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Reversible Inhibition by 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic Acid of the Plasma Membrane (Ca^{2+}+Mg^{2+})ATPase from Kidney Proximal Tubules†

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ABSTRACT: Calcium accumulation by purified vesicles derived from basolateral membranes of kidney proximal tubules was reversibly inhibited by micromolar concentrations of 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), an inhibitor of anion transport. The inhibitory effect of this compound on Ca^{2+} uptake cannot be attributed solely to the inhibition of anion transport: (Ca^{2+}+Mg^{2+})ATPase activity of the solubilized enzyme was also impaired by DIDS, indicating a direct effect on the calcium pump. The concentrations needed to attain half-maximal inhibition were 20 and 63 μM for (Ca^{2+}+Mg^{2+})ATPase and ATP-dependent Ca^{2+} transport, respectively. The rate constant of EGTA-induced Ca^{2+} efflux from preloaded vesicles was not affected by DIDS, indicating that this compound does not increase the permeability of the membrane vesicles to Ca^{2+}. In the presence of DIDS, the effects of the physiological ligands Ca^{2+}, Mg^{2+}, and ATP on (Ca^{2+}+Mg^{2+})ATPase activity were modified. The Ca^{2+} concentration that inhibited (Ca^{2+}+Mg^{2+})ATPase activity in the low-affinity range decreased from 91 to 40 μM, but DIDS had no effect on the K_m for Ca^{2+} in the high-affinity, stimulatory range. Free Mg^{2+} activated (Ca^{2+}+Mg^{2+})ATPase activity at a low Ca^{2+} concentration, and DIDS impaired this stimulation in a noncompetitive fashion. The inhibition by DIDS was eliminated when the free ATP concentration of the medium was raised from 0.3 to 8 mM, possibly due to an increase in the turnover of the enzyme caused by free ATP accelerating the $E_2 \rightarrow E_1$ transition, and leading to a decrease in the proportion of E_2 forms under steady-state conditions. Alkaline pH totally abolished the inhibition of the (Ca^{2+}+Mg^{2+})ATPase activity by DIDS, with a half-maximal effect at pH 8.3. Finally, the synthesis of ATP catalyzed by soluble enzyme, measured in the same conditions as those of ATP hydrolysis, was insensitive to DIDS up to 100 μM, indicating that this compound acts primarily in the forward direction of the catalytic cycle. Taken as a whole, these results indicate that DIDS interacts with the enzyme in the E_2 conformation, probably slowing the rate of the $E_2 \rightarrow E_1$ transition in forward cycles.

Calcium transport across the basolateral membranes of proximal tubule cells is mediated, in part, by an ATPase that is stimulated by micromolar Ca^{2+} concentrations in the presence of Mg^{2+} (Gmaj et al., 1979, 1982; De Smedt et al., 1981; Vieyra et al., 1986). Although this Ca^{2+} pumping mechanism appears to be important in regulation of intracellular Ca^{2+} concentration, little is known about the catalytic properties of the enzyme. Evidence has been presented (Gmaj et al., 1983; Vieyra et al., 1986) showing that the renal (Ca^{2+}+Mg^{2+})ATPase belongs to the P-ATPase class (Pedersen & Carafoli, 1987), passing through two principal conformational states (E_1 and E_2) during its catalytic cyclic (Figure 1).

In the erythrocyte (Waisman et al., 1981) and in the sarcoplasmic reticulum (Campbell & MacLennan, 1980), the

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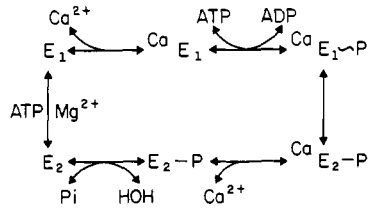

FIGURE 1: Reaction sequence of $(Ca^{2+}+Mg^{2+})$ATPases from plasma membranes [adapted from Pedersen and Carafoli (1987), Adamo et al. (1988), and Vieyra et al. (1989)].

ATP-dependent Ca^{2+} pump is stimulated by anions, and when anion exchange is blocked by inhibitors of anion transport, Ca^{2+} translocation is simultaneously inhibited. In a previous report (Vieyra et al., 1986), it was shown that Ca^{2+} uptake by vesicles derived from proximal tubule cell membranes is stimulated by phosphate. It was proposed that this activation might be caused by an asymmetrical distribution of the anionic species of phosphate, which would promote Ca^{2+}/H^+ exchange and/or that it might be associated with partial compensation of charge within the vesicles.

Since the basolateral membrane of proximal tubules contains an anion exchanger (Talor et al., 1987), it was of interest to study the effects of the anion-exchange inhibitor 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS)[1] on Ca^{2+} transport using a purified preparation of vesicles derived from basolateral plasma membranes. There is evidence that this compound may act directly on Ca^{2+}-ATPases (Campbell & MacLennan, 1980; Niggli et al., 1982; Waisman et al., 1982; Kimura et al., 1988). Recently, Pedemonte and Kaplan (1988) showed that H_2DIDS inhibits purified renal $(Na^+ + K^+)$ATPase.

The present experiments were conducted to investigate whether this compound inhibits active Ca^{2+} transport across renal membranes and whether this is achieved by a direct effect on the $(Ca^{2+}+Mg^{2+})$ATPase.

Materials and Methods

Materials. DIDS, ATP, ADP, P-enolpyruvate, ouabain, pyruvate kinase, A23187, and L-phosphatidylcholine were purchased from Sigma Chemical Co., Percoll was from Pharmacia, and other chemical reagents were of the highest purity available. ^{45}Ca was from New England Nuclear, and $[^{32}P]P_i$ was from the Brazilian Institute of Atomic Energy.

