A Novel Role of 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic Acid as an Activator of the Phosphatase Activity Catalyzed by Plasma Membrane Ca^{2+}-ATPase†

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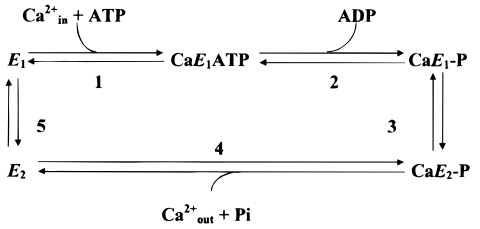
ABSTRACT: The hydrolysis of *p*-nitrophenyl phosphate catalyzed by the erythrocyte membrane Ca^{2+}-ATPase is stimulated by low concentrations of the compound 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), a classic inhibitor of anion transport. Enhancement of the phosphatase activity varies from 2- to 6-fold, depending on the Ca^{2+} and calmodulin concentrations used. Maximum stimulation of the pNPPase activity in ghosts is reached at 4−5 μM DIDS. Under the same conditions, but with ATP rather than pNPP as the substrate, the Ca^{2+}-ATPase activity is strongly inhibited. Activation of pNPP hydrolysis by DIDS is equally effective for both ghosts and purified enzyme, and therefore is independent of its effect as an anion transport inhibitor. Binding of the activator does not change the Ca^{2+} dependence of the pNPPase activity. Stimulation is partially additive to the activation of the pNPPase activity elicited by calmodulin and appears to involve a strong affinity binding or covalent binding to sulfhydryl groups of the enzyme, since activation is reversed by addition of dithiothreitol but not by washing. The degree of activation of pNPP hydrolysis is greater at alkaline pH values. DIDS decreases the apparent affinity of the enzyme for pNPP whether in the presence of Ca^{2+} alone or Ca^{2+} and calmodulin or in the absence of Ca^{2+} (with 5 μM DIDS the observed K_m shifts from 4.8 ± 1.4 to 10.1 ± 2.6, from 3.8 ± 0.4 to 7.0 ± 0.8, and from 9.3 ± 0.7 to 15.5 ± 1.1 mM, respectively). However, the pNPPase rate is always increased (as above, from 3.6 ± 0.6 to 11.2 ± 1.7, from 4.4 ± 0.5 to 11.4 ± 0.9, and from 2.6 ± 0.6 to 18.6 ± 3.9 nmol mg^{-1} min^{-1}, in the presence of Ca^{2+} alone or Ca^{2+} and calmodulin or in the absence of Ca^{2+}, respectively). ATP inhibits the pNPPase activity in the absence of Ca^{2+}, both in the presence and in the absence of DIDS. Therefore, kinetic evidence indicates that DIDS does more than shift the enzyme to the E_2 conformation. We propose that the transition from E_2 to E_1 is decreased and a new enzyme conformer, denoted E_2*, is accumulated in the presence of DIDS.

The Ca^{2+}-ATPase of the plasma membrane of erythrocytes catalyzes the ATP-energized outward Ca^{2+} transport that is responsible for maintenance of the low internal Ca^{2+} concentration necessary for cell survival. During the catalytic cycle, the enzyme alternates between two different conformations, E_1 and E_2 (*1*−*4*; see ref *5* for a recent review). The E_1 conformer (see Scheme 1) has a high affinity for Ca^{2+} and is readily phosphorylated by ATP (*4*), while E_2 has a low affinity for Ca^{2+} and can be phosphorylated by P_i (*6*, *7*). Addition of calmodulin (CaM)[1] increases the maximal velocity of ATP hydrolysis and the affinity of the enzyme for Ca^{2+} (*8*, *9*). The enzyme has a high specificity for the substrate to be hydrolyzed, since GTP, ITP, UTP, and CTP

Scheme 1: Simplified Catalytic Cycle for the Erythrocyte Ca^{2+}-ATPase



are ineffective substrates for the nucleotide triphosphatase and Ca^{2+}-transporting activities (*10*, *11*). However, under suitable conditions, the erythrocyte Ca^{2+}-ATPase can hydrolyze *p*-nitrophenyl phosphate at relatively high rates (*12*−*14*). Previous reports attributed the phosphatase activity exclusively to the E_2 conformer of the enzyme (*13*, *15*). The pNPPase activity is very low in an EGTA-containing medium, presumably because the absence of ligands favors the E_1 conformation (*16*). This activity is enhanced by addition of ATP in the presence of Ca^{2+} (*13*, *17*), by CaM (*12*, *18*), dimethyl sulfoxide (*15*), and phosphatidylserine (*19*−*21*), and by tryptic cleavage (*13*, *14*).

