

Polyols that Accumulate in Renal Tissue Uncouple the Plasma Membrane Calcium Pump and Counteract the Inhibition by Urea and Guanidine Hydrochloride

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Sorbitol and mannitol, two stereoisomeric osmolytes, inhibit the ATP-dependent Ca²⁺ transport in inside-out vesicles derived from basolateral membranes from kidney proximal tubules. This inhibition ($I_{0.5} = 400$ and 390 mM respectively) cannot be attributed to an increase in Ca²⁺ permeability, since the rate of EGTA-stimulated Ca²⁺ efflux from preloaded vesicles is not modified by these osmolytes. In the presence of 1 M sorbitol or mannitol, Ca²⁺ uptake is inhibited by 70 and 75%, respectively. Since the Ca²⁺-stimulated ATPase activity is unaffected, sorbitol and mannitol uncouple the Ca²⁺ transport from the ATPase activity. The inhibition of Ca²⁺ transport by these osmolytes is reversible, since the inhibition disappears when the vesicles are preincubated with 1 M sorbitol or mannitol and then diluted 25-fold in reaction medium to measure Ca²⁺ accumulation. On the other hand, these osmolytes protect the (Ca²⁺ + Mg²⁺)ATPase from the inhibition of Ca²⁺ transport and ATPase activity by urea and guanidinium. These data suggest that the high concentrations of polyols that renal cells accumulate during antidiuresis, may regulate Ca²⁺ transport across the plasma membrane. In addition, polyols may protect the (Ca²⁺ + Mg²⁺)ATPase from the deleterious structural effects of urea, a compound that also accumulates during antidiuresis.

Introduction

Organisms and cellular systems that are required to adapt to stress conditions such as high temperature, desiccation, or high concentrations of urea respond by concentrating one of several organic solutes such as sugars, polyols, amino acids and methylamines (Yancey *et al.*, 1982; Crowe and Crowe, 1984; Somero, 1986). This accumulation is associated with the effectiveness of these osmolytes in minimizing protein denaturation in the face of stress (Crowe and Crowe, 1984; Carpenter and Crowe, 1989; Sola-Penna and Meyer-Fernandes, 1994). In terms of function they have been classified either as compatible solutes, which have little effect on protein function, or as counteracting solutes, which offset the structural and functional effects that deleterious solutes have on cell

proteins (Bowlus and Somero, 1979; Yancey *et al.*, 1982). These studies indicate that one class of these naturally occurring compounds is able to confer stability on proteins *in vivo* without modifying their biological activity.

It has been demonstrated that some osmolytes protect against the effects of urea on the function of membrane-bound (Ca²⁺ + Mg²⁺)ATPases from sarcoplasmic reticulum and renal plasma membranes, and also modulate independently the catalytic cycle of these Ca²⁺-transporting ATPases (de Meis and Inesi, 1988; Vieyra *et al.*, 1989, 1991; Chini *et al.*, 1991, 1992). They increase the apparent affinity for Pi during the phosphorylation reaction of rabbit sarcoplasmic reticulum (Ca²⁺ + Mg²⁺)ATPase (Jorge-Garcia *et al.*, 1988; de Meis and Inesi, 1988; Chini *et al.*, 1991), and increase the rate of ATP ↔ Pi exchange catalyzed by (Ca²⁺ + Mg²⁺)ATPase from renal plasma membranes (Vieyra *et al.*, 1989, 1991). It has been postulated that they modify the equilibrium between the different conformations of the enzyme (Chini *et al.*, 1991), and that they may exert their influence through a decrease in water activity and in

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protein solvation (de Meis, 1989; Chini *et al.*, 1991).

A recent report has shown that a decrease in protein solvation promoted by carbohydrates can modify the equilibria among allosteric conformations of hemoglobin (Colombo *et al.*, 1992), an indication that the flexibility and dynamic nature of proteins are affected by protein-bound water molecules in a way that can be modified by interaction with biological solutes. We have demonstrated that trehalose uncouples the Ca^{2+} transport from ATP hydrolysis by the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase of renal plasma membranes (Sola-Penna *et al.*, 1994). This solute is accumulated in several organisms during some stress conditions, and it interacts strongly with water and with polar groups of proteins and phospholipids (Crowe and Crowe, 1984; Crowe *et al.*, 1987).