All solutions were prepared with deionized glass-distilled water. Concentrations of free and complexed species (Ca^{2+}, Mg^{2+}, ATP^{4-}, and $MgATP^{2-}$) at equilibrium were calculated by using an iterative computer program that was modified (Inesi et al., 1980; Sorenson et al., 1986) from that described by Fabiato and Fabiato (1979). $[^{32}P]P_i$ was purified as the phosphomolybdate complex with a mixture of benzene and isobutyl alcohol (Boyer & Bryan, 1967). $[\gamma\text{-}^{32}P]$ATP was prepared as described by Glynn and Chappell (1964).

Preparation of Purified Basolateral Membrane Vesicles. Basolateral membranes from sheep kidney proximal tubule cells were isolated from renal cortex by the Percoll gradient method (Grassl & Aronson, 1986) modified from that described by Scalera et al. (1980) and Sacktor et al. (1981). The final membranous preparation was resuspended in 250 mM sucrose at a concentration of 6–14 mg of protein/mL. The specific activity of the basolateral membrane $(Na^+ + K^+)$ATPase was enriched by 10–12-fold when compared to the initial homogenate.

Solubilization of Renal $(Ca^{2+}+Mg^{2+})$ATPase with Triton X-100. Solubilization of the enzyme was carried out for 20 min in an ice-cold solution containing 5 mg/mL vesicle protein, 10 mg/mL Triton X-100, 20% (v/v) glycerol, 12 mM Tris-HCl buffer (pH 8.5), and 250 mM sucrose. After solubilization, the samples were centrifuged at $106000g_{max}$ for 25 min. The supernatant was diluted to a protein concentration of 0.6 mg/mL in 250 mM sucrose and used immediately.

Measurement of ATPase Activity, Ca^{2+} Uptake, and ATP $\rightleftharpoons$ $[^{32}P]P_i$ Exchange. In all experiments, DIDS (concentrations shown in the figure legends) was mixed with the ligands and then with the protein, and the reactions were started 20 min later by adding ATP (*reversible* inhibition).[2] Incubation of vesicles or of soluble protein with DIDS in the presence of ligands for shorter or longer intervals (from 0 to 40 min) did not modify the degree of inhibition, indicating that the enzyme was not inactivated during preincubation. The temperature of the assays was 37 °C. All experiments (number in the figure legends) were performed in duplicate.

The standard assay medium (0.8 mL) for $(Ca^{2+}+Mg^{2+})$-ATPase contained 0.15 mg/mL solubilized enzyme plus 1 mg/mL L-phosphatidylcholine and 30 mM Tris-HCl (pH 7.0), 160 mM sucrose, 5 mM $MgCl_2$, 5 mM $[\gamma\text{-}^{32}P]$ATP (0.45 μCi/μmol), 0.2 mM EGTA, 1 mM ouabain, and 10 mM NaN_3. Enough $CaCl_2$ was added to give the ionized Ca^{2+} concentrations indicated in the figure legends. The reaction was started with $[\gamma\text{-}^{32}P]$ATP and stopped after 20 min by addition of 2 mL of HCl (0.1 N), and the $[\gamma\text{-}^{32}P]$ATP not hydrolyzed was removed with charcoal (Grubmeyer & Penefsky, 1981). The $[^{32}P]P_i$ released was measured in an aliquot of the supernatant obtained after centrifugation of the cold charcoal suspension in a clinical centrifuge for 20 min. Spontaneous hydrolysis of $[\gamma\text{-}^{32}P]$ATP was measured in tubes run in parallel in which the enzyme was added after the acid. The $(Ca^{2+}+Mg^{2+})$ATPase activity was the difference between the ATP hydrolysis measured in the presence and in the absence of $CaCl_2$ (EGTA, 1 mM).

Calcium uptake experiments were performed by using Millipore filtration with 0.45-μm pore size filters. The ^{45}Ca remaining in the vesicles after being washed with a 2 mM $La(NO_3)_3$ solution was counted in a liquid scintillation counter. Except when otherwise noted, the composition of the incubation medium was 30 mM Tris-HCl (pH 7.0), 160 mM sucrose, 5 mM $MgCl_2$, 5 mM ATP, 5 mM P-enolpyruvate, 0.2 mM EGTA, 0.391 mM $^{45}CaCl_2$ (0.05 ionized Ca^{2+}), 1 mM ouabain, and 10 mM NaN_3. It also contained 0.05 mg/mL pyruvate kinase and 0.2 mg/mL native vesicles. The blank values, corresponding to nonspecific binding of Ca^{2+} to the vesicles, were measured in tubes run in parallel in the absence of ATP. For Ca^{2+} efflux experiments, vesicles were preloaded with ^{45}Ca for 5 h at 37 °C in the same medium as that used for Ca^{2+} uptake, and then 2 mM EGTA, 2 mM EGTA plus 100 μM DIDS, or 20 μM ionophore A23187 was added.

ATP $\rightleftharpoons$ $[^{32}P]P_i$ exchange was assayed by measuring formation of $[\gamma\text{-}^{32}P]$ATP from ADP and $[^{32}P]P_i$ using solubilized ATPase. Assays (1 mL) contained 30 mM Tris-HCl (pH 7.0), 160 mM sucrose, 10 mM $MgCl_2$, 1 mM ATP, 0.2 mM ADP, 4 mM $[^{32}P]P_i$, 1 mM $CaCl_2$ (0.85 mM ionized Ca^{2+}), 0.1 mM ouabain, 10 mM NaN_3, and 0.15 mg/mL solubilized enzyme plus 1 mg/mL L-phosphatidylcholine, and were stopped with

[1] Abbreviations: DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; NaN_3, sodium azide; P-enolpyruvate, phospho(enol)pyruvate.