We describe here a new condition that increases the rate of pNPP hydrolysis: incubation of the enzyme with 4,4′-

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[1] Abbreviations: pNPP, *p*-nitrophenyl phosphate; PMCA, plasma membrane Ca^{2+}-ATPase; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; DTT, dithiothreitol; CaM, calmodulin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS). This compound is a potent inhibitor of anion transporters in different cell systems (*22*−*25*), and has been used to study the mechanism of toxicity by thiols and disulfides, two major environmental pollutants (*26*). DIDS has also been shown to inhibit ATP hydrolysis by plasma membrane Ca^{2+}-ATPases (*27*−*31*). Due to its effect on the anion transport, it is not clear whether this inhibition could be attributed to a direct effect of DIDS on the enzyme or to changes in electric charge compensation necessary for a continued Ca^{2+} transport (*28*, *30*, *32*). We show here that DIDS binds directly and irreversibly to the enzyme and causes a concomitant increase in the pNPPase activity. It is suggested that preincubation with DIDS drives the equilibrium toward an enzyme form denominated E_2*.

EXPERIMENTAL PROCEDURES

Preparation of Erythrocyte Membrane Ghosts. Pig erythrocyte ghosts were prepared from fresh blood according to ref *33*. The protein concentration was estimated by the method of Lowry et al. (*34*).

Purification of the Ca^{2+}-ATPase. The Ca^{2+}-ATPase was purified from red cell membrane ghosts by solubilization with polydocanol and affinity chromatography through a CaM−Sepharose 4B column, according to the method of Caroni et al. (*35*) as modified by Pasa et al. (*36*). On SDS−PAGE (*37*), silver-staining (*38*) of this preparation reveals a single band of 135−145 kDa.

ATPase Activity. Erythrocyte membrane Ca^{2+}-ATPase activity was measured at 37 °C in assay medium containing 120 mM KCl, 5.5 mM $MgCl_2$, 30 mM Tris-HCl (pH 7.4), 1 mM ouabain, 1 mM EGTA, 1 μM free Ca^{2+}, and 200 μg/mL membrane protein, in the presence or absence of 2 μg/mL CaM, and varying the concentrations of DIDS. The reactions were started with the addition of 2 mM [γ-^{32}P]-ATP (final concentration) and quenched after 30 min with 1 volume of activated charcoal suspended in 0.1 N HCl. The [^{32}P]P_i released to the supernatant was quantified by the method of Grubmeyer and Penefsky (*39*), as described by Carvalho-Alves and Scofano (*40*). Stock solutions of DIDS (1 mM) were freshly prepared in water, assuming a molar extinction coefficient of 3.45×10^4 M^{-1} cm^{-1} at 285 nm.

pNPPase Activity. pNPP hydrolysis by the erythrocyte ghosts was measured in a medium similar to that used for measuring the ATPase, except that ATP was omitted and (unless otherwise stated) reactions were started by the addition of pNPP to a final concentration of 3 mM. When purified enzyme was used, ouabain was omitted. After 60 min at 37 °C, the reactions were quenched with 0.2 volume of 0.3 N NaOH, followed by 2.8 volumes of deionized water. The samples were spun in a clinical or an Eppendorf centrifuge to clear off ghost debris, and the pNP in the supernatant was estimated spectrophotometrically at 425 nm, by use of an extinction coefficient of 1.1×10^4 M^{-1} cm^{-1}.

Ca^{2+} Dependence of the pNPPase Activity. The Ca^{2+} dependence of the pNPPase activity was measured in medium containing 30 mM Tris-HCl (pH 7.4), 5.5 mM $MgCl_2$, 120 mM KCl, 1 mM ouabain, 1 mM EGTA, different amounts of $CaCl_2$ to result in the various free Ca^{2+} concentrations, either in the presence or absence of 2 μg/mL CaM, and either 5 μM DIDS (ghosts) or 1 μM DIDS (purified enzyme). Free Ca^{2+} concentrations were calculated according to Fabiato and Fabiato (*41*), with the Ca-EGTA constants of Schwarzenbach et al. (*42*). The hydrolysis of pNPP was quantified as described above. The $K_{0.5}$ for activation of the pNPPase activity by Ca^{2+} was calculated with the nonlinear regression program Enzfitter (Elsevier Biosoft, London).