Kidney is the mammalian organ that accumulates several osmolytes under specific conditions such as antidiuresis. The accumulation of very high concentrations of urea (0.4–0.9 M, Knepper and Rector, 1991) would be lethal to this tissue. However, almost in parallel with the increase in urea, counteracting compounds, such as methylamines, also accumulate in this tissue (Yancey and Burg, 1989). The parallel accumulation of this class of compounds has been associated with a protection against the deleterious effects of urea in renal tissue (Yancey and Burg, 1989). Another family of compounds that are accumulated in parallel with urea is that of polyols, especially sorbitol, mannitol and inositol (Bagnasco *et al.*, 1986). It is of interest to note that these osmolytes are the most effective, among a series of polyhydric alcohols, in stabilizing oligomeric enzymes and in enhancing the re-assembly of denatured subunits in the presence of high urea concentrations (Shifrin and Parrott, 1975). The counteracting solutes represented by the methylamine and polyol families are found in large amounts in the renal medulla (Bagnasco *et al.*, 1986), and they are also present in the renal cortex (Gullans *et al.*, 1988).

The present report is aimed to investigate whether compounds of the polyol family that accumulate in renal cells in antidiuresis conditions can, like the methylamines, counteract the inhibitory effects of urea and guanidinium on the activity of the Ca^{2+} pump of the basolateral plasma membranes of kidney proximal tubule cells. Our

results show that sorbitol and mannitol uncouple ATP hydrolysis and Ca^{2+} transport without increasing the Ca^{2+} permeability of the membrane, and that they protect the pump from inhibition by urea and guanidinium hydrochloride.

Materials and Methods

Preparation of purified vesicles derived from basolateral membranes

Basolateral membrane vesicles were isolated from sheep kidney proximal tubules by the Percoll gradient method (Grassl and Aronson, 1986). Compared with the initial homogenate this membrane fraction was enriched 9–12-fold in the specific activity of the basolateral membrane marker $(\text{Na}^+ + \text{K}^+)\text{ATPase}$. Protein concentrations were determined using the Folin phenol reagent (Lowry *et al.*, 1951) and bovine serum albumin as a standard.

^{45}Ca Uptake

Except when otherwise noted the basic medium contained, in a final volume of 0.5 ml, 30 mM Tris (tris(hydroxymethyl)aminomethane)-HCl buffer (pH 8.5), 5 mM ATP, 5 mM MgCl_2 , 1 mM ouabain, 10 mM NaN_3 , 0.1 mM EGTA ([ethylenebis(oxyethylene)nitrilo]tetraacetic acid) and 0.1 mM ^{45}Ca - CaCl_2 (10 μM free Ca^{2+}). The apparent $K_{\text{Ca,EGTA}}$ used was 2.8×10^7 (Fabiato and Fabiato, 1979). The experiments were carried out at 37 °C. Concentrations of osmolytes and other modifications in the medium are specified in the corresponding figure legends. ^{45}Ca 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 ^{45}Ca remaining in the vesicles was counted in a liquid scintillation counter after the filters were washed twice with 10 ml of a cold solution containing 2 mM $\text{La}(\text{NO}_3)_3$, 100 mM KCl, and 20 mM MOPS (4-morpholinepropanesulfonic acid)-Tris (pH 7.0).

$(\text{Ca}^{2+} + \text{Mg}^{2+})\text{ATPase}$ activity

ATPase activity was measured in the same conditions as Ca^{2+} uptake, except that the reaction

was quenched with acid, followed by adsorption of the non-hydrolyzed [γ -³²P]ATP on charcoal (Grubmeyer and Penefsky, 1981). Following centrifugation at 4000×g for 30 min, an aliquot of the supernatant was withdrawn to measure the amount of ³²Pi released. Spontaneous hydrolysis of [γ -³²P]ATP was measured in tubes run in parallel in which the enzyme was added after the acid. The (Ca²⁺+Mg²⁺)ATPase activity was the difference between the ATP hydrolysis measured in the presence of CaCl₂ and in its absence (EGTA 1 mM). ³²Pi was counted in a liquid scintillation counter.