[2] Reversible inhibition was that measured in the presence of all ligands of the enzyme, as defined previously for $(Na^+ + K^+)$ATPase (Pedemonte & Kaplan, 1988).

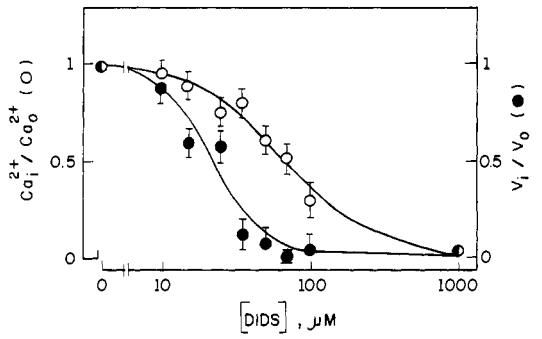

FIGURE 2: Reversible inhibition by DIDS of Ca^{2+} uptake and (Ca^{2+}+Mg^{2+})ATPase activity. Ca^{2+} uptake (○): The membrane vesicles were incubated for 4 h in the assay media described under Materials and Methods plus 50 μM ionized Ca^{2+}, in the presence of the concentrations of DIDS shown on the abscissa. The equation of the line that was adjusted to the experimental points is described in the text (eq 1). $[Ca^{2+}_i]$ and $[Ca^{2+}_0]$ are the steady-state levels of intravesicular Ca^{2+} in the presence and in the absence of the inhibitor, respectively. The steady-state level of Ca^{2+} accumulated in the absence of DIDS ($[Ca^{2+}_0]$) was 6.5 ± 1.5 nmol mg^{-1}. Data are means ± SE of four experiments with different preparations. (Ca^{2+}+Mg^{2+})ATPase (●): The hydrolytic activity of solubilized enzyme was measured as indicated under Materials and Methods (50 μM ionized Ca^{2+}) in the presence of DIDS concentrations shown on the abscissa. The line adjusted to the experimental points was fit by nonlinear regression using eq 2 (see text). The (Ca^{2+}+Mg^{2+})ATPase activity in the absence of DIDS (v_0) was 7.7 ± 0.6 nmol mg^{-1} min^{-1}. Data are means ± SE of six experiments with different preparations.

0.5 mL of 20% trichloroacetic acid. After centrifugation at 4 °C at 4000*g* for 20 min, an aliquot of the supernatant was used for measurement of [γ-^{32}P]ATP formed, after extraction of the excess [^{32}P]P_i as the ammonium phosphomolybdate complex with a mixture of benzene and isobutyl alcohol (de Meis & Carvalho, 1974). Blanks in which the enzyme was added after the acid were processed identically and subtracted from the total counts in the aqueous phase of the last extraction. Ca^{2+}-dependent ATP ⇌ [^{32}P]P_i exchange was the difference between ATP synthesis measured in the presence and in the absence of $CaCl_2$ (1 mM EGTA; less than 20% of the values obtained with Ca^{2+}).

Analysis of the Data. Kinetic parameters were derived from the experimental data by using the statistical curve-fitting package Enzfitter (Elsevier-Biosoft, Cambridge, U.K.) and are means of those obtained by fitting the data of each experiment. Differences were evaluated for statistical significance by using Student's *t* test for paired or unpaired data, as required.

Results

Effects of DIDS on Ca^{2+} Transport and (Ca^{2+}+Mg^{2+})-ATPase Activity. At micromolar concentrations, DIDS inhibited calcium transport in native vesicles and the hydrolytic activity of solubilized (Ca^{2+}+Mg^{2+})ATPase. Figure 2 (open circles) shows the dose–response curve of DIDS on steady-state Ca^{2+} accumulation by the vesicles. The function that was fit to the experimental data is

$$[Ca^{2+}_i]/[Ca^{2+}_0] = K_i/(K_i + [DIDS]^n) \quad (1)$$

in which $[Ca^{2+}_i]$ and $[Ca^{2+}_0]$ are the steady-state levels of Ca^{2+} accumulated in the presence and absence of DIDS, respectively, *n* is an index of cooperativity for the inhibitory effects of DIDS (1.4 ± 0.1), and $I_{50} = K_i^{1/n}$ is the concentration of DIDS that gives half-maximal inhibition of Ca^{2+} uptake (62.9 ± 3.9 μM).

The addition of 100 μM DIDS, a concentration that inhibited Ca^{2+} accumulation by 70%, did not affect the rate constant of EGTA-induced Ca^{2+} efflux from vesicles preloaded with Ca^{2+} for 5 h in the experimental conditions described in the legend of Figure 2. This Ca^{2+}, however, can be rapidly and completely released from the vesicular lumen by 20 μM A23187. Upon addition of 2 mM EGTA, the efflux of Ca^{2+} could be described by the sum of two exponential functions: $[Ca^{2+}_t] = [Ca^{2+}_{0(fast)}] \exp(-k_1 t) + [Ca^{2+}_{0(slow)}] \exp(-k_2 t)$. The rate constant of the faster component could not be resolved with the technique employed. The slower component of EGTA-induced Ca^{2+} efflux (k_2 = 0.011 min^{-1}) was not altered by adding 100 μM DIDS together with the EGTA (k_2 = 0.012 min^{-1}).