pH Dependence of pNPP Hydrolysis after Preincubation with DIDS. Ghosts were preincubated at room temperature for 30 min in medium (typically 1.4 mL) containing 30 mM Tris-HCl (pH 7.4), 120 mM KCl, 5.5 mM $MgCl_2$, 1 mM ouabain, 1 mM EGTA and no added $CaCl_2$, and 1 mg/mL ghost protein, with or without addition of 5 μM DIDS. The suspensions were centrifuged for 30 min at 10 000 rpm in an SS-34 rotor at 4 °C, and the supernatants were discarded. The resulting pellets were rinsed once and resuspended in water to a protein concentration of 2 mg/mL. These samples were aliquoted and activity was determined in medium containing 30 mM buffer at different pH values (6.0−8.0), 120 mM KCl, 5.5 $MgCl_2$, 1 mM EGTA, 1 mM ouabain, 0.2 mg/mL protein, and 3 mM pNPP. Hydrolysis was quenched after 60 min at 37 °C and the pNP produced was measured as above. The buffers used were MOPS−HCl (pH 6.0, 6.5, and 7.0) and Tris-HCl (pH 7.4 and 8.0). In another set of experiments, both the preincubation and the activity measurements were done at identical pH values.

Treatment of Ghosts with DTT after Pretreatment with DIDS. Erythrocyte ghosts were preincubated with or without 5 μM DIDS, pelleted, and resuspended as described above. However, in the last step the ghosts were diluted in medium containing 5 mM DTT, at pH 7.4, with 1 mM EGTA and no added $CaCl_2$. The hydrolysis of pNPP was assayed as above.

Treatment of Right-Side-Out Erythrocyte Ghosts with DIDS. Resealed right-side-out erythrocyte ghosts were prepared according to Richards and Eisner (*43*). These ghosts were labeled for 30 min with DIDS either before or after a freeze−thaw cycle, followed by centrifugation and resuspension in assay medium without DIDS. The preincubation medium contained 30 mM Tris-HCl (pH 7.4), 120 mM KCl, 5.5 mM $MgCl_2$, 1 mM ouabain, 0.2 mM EGTA, approximately 1 mg/mL membrane protein, and either none, 5 μM, or 10 μM DIDS. Samples of the resuspended ghosts were diluted 5-fold in medium supplemented either with 50 μM $CaCl_2$, 2 μg/mL CaM, and 2 mM ATP or with 3 mM pNPP in order to measure the ATPase and pNPPase activities.

Reagents. ATP (disodium salt), pNPP (dicyclohexylammonium salt), DTT, DIDS (free acid), EGTA, MOPS, Tris, ouabain, CaM, and CaM−Sepharose 4B were from Sigma. All other reagents used were of analytical grade.

RESULTS

Incubation of red cell membranes with increasing concentrations of DIDS leads to two opposed effects: the Ca^{2+}-dependent ATPase activity is impaired, while the pNPPase activity is gradually increased, in a mirror image curve up to 5 μM DIDS. This behavior is observed both for the CaM-stimulated activities (Figure 1A) and for the basal Ca^{2+}-dependent ones (data not shown). Higher concentrations of DIDS added to the medium progressively eliminated the

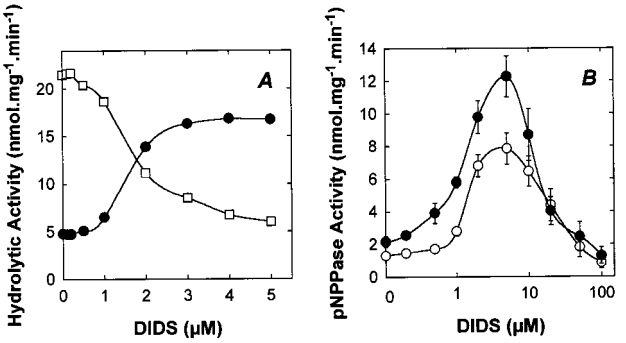


FIGURE 1: Comparison of the effects of DIDS on the Ca^{2+}-ATPase and pNPPase activities of erythrocyte ghosts. (A) Erythrocyte ghosts were incubated with increasing concentrations of DIDS and the activities were measured in the presence of 0.2 mg/mL ghosts protein, 30 mM Tris-HCl (pH 7.4), 5.5 mM $MgCl_2$, 120 mM KCl, 1 mM ouabain, 1 mM EGTA, 2 μg/mL CaM, and $CaCl_2$ to give 1 μM free Ca^{2+}, either with 2 mM ATP (□) or 3 mM pNPP (●) as substrate. (B) The pNPPase activity of ghosts was measured in the presence of increasing concentrations of DIDS in the absence (○) or presence (●) of CaM and with 1 μM free Ca^{2+} as in panel A. Values represent (A) a typical experiment and (B) mean ± SE (n = 5).