Reagents

ATP, ouabain, phosphoenolpyruvate, pyruvate kinase, EGTA, Tris, sorbitol, mannitol, urea, and guanidinium hydrochloride were purchased from Sigma. ³²Pi was from the Brazilian Institute of Energy and Nuclear Research and ⁴⁵Ca was obtained from New England Nuclear (MA). Distilled water deionized by the MilliQ system of resins (Millipore Corp., U.S.A.) was used in the preparation of

all solutions. [γ -³²P]ATP was prepared as described by Maia *et al.* (1983).

Results

Effects of sorbitol and mannitol on Ca²⁺ uptake and on (Ca²⁺+Mg²⁺)ATPase activity catalyzed by inside-out vesicles derived from basolateral membranes of kidney proximal tubules

Fig. 1 shows that sorbitol (Fig. 1A) and mannitol (Fig. 1B) inhibit Ca²⁺ uptake in a dose-dependent manner, with $I_{0.5} \approx 0.4$ M in both cases (empty circles). This effect is specific for the active Ca²⁺ transport since the ATP-independent Ca²⁺ binding is not modified by the addition of sorbitol or mannitol (data not shown). The same figures show (filled circles) that the ATP hydrolysis stimulated by micromolar Ca²⁺ concentrations and measured under the same conditions as Ca²⁺ transport is not inhibited by sorbitol (Fig. 1A) nor by mannitol (Fig. 1B). The uncoupling effect of sorbitol and mannitol on ATP-dependent Ca²⁺ transport is completely reversible. The inhibition of Ca²⁺ uptake disappears if

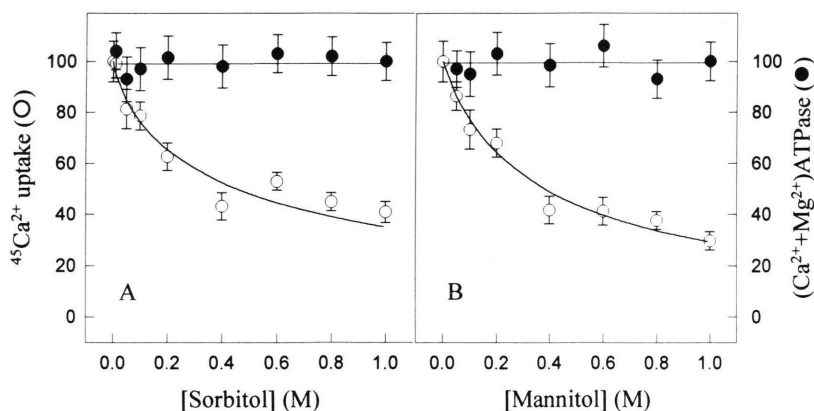


Fig. 1. Uncoupling by sorbitol and mannitol of ATP-dependent Ca²⁺ uptake and (Ca²⁺+Mg²⁺)ATPase activity. ATP-dependent Ca²⁺ accumulation (○) and (Ca²⁺+Mg²⁺)ATPase activity (●) were measured after 30 min in the experimental conditions indicated under Materials and Methods, in the presence of the sorbitol (A) or mannitol (B) concentrations shown on the abscissas. The amount of Ca²⁺ accumulated was 5.2 ± 0.5 nmol·mg⁻¹ protein ($n = 4$), and (Ca²⁺+Mg²⁺)ATPase activity in the absence of solutes was that indicated in a preceding paper (26 ± 2 nmol·mg⁻¹·min⁻¹; Sola-Penna *et al.*, 1994). ATP hydrolysis in the absence of Ca²⁺ (1 mM EGTA) was the same at all solute concentrations (not shown). Bars correspond to standard errors calculated from the absolute activity values of four experiments and converted to percentage of the control value. Control experiments showed a linear relationship of Ca²⁺ transport and ATP hydrolysis with time in the interval chosen.

