the absence and presence of 15% glycerol, a concentration that promotes 40–50% inhibition of Ca^{2+} transport with both Ca^{2+} concentrations (Figs 4 and 5). The time course of $[^{45}Ca^{2+}]Ca^{2+}$ incorporation after addition of a pulse of $^{45}Ca^{2+}$ shows that glycerol decreases the velocity of influx by 40–50% (Figs 6A and 6B), *i. e.* the same degree of inhibition observed in Figs 4 and 5.

Discussion

The stabilization of protein structures by glycerol has been attributed to its ability to reduce the

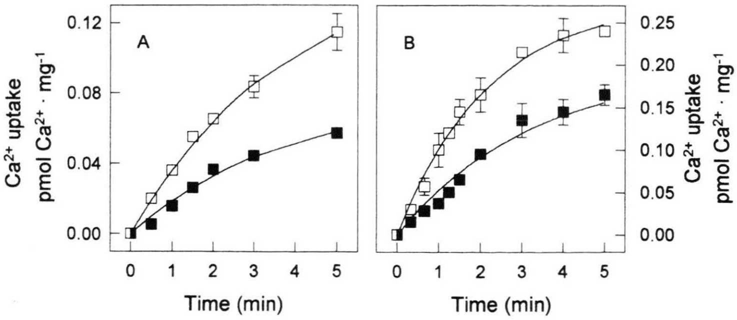

Fig. 6. Time course of the unidirectional Ca^{2+} influx in the absence and presence of glycerol. Vesicles were preloaded in the same experimental conditions described for the experiments shown in Fig. 4, with 0.07 µM (A) or 17 µM (B) free Ca^{2+}, except that non-radioactive Ca^{2+} and 5 mg protein/ml were used. After 5 h (zero time on the abscissae) the samples were diluted with the same solutions containing 15% (v/v) (■) or no (□) glycerol to obtain a final protein concentration of 0.2 mg/ml, and immediately supplied with $^{45}Ca^{2+}$ ($\approx 10^7$ cpm/ml; essentially carrier free). $[^{45}Ca]Ca^{2+}$ incorporation (in pmol Ca^{2+}/mg) was measured at the times indicated on the abscissae as described under Materials and Methods. The curves were drawn by hand.

interactions between protein and water (Gekko and Timasheff, 1981a, 1981b). In the sarcoplasmic reticulum Ca^{2+}-ATPase, another ion-transporting enzyme, it has been shown that sugars decrease the $K_{0.5}$ for Pi (Chini *et al.*, 1991). This observation suggests that polyols stabilize the E_2 conformation (Fig. 1). This conformation is described as having the catalytic site in a less hydrated state (de Meis *et al.*, 1980). Arakawa and Timasheff (1982) postulated that polyols, in general, lower the amount of water solvating proteins without interacting directly with the protein surface, a phenomenon that they called preferential hydration.

The activation by calmodulin of the erythrocyte plasma membrane (Ca^{2+}+Mg^{2+})ATPase can be mimicked by organic solvents, including glycerol (Benaim and de Meis, 1989, 1990). Although the activation of the renal plasma membrane (Ca^{2+}+Mg^{2+})ATPase by calmodulin appears to differ in some respects from the activation observed in the erythrocyte enzyme (Gopinath and Vincenzi, 1977; Jarrett and Penniston, 1977; Coelho-Sampaio *et al.*, 1991), the results presented in this report show that glycerol mimics the effects of calmodulin on the Ca^{2+} concentration dependence of the solubilized renal (Ca^{2+}+Mg^{2+})ATPase (Coelho-Sampaio *et al.*, 1991). This observation provides further evidence for the existence of a very-high-affinity Ca^{2+} site in this enzyme, one that would be enhanced when specific hydrophobic domains are exposed to the medium.

In addition to its very high affinity for Ca^{2+} ($K_{0.5} \leq 10^{-9}$ M) the new site also pumps Ca^{2+} at a low rate (Fig. 4, empty circles). At low Ca^{2+} concentrations, however, its coupling ratio measured in intact vesicles is decreased by glycerol (Fig. 5A), suggesting that the intramolecular communication between the very-high-affinity Ca^{2+} sites and the domain(s) that interact with ATP is sensitive to a hydrophobic milieu. The existence of two stimulatory components with Ca^{2+} affinities that differ by more than two orders of magnitude (Fig. 2) and are associated with different transport capabilities (Fig. 4), may indicate that Ca^{2+} combines with the pump at two different classes of sites. Since the site with the highest affinity must be saturated at the low cytosolic Ca^{2+} activity currently accepted for epithelial cells (Lee *et al.*, 1980; Poggioli *et al.*, 1992), it is likely that only the site with affinity varying between 0.2–1.2 μM is affected by Ca^{2+} fluctuations under physiological and pathological conditions.

The results of Figs. 3 to 5 show that Ca^{2+}-stimulated ATP hydrolysis and ATP-driven Ca^{2+} flux are uncoupled by 0.5–15% (v/v) glycerol when the free Ca^{2+} concentration is too low to saturate the second stimulatory component of the Ca^{2+} curve (Fig. 2). This indicates that, despites its similarity with calmodulin in exposing a very high affinity component (Benaim and de Meis, 1989, 1990; Coelho-Sampaio *et al.*, 1991) the polyol differs from calmodulin in another respect, since the peptide stimulates both ATP hydrolysis and Ca^{2+} transport (Gopinath and Vincenzi, 1977; Jarrett and Penniston, 1977). Fig. 6 shows that $[^{45}Ca^{2+}]Ca^{2+}$ uptake by vesicles loaded with nonradioactive Ca^{2+} is inhibited by glycerol at both free Ca^{2+} concentrations. Since glycerol does not alter the passive efflux (inset of Fig. 4), it may be concluded that inhibition of Ca^{2+} accumulation is due to the inhibition of unidirectional Ca^{2+} influx.

The effects of glycerol concentrations higher than 10% (v/v) on ATP hydrolysis depend on the Ca^{2+} concentration. With the use of solubilized enzyme, the polyol stimulates hydrolysis with 0.07 μM free Ca^{2+} throughout the concentration range studied (Fig. 3, empty circles), an effect that is also found with native vesicles and 15% (v/v) glycerol (Fig. 5A). In contrast, the polyol is strongly inhibitory with 17 μM free Ca^{2+} (Fig. 3, filled circles and Fig. 5B). Interestingly, glycerol inhibits the unidirectional Ca^{2+} influx to the same extent, regardless of whether the Ca^{2+} sites are saturated or not (Fig. 6). It may be concluded, therefore, that the interaction of the polyol with the enzyme domains involved in energy transduction, and in contrast with the domains involved in the Ca^{2+} translocation itself, depends on the conformational state induced by the occupancy of the Ca^{2+} sites.

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