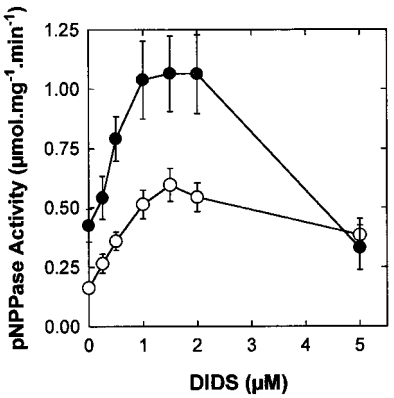


FIGURE 2: Effects of DIDS on the pNPPase activity of purified erythrocyte PMCA. The purified enzyme (2 μg/mL) was incubated with increasing concentrations of DIDS in medium with 30 mM Tris-HCl (pH 7.4), 5.5 mM $MgCl_2$, 120 mM KCl, 3 mM pNPP, 1 mM EGTA, and 1 μM free Ca^{2+} with either none (○) or 2 μg/mL CaM (●). Values are given as mean ± SE (n = 4).

stimulated pNPPase activity (Figure 1B), both in the presence and in the absence of CaM.

Evidence has been presented that the transport of Ca^{2+} by the red cell Ca^{2+} pump is electrogenic and depends either on the counter transport of H^+ through the pump (*30*) or on the cotransport of anions through specific transport proteins (*22*, *28*, *30*, *44*). To avoid any possible interference of anion transporters (or other ATPases present in ghosts) on the effects we observed, the experiments described above were repeated with a highly purified detergent-solubilized Ca^{2+}-ATPase, rid of other proteins according to silver staining on PAGE. The enhancement of the pNPPase activity is also obtained with the purified enzyme, although the activation is shifted to even lower concentrations (approximately 2 μM) of DIDS in the assay medium (Figure 2). This shift on the DIDS concentration dependence is probably due to the virtual elimination of other erythrocyte proteins that could bind DIDS, like the band III protein (*28*), and to a higher DIDS/phospholipid ratio, resulting in higher free stilbene concentrations. The same shift is obtained for inhibition of the

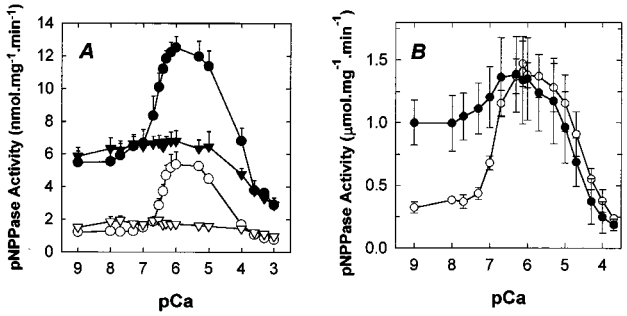


FIGURE 3: Ca^{2+} dependence of the pNPPase activity of erythrocyte Ca^{2+}-ATPase incubated with DIDS. (A) The pNPPase activity was assessed in medium similar to Figure 2 but with 0.2 mg/mL ghost protein, 1 mM ouabain, and different additions of $CaCl_2$ to result in the indicated free Ca^{2+} concentrations, either in the presence (solid symbols) or absence (open symbols) of 5 μM DIDS, and with (circles) or without (triangles) 2 μg/mL CaM. (B) pNPPase activity was assayed essentially as in panel A, but purified protein was used at a 2 μg/mL concentration, and ouabain was omitted. Curves (●, ○) were done in the presence of 2 μg/mL CaM, but only 1 μM DIDS (●) was used. Values represent mean ± SE in both panels (n = 3).