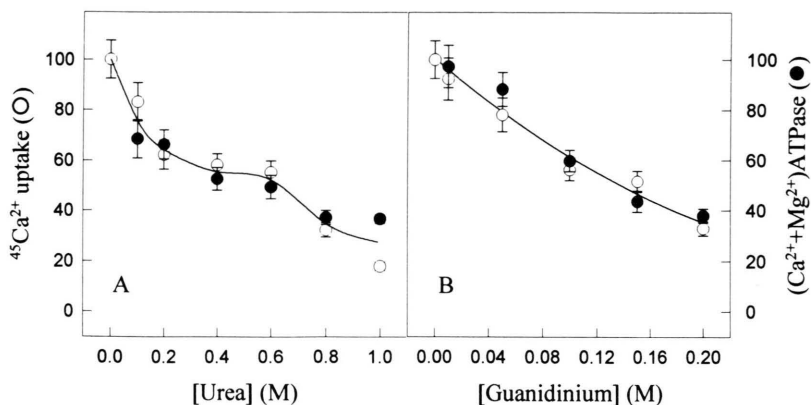


Fig. 2. Inhibition of Ca²⁺ uptake and ATPase activity by urea and guanidinium hydrochloride. ATP-dependent Ca²⁺ uptake (○) and (Ca²⁺+Mg²⁺)ATPase activity (●) were measured after 30 min in the same conditions indicated under Materials and Methods, in the presence of the urea (A) or guanidinium hydrochloride (B) concentrations shown on the abscissas. Bars correspond to standard errors calculated from the absolute activity values of four experiments and converted to percentage of the control value.

the vesicles are diluted 25-fold in an osmolyte-free medium after incubation for 60 min in the presence of 1 M of the organic solutes (data not shown).

Effects of urea and guanidinium hydrochloride on Ca²⁺ uptake and on (Ca²⁺+Mg²⁺)ATPase activity

Fig. 2A shows that urea inhibits Ca²⁺ uptake in a dose-dependent manner, essentially in parallel with the inhibition of Ca²⁺-ATPase activity (empty and filled circles). The curve is biphasic, with a plateau between 0.2 and 0.6 M, a result that may be an indication that two different mechanisms are involved. Guanidinium hydrochloride, another agent considered to be a potent denaturant, inhibits both Ca²⁺ transport and ATP hydrolysis in a monophasic manner with an $I_{0.5} \approx 130$ mM (Fig. 2B). These results show that solutes of this type – in contrast to sorbitol and mannitol – do not uncouple Ca²⁺ transport from ATP hydrolysis. In common with other counteracting solutes such as methylamines, polyols appear to act on specific domains of energy transducing enzymes (Sola-Penna *et al.*, 1994; Coelho-Sampaio *et al.*, 1994), whereas urea and guanidinium hydrochloride promote a global inhibition of the pump. Their effects may be related to their ability to unfold the ATPase molecule as a whole (Mashino and Fridovich, 1987).

Effect of polyols, urea, and guanidinium on Ca²⁺ permeability

Inhibition of the ATP-dependent Ca²⁺ transport by sorbitol and mannitol might be associated with

an increase in the Ca²⁺ permeability of the membrane. However, it was found that a polyol concentration that promotes 50% inhibition of Ca²⁺ accumulation does not increase the rate constant for EGTA-induced Ca²⁺efflux from vesicles pre-