Stilbene derivatives with isothiocyanate groups are also able to inactivate (Na^++K^+)ATPase, i.e., to promote irreversible inhibition (Pedemonte & Kaplan, 1988). To establish whether or not (Ca^{2+}+Mg^{2+})ATPase is inactivated when incubated with DIDS in the presence of ligands, the following experiment was performed. The vesicles (2 mg/mL protein) were preincubated for 20 min at room temperature in the medium described in the legend to Figure 2, in the absence or in the presence of 200 μM DIDS, a concentration that almost abolished Ca^{2+} transport. Then, alliquots were diluted 20-fold in the same solution with no DIDS, and including 1 mg/mL bovine serum albumin. After addition of 5 mM ATP, Ca^{2+} uptake was measured as indicated under Materials and Methods. The pumping activity of treated and untreated vesicles was identical within experimental error, showing that the enzyme is not inactivated by DIDS in the presence of its ligands.

In order to determine whether the inhibitory effect of DIDS on Ca^{2+} transport was caused by a direct interaction with the Ca^{2+} pump, the rate of (Ca^{2+}+Mg^{2+})ATPase activity was measured at different DIDS concentrations. As is the case for Ca^{2+} uptake, (Ca^{2+}+Mg^{2+})ATPase activity of the soluble enzyme was inhibited by DIDS (Figure 2, filled circles). However, the hydrolytic activity appears to be more sensitive than pumping activity, and the cooperativity of the inhibition is also more pronounced with the Triton X-100 treated enzyme (I_{50} = 19.9 ± 2.3 μM, $p < 0.001$; n = 2.3 ± 0.3, $p < 0.02$, with respect to the values for Ca^{2+} uptake). The values for these parameters were determined by the equation:

$$v_i/v_0 = K_i/(K_i + [DIDS]^n) \quad (2)$$

where v_i and v_0 are the velocities of ATP hydrolysis in the presence and in the absence of DIDS, respectively. The inhibition of soluble (Ca^{2+}+Mg^{2+})ATPase activity by DIDS strongly suggests that the mechanism by which this compound inhibits Ca^{2+} accumulation in the vesicles may be ascribed to a direct effect on the Ca^{2+} pump rather than to a primary blockade of anion exchange with subsequent impairment of Ca^{2+} transport.

Dependence of DIDS Inhibition on Free Ca^{2+} Concentration. The effects of H_2DIDS on the (Na^++K^+)ATPase were shown to be modified by the binding of specific ligands (Pedemonte & Kaplan, 1988). Since DIDS had a direct effect on the (Ca^{2+}+Mg^{2+})ATPase, it was also of interest to see whether the physiological ligands of the Ca^{2+} pump affected the inhibition of the enzyme by DIDS. In a previous study (Vieyra et al., 1986), the Ca^{2+} concentration dependent of a solubilized preparation of rabbit renal (Ca^{2+}+Mg^{2+})ATPase revealed the existence of two Ca^{2+} binding sites: one stimulatory, of high affinity (K_m = 0.6 μM), that is probably located at the cytosolic surface of the membrane, and another with low affinity for Ca^{2+}, probably facing the peritubular space. The progressive occupancy of this second site leads to inhibition of the (Ca^{2+}+Mg^{2+})ATPase activity (Vieyra et al., 1986, 1989). In

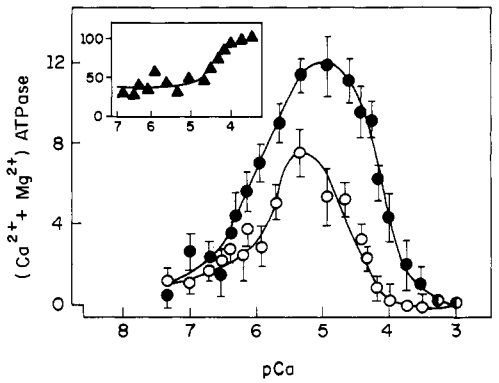

FIGURE 3: Effects of DIDS on the Ca^{2+} concentration dependence of $(Ca^{2+}+Mg^{2+})$ATPase activity. The hydrolytic activity of the solubilized enzyme was measured in the absence (●) or in the presence of 25 μM DIDS (○) in the assay conditions described under Materials and Methods. $CaCl_2$ was added to give free Ca^{2+} concentrations between 0.05 μM and 1 mM. The lines through the data points (means ± SE of four determinations with different preparations) were drawn by eye. Units on the ordinate are nanomoles per milligram per minute. Inset: Percentage of inhibition calculated from the data shown in the principal panel. The line was fit by nonlinear regression to the points according to the empirical equation: $(1 - v_i/v_0)100 = A + (100 - A)[Ca^{2+}]^n/(K + [Ca^{2+}]^n)$. In this equation, A (39.6) is the constant percentage of inhibition at the high-affinity free Ca^{2+} concentration range (up to 5 μM), n (1.6) is a cooperative index, and K is the free Ca^{2+} concentration (46.3 μM) at which inhibition by 25 μM DIDS is half-maximal.

the next experiments, we examined the effects of DIDS on $(Ca^{2+}+Mg^{2+})$ATPase over a Ca^{2+} concentration range that allows occupancy of one or both Ca^{2+} binding sites (Figure 3). It can be seen that hydrolytic activity was more affected by DIDS as the Ca^{2+} concentration increased to the low-affinity range (inset to Figure 3).