ATPase activity (not shown), consistently with the results described by Niggli et al. (*27*) that found that the K_i for DIDS is dependent on the amount of liposomes added to the reaction medium. Furthermore, when right-side-out erythrocyte ghosts were pretreated with 5 or 10 μM DIDS, conditions supposed to label specifically the band III protein, no effects of DIDS were observed on the Ca^{2+}-ATPase or pNPPase activities of the ghosts measured after membrane disruption (data not shown). This is indicative that the effects observed under our conditions are due neither to labeling of band III nor to labeling at the extracellular portions of the plasma membrane Ca^{2+} pump.

Addition of DIDS does not change the apparent Ca^{2+} dependence for pNPP hydrolysis in the presence of CaM (Figure 3). Hydrolysis of pNPP by the erythrocyte PMCA in the presence of CaM is stimulated by Ca^{2+} due to the binding of Ca^{2+} to this regulatory protein (*12*, *13*, *45*). Thereafter, the binding of the Ca^{2+}−CaM complex to the ATPase is responsible for the enhancement of the pNPPase activity (*13*, *45*, *46*). At higher Ca^{2+} concentrations, the pNPPase is inhibited due to the binding of Ca^{2+} directly to the Ca^{2+}-ATPase, leading to a Ca^{2+}-bound form unable to hydrolyze pNPP. A maximum of activity is observed at pCa 6 for the CaM-activated ghosts, both in the absence and in the presence of DIDS. In the presence of DIDS the maximal activity is increased more than 2-fold, showing that DIDS does not impair CaM binding and that their effects are cumulative. The $K_{0.5}$ for Ca^{2+} of the rising part of the curve in the absence of DIDS was found to be 0.25 μM, with a Hill coefficient of 1.4. The same parameters for the enzyme treated with 5 μM DIDS are 0.10 μM and 1.4, respectively. In both cases, higher concentrations of Ca^{2+} lead to inhibition of the enzyme. The $K_{0.5}$ for Ca^{2+} of the inhibitory component of the curve could not be calculated accurately due to uncertainties about the end of each curve. We observed essentially the same behavior when purified enzyme was assayed, since the apparent Ca^{2+} dependence of pNPP hydrolysis is maintained. Moreover, DIDS does not impair CaM binding, although in these experiments the same maximal activity was obtained both for activation by CaM

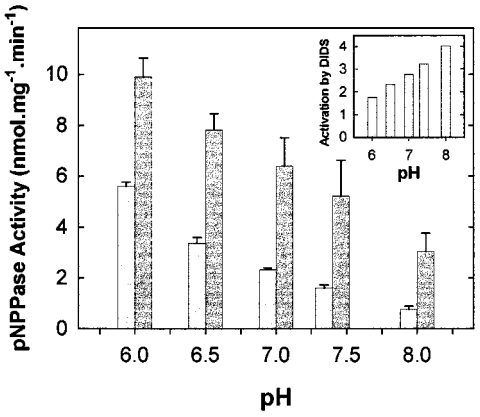


FIGURE 4: pH dependence of the pNPPase activity of ghosts preincubated with 5 μM DIDS at pH 7.4. After preincubation with (dark gray) or without (light gray) 5 μM DIDS at pH 7.4, ghosts were pelleted and resuspended as stated under Experimental Procedures. The pNPPase activity was then assessed at the pH indicated, in medium with 3 mM pNPP, 30 mM Tris-HCl (pH 7.4), 5.5 mM $MgCl_2$, 120 mM KCl, 0.2 mg/mL ghost protein, 1 mM ouabain, and 1 mM EGTA without addition of $CaCl_2$. Values are mean ± SE ($n = 3$). The inset shows the enhancement elicited by DIDS relative to the control at each pH.

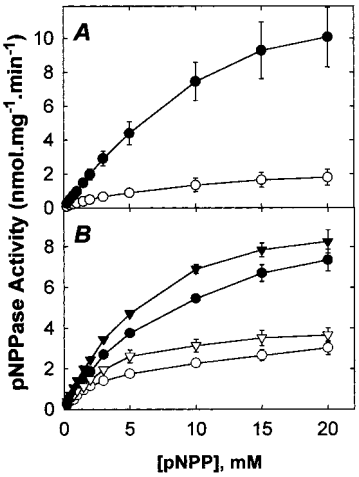


FIGURE 5: pNPP concentration dependence for hydrolysis by the erythrocyte Ca2+-ATPase in the presence of DIDS. (A) pNPPase activity was measured as in Figure 3, but with 1 mM EGTA and no $CaCl_2$, and varying concentrations of pNPP, either in the presence (●) or absence (○) of 5 μM DIDS. (B) Same as in panel A but $CaCl_2$ was added to give 1 μM free Ca^{2+}, with (▼, ▽) or without (●, ○) addition of 2 μg/mL CaM. In both panels, $MgCl_2$ was increased together with the substrate in order to maintain a free Mg^{2+} concentration of approximately 5 mM. Data show means ± SE ($n = 3$).

alone and for activation by CaM plus DIDS (Figure 3B). The CaM-independent activity of ghosts was also stimulated by 5 μM DIDS by a factor of 3−4-times the control, and essentially the same effect was observed in the whole Ca^{2+} range studied (Figure 3A).