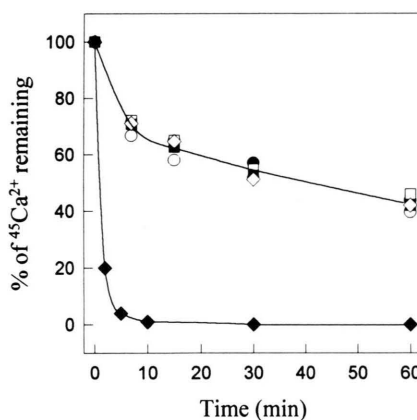


Fig. 3. Time course of Ca²⁺ efflux from preloaded vesicles in the absence and presence of osmolytes. Vesicles were preloaded for 5 h in the experimental conditions described in the legend of Fig. 1, plus phospho(enol)pyruvate and pyruvate kinase in order to regenerate ATP from the ADP formed during reaction. Ca²⁺ remaining in the vesicles was measured at the times indicated on the abscissa after addition of a concentrated medium to obtain final concentrations of 2 mM EGTA plus either: no other addition (○), 400 mM sorbitol (●), 400 mM mannitol (□), 400 mM urea (■), 150 mM guanidinium hydrochloride (◇), or 10 μM A 23187 (◆). ⁴⁵Ca²⁺ inside the vesicles at the beginning of the efflux period was 15.0 ± 2.6 nmol·mg⁻¹ protein ($n = 8$), and standard errors were $\leq 20\%$ of absolute values. Except when the ionophore is present, there is no statistical difference between the values obtained in the absence or presence of solute.

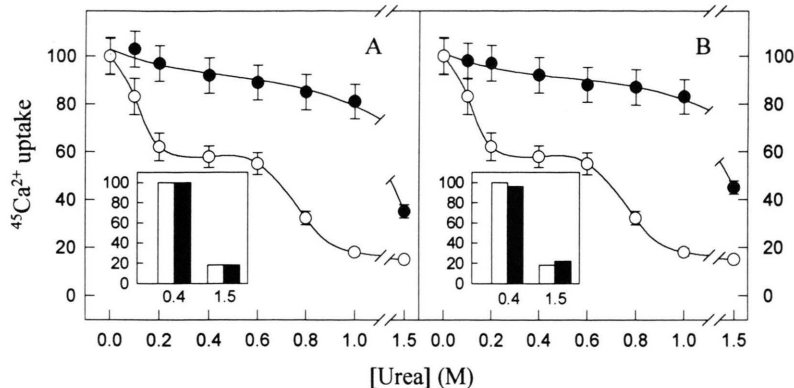


Fig. 4. Sorbitol and mannitol protection against urea inhibition of Ca^{2+} uptake. Ca^{2+} uptake was carried out in the same conditions as in Fig. 2 in the presence of the urea concentrations indicated on the abscissa, and in the absence (\circ) or in the presence (\bullet) of 0.4 M sorbitol (A) or mannitol (B). The total amount of Ca^{2+} accumulated (mean \pm standard error) was $5.2 \pm 0.5 \text{ nmol} \cdot \text{mg}^{-1}$ protein ($n = 4$) in the absence of polyols, $2.2 \pm 0.3 \text{ nmol} \cdot \text{mg}^{-1}$ protein in the presence of 0.4 M sorbitol (see Fig. 1 A, empty circles), and $2.2 \pm 0.3 \text{ nmol} \cdot \text{mg}^{-1}$ protein in the presence of 0.4 M mannitol (see Fig. 1 B, empty circles). Bars correspond to standard errors calculated from the absolute activity values of four experiments and converted to percentage of the control value. Inset: vesicles were preincubated for 30 min in the presence of 0.4 or 1.5 M urea, as shown on the abscissa or in the presence of 0.4 or 1.5 M urea plus 0.4 M sorbitol (A) or 0.4 M mannitol (B). Then the samples were diluted 25-fold with the reaction medium indicated under Materials and Methods for Ca^{2+} uptake experiments. Results are expressed as percent of controls that were preincubated and diluted under the same conditions but without urea or polyols.

loaded with Ca^{2+} (Fig. 3). The same figure shows a similar lack of effect of urea and guanidinium on the passive Ca^{2+} efflux. Since the effects of both classes of solutes on Ca^{2+} uptake can not be attributed to an increase in membrane permeability for Ca^{2+} , it may be concluded that they inhibit the unidirectional ATP-driven Ca^{2+} influx.