The kinetic parameters for $(Ca^{2+}+Mg^{2+})$ATPase in the high-affinity Ca^{2+} range were calculated by using the equation:

$$v = V_{max}[Ca^{2+}]^n/(K_m + [Ca^{2+}]^n) \quad (3)$$

The data obtained for Ca^{2+} concentrations in the low-affinity inhibitory range (above 10 μM) were fit with the equation:

$$v = v_0K_i/(K_i + [Ca^{2+}]^n) \quad (4)$$

in which v_0 corresponds to the V_{max} of the high-affinity and stimulatory component, and the Ca^{2+} concentration that produced half-maximal inhibition (I_{50}) was calculated by $K_i^{1/n}$.

The kinetic parameters for the high- and low-affinity components are summarized in Table I. It can be seen that neither the K_m for Ca^{2+} activation nor the n_1 value was modified upon addition of 25 μM DIDS, although V_{max} was decreased by 50%. On the other hand, the Ca^{2+} affinity of the low-affinity component was increased by DIDS: the Ca^{2+} concentration that caused half-maximal inhibition of the $(Ca^{2+}+Mg^{2+})$ATPase activity in the presence of DIDS was less than half of that required in its absence (Table I).

Dependence on Free ATP Concentration of the Inhibition by DIDS of $(Ca^{2+}+Mg^{2+})$ATPase Activity. There is evidence that stilbene derivatives interact with $(Na^+ + K^+)$ATPase at the nucleotide binding domain (Pedemonte & Kaplan, 1988, 1990). Therefore, it was of interest to study the influence of ATP on the reversible inhibition by DIDS of $(Ca^{2+}+Mg^{2+})$ATPase activity.

In Table II, it is shown that increasing concentrations of free ATP abolished the inhibition promoted by 15 μM DIDS in a medium in which the concentration of the $MgATP^{2-}$ complex was maintained in the range 0.71–0.99 mM, and the free Mg^{2+} concentration was less than the $K_{0.5}$ for activation (see below) throughout the range of free ATP concentrations studied. Reversible inhibition promoted by DIDS was counteracted by increasing ATP concentration along a Michaelis–Menten-like curve (not shown) with $K_{0.5}$ = 1.1 mM. This effect is not due to an interaction of DIDS with ATP, since there was no modification in the absorption of DIDS at 340 nm with ATP concentrations varying between 0 and 10 mM (data not shown).[3]

Table I: Effects of DIDS on the Ionized Ca^{2+} Concentration Dependence of $(Ca^{2+}+Mg^{2+})$ATPase Activity[a]

	control	+25 μM DIDS[b]
high-affinity component[c]		
K_m (μM)	0.9 ± 0.1	0.8 ± 0.2 (NS)
V_{max} (nmol mg^{-1} min^{-1})	12.2 ± 1.4	6.4 ± 1.1 ($p < 0.001$)
n_1	1.3 ± 0.4	1.1 ± 0.2 (NS)
low-affinity component[c]		
I_{50} (μM)	91.0 ± 21.0	40.6 ± 4.8 ($p < 0.001$)
n_2	2.7 ± 0.6	2.7 ± 0.4 (NS)

[a] $(Ca^{2+}+Mg^{2+})$ATPase assays were performed as indicated under Materials and Methods. The kinetic parameters (K_m, V_{max}, n_1, I_{50}, and n_2) for the best fit of the ionized Ca^{2+} concentration dependence were calculated by eq 3 and 4. For definition of these parameters, see the text. Values are means ± SE of four experiments. [b] Statistical differences between control and DIDS-treated groups were ascertained as indicated under Materials and Methods; NS, not significant ($p > 0.05$). [c] High-affinity component and low-affinity component correspond to the stimulatory and inhibitory phases of the curves shown in Figure 3.

Table II: Free ATP Concentration Effect on the Inhibition of $(Ca^{2+}+Mg^{2+})$ATPase Activity by DIDS[a]

	$(Ca^{2+}+Mg^{2+})$ATPase activity (nmol mg^{-1} min^{-1})	
free [ATP][b] (mM)	control	+15 μM DIDS
0.3	3.5 ± 0.4	1.0 ± 0.7 (71)[c]
1.4	6.4 ± 0.6	3.7 ± 0.7 (42)
3.5	6.1 ± 0.5	5.8 ± 1.5 (5)
7.9	8.5 ± 1.4	9.7 ± 1.9 (ni)[d]

[a] $(Ca^{2+}+Mg^{2+})$ATPase assays were performed as indicated under Materials and Methods and under Results. Values shown are mean ± SE of four experiments. [b] Enough ATP was added to give the free ATP concentrations shown, calculated as described under Materials and Methods. [c] Percent with respect to the control. [d] No inhibition was found.

Effect of DIDS on Stimulation of the $(Ca^{2+}+Mg^{2+})$ATPase by Free Mg^{2+}. It has been shown that free Mg^{2+} is a requirement for the activation of $(Ca^{2+}+Mg^{2+})$ATPases, including the renal Ca^{2+} pump (Palmer & Posey, 1970; Moore et al., 1974; Garrahan et al., 1976; Souza & de Meis, 1976; Adamo et al., 1990; Zhang & Kraus-Friedmann, 1990). For the erythrocyte $(Ca^{2+}+Mg^{2+})$ATPase, it has been proposed that free Mg^{2+} can accelerate the $E_2 \rightarrow E_1$ transition (Adamo et al., 1990) as well as ATP does (Adamo et al., 1988). Thus, in the following experiments, we examined the effects of DIDS on the response of $(Ca^{2+}+Mg^{2+})$ATPase to increasing free Mg^{2+} concentrations.