Protection by polyols against inhibition of Ca^{2+} transport and ATP hydrolysis

As shown in Fig. 2 A and 2 B, urea and guanidinium hydrochloride do not uncouple Ca^{2+} transport from ATP hydrolysis. Instead, and as mentioned above, they inhibit both reactions as a result of

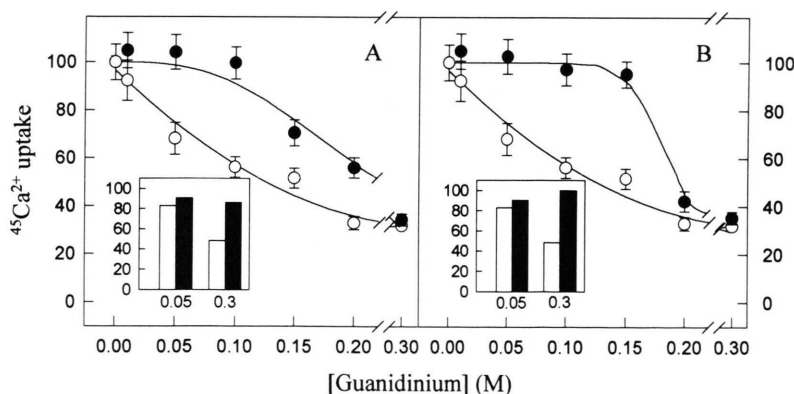


Fig. 5. Sorbitol and mannitol protection against guanidinium hydrochloride inhibition of Ca^{2+} uptake. Ca^{2+} uptake was carried out in the same conditions as in Fig. 2 in the presence of the guanidinium hydrochloride concentrations indicated on the abscissa, in the absence (\circ) or in the presence (\bullet) of 0.4 M sorbitol (A) or mannitol (B). Bars correspond to standard errors calculated from the absolute activity values of four experiments and converted to percentage of the control value. Inset: the experimental procedures and symbols were the same described in the legend to Fig. 4 (inset), except that guanidinium hydrochloride (0.05 and 0.3 M) was used instead of urea.

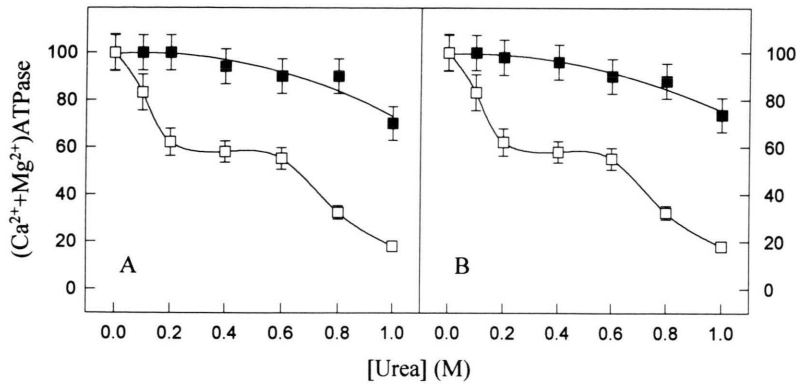


Fig. 6. Sorbitol and mannitol protection against urea inhibition of the $(\text{Ca}^{2+} + \text{Mg}^{2+})\text{ATPase}$ activity. $(\text{Ca}^{2+} + \text{Mg}^{2+})\text{ATPase}$ activity were measured after 30 min in the same conditions indicated under Materials and Methods, in the absence (□) or in the presence (■) of 0.4 M sorbitol (A) or 0.4 M mannitol (B). The velocity of ATP hydrolysis in the control was $26 \pm 2 \text{ nmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ as indicated in a preceding paper (Sola-Penna *et al.*, 1994). Bars correspond to standard errors calculated from the absolute activity values of four experiments and converted to percentage of the control value.

their direct interaction with the structure of the $(\text{Ca}^{2+} + \text{Mg}^{2+})\text{ATPase}$. Fig. 4A and 4B, respectively, show that sorbitol and mannitol counteract the urea-induced inhibition of Ca^{2+} transport. Significant protection is also conferred by these two polyols in the guanidinium-induced inhibition (Fig. 5A and 5B). It should be mentioned that the Ca^{2+} transport by the vesicles is completely restored when they are diluted 25-fold with urea-free medium after incubation with 0.4 M urea but not after incubation with 1.5 M urea (inset to Fig. 4A and 4B, empty bars). In addition neither sorbitol nor mannitol modifies the complete restoration of pumping activity of vesicles preincubated with 0.4 M urea, nor are they able to counteract the inhibition promoted by 1.5 M urea (inset to Fig. 4A and 4B, filled bars). With guanidinium chloride, which inhibits the enzyme with a simple

monotonic concentration profile (Fig. 2B), both polyols are able to preserve the functional state of the pump when they are present in the preincubation medium even in the presence of a high concentration of the denaturing agent. Although Ca^{2+} uptake is inhibited when assayed after preincubation of the vesicles in the presence of guanidinium chloride with no sorbitol or mannitol (inset to Fig. 5A and 5B, empty bars), the pumping activity attains the control value if the polyols are also present in the preincubation media (inset to Fig. 5A and 5B, filled bars).

Finally, it can be seen that 0.4 M sorbitol (Fig. 6A and 7A) or mannitol (Fig. 6B and 7B), is as effective in counteracting also inhibitory effects of urea (Fig. 6) and guanidinium hydrochloride (Fig. 7) on the $(\text{Ca}^{2+} + \text{Mg}^{2+})\text{ATPase}$ activity as they are for Ca^{2+} transport. Thus, although

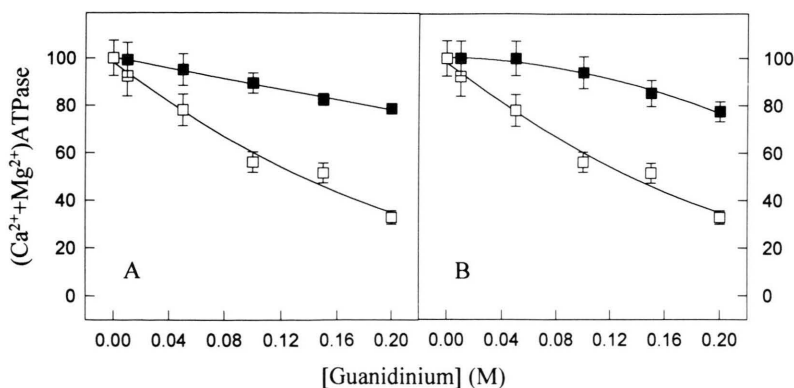


Fig. 7. Sorbitol and mannitol protection against guanidinium hydrochloride inhibition of the $(\text{Ca}^{2+} + \text{Mg}^{2+})\text{ATPase}$ activity. $(\text{Ca}^{2+} + \text{Mg}^{2+})\text{ATPase}$ activity were measured after 30 min in the conditions indicated under Materials and Methods, in the absence (□) or in the presence (■) of 0.4 M sorbitol (A) or 0.4 M mannitol (B). Bars correspond to standard errors calculated from the absolute activity values of four experiments and converted to percentage of the control value.

these polyols uncouple Ca²⁺ transport from ATP hydrolysis (Fig. 1), they are able to confer protection against the inhibitory effects of urea and guanidinium hydrochloride on the (Ca²⁺ + Mg²⁺)-ATPase.

Discussion

The protective role of organic solutes such as methylamines against protein damage by urea in biological systems (Yancey *et al.*, 1982; Somero, 1986; Mashino and Fridovich, 1987; Jorge-Garcia *et al.*, 1988; Vieyra *et al.*, 1989, 1991) has been attributed to their ability to preserve the structural and functional integrity of proteins, probably because they promote compaction of the protein structure (Mashino and Fridovich, 1987). In this work, we show that polyols that are accumulated in renal cells at high concentrations under specific physiological conditions (Bagnasco *et al.*, 1986) modulate the coupling ratio of the renal plasma membrane (Ca²⁺ + Mg²⁺)-ATPase and confer protection against protein damage by urea and guanidinium hydrochloride.