In the presence of a fixed $MgATP^{2-}$ concentration (100 μM), the $(Ca^{2+}+Mg^{2+})$ATPase activity was stimulated by increasing concentrations of free Mg^{2+} following a Michaelis–Menten-like curve (Figure 4):

$$v = v_0 + V_{max}[Mg^{2+}]/(K_{0.5} + [Mg^{2+}]) \quad (5)$$

where v_0 represents the velocity extrapolated to zero free Mg^{2+}.

When 15 μM DIDS was added, the V_{max} of the component activated by free Mg^{2+} decreased from 4.0 to 1.4 nmol mg^{-1}

[3] The absorption of DIDS at its maximum (205 nm) was not recorded in the presence of ATP due to absorption by the nucleotide at this wavelength.

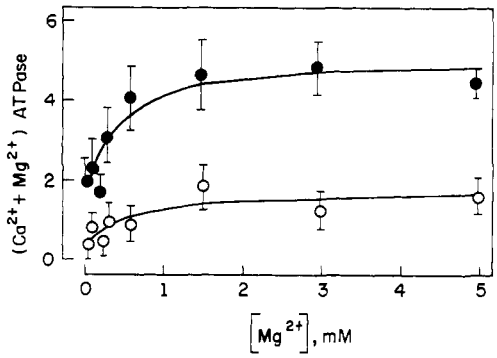

FIGURE 4: Effect of DIDS on the activation by free Mg^{2+} of $(Ca^{2+}+Mg^{2+})$ATPase activity. The assay media were those described under Materials and Methods with ionized $[Mg^{2+}]$ varying as shown on the abscissa. The concentrations of free Ca^{2+} and $MgATP^{2-}$ were kept fixed at 12 and 100 μM, respectively. The data (means ± SE of five experiments) represent the ATPase activity in nanomoles per milligram per minute measured in the absence (●) or in the presence of 15 μM DIDS (○). Equation 5 (see text) was fit to the points by nonlinear regression to obtain the smooth curves.

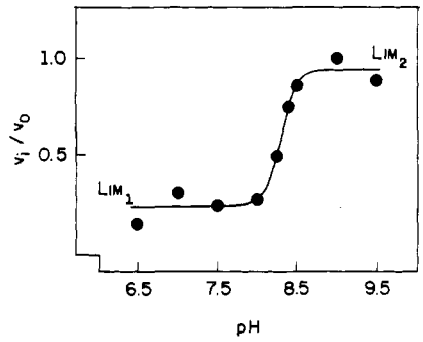

FIGURE 5: pH dependence of the reversible inhibition by DIDS of $(Ca^{2+}+Mg^{2+})$ATPase activity. Assay for ATPase activity was as indicated in the legend of Figure 2, except that buffer was Mes–Tris (40 mM), adjusted to pH values between 6.5 and 9.5 with HCl or Tris base. Different concentrations of $CaCl_2$ were used to give a constant concentration of ionized Ca^{2+} (12 μM) at each pH value. Each value represents the ratio between the $(Ca^{2+}+Mg^{2+})$ATPase activity measured in the absence and that measured in the presence of 25 μM DIDS. The continuous line is the fit by nonlinear regression of eq 6 to the points, with an n value of 4.3, upper and lower asymptotes of 0.24 and 0.93, and pK' = 8.3. Values are means of two experiments with different preparations.

min^{-1}, whereas the $K_{0.5}$ for Mg^{2+} activation remained essentially the same (367 and 467 μM, in the absence and in the presence of DIDS, respectively).

Effect of pH on the Inhibition of $(Ca^{2+}+Mg^{2+})$ATPase Activity by DIDS. The experiments of Figure 5 were performed to investigate whether the dissociation of side-chain groups of the enzyme influences the inhibition by DIDS. The experimental points were fit by the equation:

$$v_i/v_0 = [\mathrm{lim}_1 + \mathrm{lim}_2(10^{\mathrm{pH}-\mathrm{p}K'})^n]/[(10^{\mathrm{pH}-\mathrm{p}K'})^n + 1] \quad (6)$$

in which v_i and v_0 are the velocities in the presence of 25 μM DIDS and in its absence at each pH value, respectively, lim_1 and lim_2 represent the lower and the upper asymptotic values of the v_i/v_0 ratio, n is a cooperativity index, and pK' is the medium pH at which the influence of 25 μM DIDS was changed by 50%. It can be observed that alkalinization of the medium protects the enzyme from DIDS inhibition. This effect occurred within a narrow pH interval, and from the calculated pK' value of 8.3, it may be concluded that protonated residues whose side-chain groups half-dissociate at this pH value are involved in the reversible binding of DIDS to the enzyme.

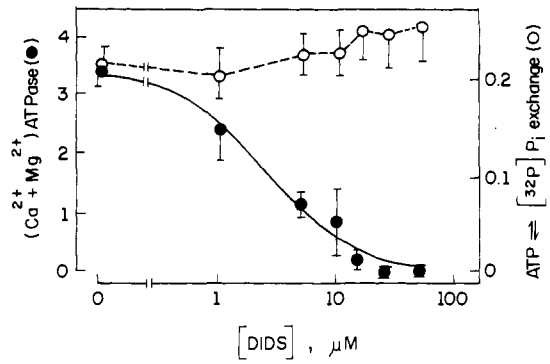

FIGURE 6: Effects of DIDS on ATP hydrolysis and ATP synthesis catalyzed simultaneously by $(Ca^{2+}+Mg^{2+})$ATPase in the absence of a Ca^{2+} gradient. Assays were performed as indicated under Materials and Methods for ATPase activity (●) and ATP ⇌ $[^{32}P]P_i$ exchange (○). Triton X-100 solubilized $(Ca^{2+}+Mg^{2+})$ATPase, 0.2 mM ADP, and 1 mM $CaCl_2$ (850 μM ionized Ca^{2+}) were used in both series of experiments. Units on both ordinates are nanomoles per milligram per minute. The continuous line is the fit by nonlinear regression of eq 2 to the points. The dashed line was drawn by hand. Data are means ± SE of two experiments.

Effects of DIDS on the ATP ⇌ $[^{32}P]P_i$ Exchange Reaction. The $(Ca^{2+}+Mg^{2+})$ATPase of the basolateral membrane from proximal tubule can catalyze simultaneous cycles of ATP hydrolysis and ATP synthesis (Vieyra et al., 1989). To determine whether or not DIDS affects both ATP hydrolysis and ATP synthesis, the effects of this compound on the Ca^{2+}-dependent ATP ⇌ $[^{32}P]P_i$ exchange were compared with those on ATP hydrolysis catalyzed by the soluble enzyme in the same experimental conditions. The results of Figure 6 show that ATP synthesis is insensitive to inhibition by DIDS. In a medium containing 25 μM DIDS, $(Ca^{2+}+Mg^{2+})$ATPase activity was completely inhibited whereas the Ca^{2+}-dependent ATP ⇌ $[^{32}P]P_i$ exchange remained unmodified up to 100 μM drug. Interestingly, with the high ionized Ca^{2+} concentration used (850 μM), needed to activate the exchange in the absence of a Ca^{2+} gradient (Knowles & Racker, 1975; de Meis & Tume, 1977; Ratkje & Shamoo, 1980), and with a simultaneous very low free ATP concentration (12 μM), the sensitivity to DIDS inhibition of ATP hydrolysis was greater (I_{50} = 2.5 ± 0.8 μM) than with 50 μM ionized Ca^{2+} and 600 μM free ATP (I_{50} = 19.9 μM; see Figure 2, filled circles).

Discussion

The negatively charged amino-reactive reagent DIDS and its dihydro derivative H_2DIDS have been widely used in the study of both anion exchange (Zaki et al., 1975; Lepke et al., 1976; Cabantchik et al., 1978; Kasai & Taguchi, 1981) and the relationship between anion fluxes and those of other ionic species, including Ca^{2+} (Campbell & MacLennan, 1980; Waisman et al., 1981, 1982; Romero & Ortiz, 1988). However, these compounds appear to operate in different systems in a way that is not directly related to anion translocation (Campbell & MacLennan, 1980; Niggli et al., 1982; Waisman et al., 1982; Kimura et al., 1988; Pedemonte & Kaplan, 1988). This work shows that DIDS promotes reversible inhibition of the $(Ca^{2+}+Mg^{2+})$ATPase from plasma membrane of kidney proximal tubules when it is added to the enzyme-containing solution in the presence of Ca^{2+} and Mg^{2+}, the reaction being initiated by the addition of ATP.

With intact vesicles (Figure 2), the interactions of DIDS with the Ca^{2+} pump probably occurs at the cytosolic (extravesicular) aspect of the membrane. Previous studies have shown that the inhibitory effects of DIDS on the $(Ca^{2+}+Mg^{2+})$ATPase activity of red cells are only observed if the

cytosolic face of the membrane is exposed to the compound (Waisman et al., 1982; Romero & Ortiz, 1988).

Kimura et al. (1988) have found that DIDS inhibits (Ca^{2+}+Mg^{2+})ATPase and also promotes Ca^{2+} release from hepatic microsomes preloaded with Ca^{2+}. With purified vesicles derived from basolateral membranes from kidney proximal tubules, there was no effect of DIDS on Ca^{2+} permeability, and, therefore, inhibition of ATP-dependent Ca^{2+} uptake in this preparation (Figure 2, open circles) can be ascribed to an effect on the anion transporter present in this membrane (Talor et al., 1987) and/or to a direct influence on (Ca^{2+}+Mg^{2+})ATPase. Inhibition of anion transport might inhibit Ca^{2+} uptake if it decreased the internal concentration of H^+, H^+ being required for export in exchange for Ca^{2+} (Tsukamoto et al., 1988). Although this possibility was not explored in the presence series of experiments, it is clear (Figure 2, filled circles) that DIDS inhibits (Ca^{2+}+Mg^{2+})-ATPase activity of the solubilized enzyme, a preparation that does not support ionic gradients. It is also more potent as an inhibitor of hydrolysis than as an inhibitor of Ca^{2+} uptake, which does not favor the hypothesis that there might be summing of two effects in the latter case.

The reversible inhibition of Ca^{2+} uptake by intact vesicles showed cooperativity (n = 1.4), suggesting that more than one molecule of DIDS is involved at several enzyme sites. DIDS' inhibition of the (Ca^{2+}+Mg^{2+})ATPase activity catalyzed by soluble enzyme appears to be a more cooperative phenomenon (n = 2.3; $p < 0.02$ compared to data for Ca^{2+} uptake). This value, together with a lower I_{50} for inhibition of the activity of solubilized enzyme (compare open and filled circles in Figure 2), indicates that solubilization, with partial removal of the natural lipid environment, may alter the ATPase preparation in a manner that increases its interactions with DIDS. It may be that these sites are in a protein domain near the phospholipid moiety of the membrane. It has been shown that inhibition by DIDS of reconstituted (Ca^{2+}+Mg^{2+})ATPase from red cells depends on the amount of liposomes added (Niggli et al., 1982).