In renal plasma membranes the uncoupling of ATP-dependent Ca²⁺ transport and ATP hydrolysis (Fig. 1 A and 1 B) may reflect an interaction of sorbitol and mannitol with the enzyme phospholipid environment. Recently we have shown that trehalose also uncouples this transport system (Sola-Penna *et al.*, 1994). This phenomenon may be related to the occurrence of strong interactions between polar groups of phospholipids and the hydroxyl groups of trehalose (Crowe and Crowe, 1984; Crowe *et al.*, 1984; Sola-Penna *et al.*, 1994). Since the chemical properties of the polyols used in this report are similar to those of trehalose, the sorbitol- and mannitol-induced uncoupling of ATP hydrolysis and Ca²⁺ transport in renal plasma membranes may also be related to a decrease in membrane mobility promoted by interactions of the osmolytes with membrane phospholipids (Crowe and Crowe, 1988; Sola-Penna *et al.*, 1994). Since the Ca²⁺ binding site in different Ca²⁺-ATPases is located in a hydrophobic region (Shull and Greb, 1988; Clarke *et al.*, 1989; Carafoli, 1991, 1992), interactions between these polyols and the phospholipids around the Ca²⁺-binding domain might impair conformational changes of the enzyme associated with cation translocation. These

results led to the conclusion that the long-range intramolecular linkage between Ca²⁺-activated ATP hydrolysis and Ca²⁺ transport is dissociated by the polyols.

The interaction of polyols with phospholipids around the ATPase may also increase the compaction of protein structure. Such an effect would confer protection against protein unfolding by urea and guanidinium hydrochloride (Robinson and Jencks, 1965), counteracting by this mechanism the inhibition of Ca²⁺ transport and ATP hydrolysis (Fig. 4–7). Since thermodynamic studies have shown that mannitol at concentrations up to about 0.3 M has a structuring effect on water, while sorbitol has a structure-breaking effect (Stern and O'Connor, 1972), the uncoupling of Ca²⁺ transport and ATP hydrolysis can not be explained by interaction of these compounds with water molecules.

The uncoupling promoted by sorbitol and mannitol (Fig. 1) suggests that in tissues where polyols occurs (Balaban and Knepper, 1983; Bagnasco *et al.*, 1986; Balaban and Burg, 1987; Gullans *et al.*, 1988), then may regulate physiological transport processes mediated by ATPases, including the plasma membrane (Ca²⁺ + Mg²⁺)-ATPase responsible for the fine-tuned regulation of cytosolic Ca²⁺ concentrations (Carafoli, 1991, 1992). During antidiuresis, rabbits accumulate sorbitol and mannitol in the inner medulla (Bagnasco *et al.*, 1986), where the concentration of urea can vary between 0.4 M and 0.9 M (Knepper and Rector, 1991), and there is evidence that Ca²⁺ ions are involved in the process of intracellular osmoregulation (McCarty and O'Neil, 1992; Bagnasco *et al.*, 1993). Since urea appears in the urine whereas polyols do not, it may be that the latter compounds play a significant role in both maintenance of intracellular osmotic balance (Bagnasco *et al.*, 1986) and stabilization of ion-transporting ATPases in the presence of physiologically high urea concentrations.

Protection by sorbitol and mannitol against the effect of urea is almost complete in the first part of the inhibition curve (Fig. 4 A and 4 B), and appears to decrease above 1 M urea. Since the inhibition by 0.4 M urea is completely reversible whereas that promoted by 1.5 M is not, either in the absence or in the presence of one of the carbohydrates (inset to Fig. 4 A and 4 B), it may be concluded that counteracting effects of polyols occur

only in the urea concentration range that is usually found in physiological conditions (Knepper and Rector, 1991).

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