In Figure 3 and Table I, it can be seen that DIDS modifies the interaction of the enzyme with Ca^{2+}. At the level of the high-affinity component, DIDS acts as a noncompetitive inhibitor, as in the case of reversible inhibition of (Na^++K^+)-ATPase by H_2DIDS (Pedemonte & Kaplan, 1988). In contrast, when the low-affinity, inhibitory Ca^{2+} component was studied (Figure 3), DIDS promoted a significant decrease in the I_{50} for Ca^{2+} (Table I). Since it is accepted that (Ca^{2+}+-Mg^{2+})ATPases in the E_2 conformation have Ca^{2+} sites in a low-affinity state (Tanford, 1983, 1984; Stahl & Jencks, 1987) the observation of Table I may indicate that DIDS binds to the enzyme in its E_2 conformation. This conclusion is reinforced by the data from Figure 6, where the ionized [Ca^{2+}] is 850 μM and free [ATP] is 12 μM, and the affinity of the enzyme for DIDS is 1 order of magnitude higher than in media containing 50 μM ionized Ca^{2+} and 600 μM free ATP (compare with Figure 2, filled circles).

In Table II, it can be seen that free ATP protects (Ca^{2+}+-Mg^{2+})ATPase activity against reversible inhibition by DIDS. The $K_{0.5}$ obtained (1.1 mM) is remarkably similar to the low-affinity K_m for ATP (0.7 mM) obtained for ATP hydrolysis using solubilized renal (Ca^{2+}+Mg^{2+})ATPase (Coelho-Sampaio et al., 1991). It was recently reported that H_2DIDS binds to purified renal (Na^++K^+)ATPase in a protein domain that contains the ATP binding site (Pedemonte & Kaplan, 1988, 1990) whose highly conserved amino acid sequence is also shared by (Ca^{2+}+Mg^{2+})ATPases (MacLennan et al., 1985; Serrano et al., 1986; Brandl et al., 1986; Filoteo et al., 1987; Xu, 1989). Interaction of the inhibitor with the nucleotide binding site, suggested by the data of Table II, is in line with the observation that radiolabeled H_2DIDS binds to one of the cytoplasmic loops of (Na^++K^+)ATPase (Pedemonte & Kaplan, 1990). It is of interest, however, that inactivation of (Na^++K^+)ATPase by H_2DIDS, but not its reversible inhibition, is affected by ATP (Pedemonte & Kaplan, 1988).

The results of Figure 4 confirm previous data showing that free Mg^{2+} stimulates renal (Ca^{2+}+Mg^{2+})ATPase (Vieyra et al., 1986). Recently, it has been proposed that ionized Mg^{2+} accelerates the $E_2 \rightarrow E_1$ transition of the (Ca^{2+}+Mg^{2+})ATPase from red cells, with a $K_{0.5}$ that is in the submillimolar range (Adamo et al., 1990). Since DIDS behaves as a noncompetitive inhibitor with respect to free Mg^{2+} (Figure 4), it may be that the latter binds to an enzyme domain different from that of DIDS, and perhaps different from that of ATP. There is evidence that velocities of partial reactions of plasma membrane (Ca^{2+}+Mg^{2+})ATPase are independently modified by ATP and Mg^{2+} (Adamo et al., 1988, 1990).

Reversible inhibition of (Ca^{2+}+Mg^{2+})ATPase by DIDS was also counteracted in a complex way when medium pH was raised (Figure 5). Although the amino acid residues involved in DIDS binding domains are not unequivocally identified (Pedemonte & Kaplan, 1990), the pK' value of 8.3 is in agreement with deprotonation, in a hydrophobic environment, of an ϵ-NH_2-lysine residue (Pedemonte & Kaplan, 1988) that can interact with the negatively charged DIDS. Moreover, the high cooperativity index for the effect of pH (n = 4.3; Figure 5) could be considered as indicative of juxtaposition of positively charged groups near the DIDS binding site. Several positively charged amino acids have been identified in the nucleotide binding domain of P-ATPases (MacLennan et al., 1985; Xu, 1989), and there is strong evidence concerning the hydrophobic characteristics of the phosphorylation site in the E_2 conformation (Chiesi et al., 1984; de Meis & Inesi, 1985; Pedersen & Carafoli, 1987; de Meis et al., 1988). Although there is a possibility that isothiocyano groups of DIDS can react with sulfhydryl groups of the enzyme, as proposed for the (Ca^{2+}+Mg^{2+})ATPase from red cells (Niggli et al., 1982), such an interaction would not promote irreversible inhibition in the experimental conditions of this study. As described above, the effects of DIDS were completely abolished upon dilution of the vesicles preincubation with the inhibitor in the experimental conditions of Figure 2.

Finally, the results of Figure 6 showing that the reversal of (Ca^{2+}+Mg^{2+})ATPase promoted by millimolar Ca^{2+} concentrations in the absence of a Ca^{2+} gradient is insensitive to DIDS inhibition, whereas forward cycles are strongly impaired, may mean that DIDS binds to a subconformation of E_2 which is not present in a significant amount when the pump moves in the backward direction. It may be that reversal occurs through a parallel route catalyzed by another subconformation of E_2 that would be insensitive to DIDS inhibition up to 100 μM. This form, after phosphorylation by medium [^{32}P]P_i, could lead to formation of [γ-^{32}P]ATP after binding of Ca^{2+} to the low-affinity Ca^{2+} binding site of the low-energy phosphoenzyme E_2–P (Tanford, 1983, 1984; Stahl & Jencks, 1987). It is noteworthy that H_2DIDS treatment of (Na^++K^+)ATPase did not affect phosphorylation by [^{32}P]P_i (Pedemonte & Kaplan, 1988).

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