The effects of DIDS on the conformational states of the Ca^{2+}-ATPase were further assessed by measuring the pNPPase activity at various pH values. In these experiments, ghosts were preincubated with 5 μM DIDS at pH 7.4 and then pelleted and washed (see Experimental Procedures). Activity was then measured in medium buffered at different pH values (from 6.0 to 8.0). The effects of DIDS on the ATPase (not shown) and pNPPase activities were not modified by this washing step (Figure 4), suggesting either binding with high affinity (but see Figure 1) or, more probably, covalent binding of DIDS to the enzyme (see below). Alkalinization is supposed to drive the enzyme to the E_1 conformation (*15*, *46*, *47*). Accordingly, the pNPPase activity, which is catalyzed by the E_2 form, decreased concomitantly with alkalinization of the assay medium (Figure 4). However, the activation of the pNPPase activity elicited by DIDS (inset, Figure 4) was enhanced at the higher pH values. This suggests that the binding of DIDS has impeded the ability of the enzyme to adopt the E_1 conformation, thus favoring the pNPPase activity.

A possible explanation for our data would be that DIDS displaced the equilibrium $E_1 \leftrightarrow E_2$ toward E_2. To test this possibility we measured the effect of DIDS on the affinity of the enzyme for pNPP. In the absence of Ca^{2+}, DIDS increased the maximal velocity of pNPP hydrolysis (Figure 5A). Unexpectedly, however, this treatment decreased rather than increased the apparent affinity of the enzyme for pNPP (from 9.3 ± 0.7 mM in the absence to 15.5 ± 1.1 mM in the presence of DIDS; Figure 5A and Table 1). In a reaction medium containing Ca^{2+} (with or without added CaM), similar effects were observed (Figure 5B and Table 1). These data suggest a change in the catalytic efficiency of the enzyme and cannot be attributed merely to a shift toward E_2 in the equilibrium between the E_1 and E_2 conformers (Scheme 1).

It is shown in Figure 1A that the rate of ATP hydrolysis was decreased by DIDS concurrently with the increase in pNPP hydrolysis. It is well-known that, in the absence of DIDS, pNPP and ATP compete for the low-affinity substrate site of the Ca^{2+}-ATPase (*13*, *17*). When the Ca^{2+}-ATPase was incubated with DIDS, the pNPPase activity was still inhibited by ATP at a concentration similar to that observed in the absence of DIDS (Figure 6). These data show that DIDS did not impair the binding of ATP but rather decreased the enzyme catalytic efficiency of the enzyme to hydrolyze this substrate.

Niggli et al. (*27*) suggested that inhibition of the erythrocyte Ca^{2+}-ATPase by DIDS might be due to modification of essential sulfhydryl groups. We thus studied the effect of the addition of 5 mM DTT to ghosts previously treated with 5 μM DIDS, after they were pelleted, washed, and diluted with reaction medium. We found that the activation of the pNPPase activity by DIDS in the absence of Ca^{2+} was almost completely reversed by the addition of DTT (Figure 7). This result has two implications: (i) DIDS is not covalently bound to amino groups of the erythrocyte Ca^{2+}-ATPase through the stilbene's isothiocyanate groups, since this type of covalent link (an *N*,*N*′-disubstituted thiourea derivative) would not be cleaved by thiol reducing agents, and (ii) DIDS would react with sulfhydryl groups of the enzyme, in such a way that the resulting product (an *S*-ester of dithiocarbamic acid) is susceptible to cleavage by DTT (*48*, *49*).

DISCUSSION

We describe here a new activator of pNPP hydrolysis by the erythrocyte PMCA: 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS). DIDS binds directly and irreversibly to the enzyme (Figures 2 and 4), both in the presence and in the absence of calmodulin, and increases the pNPPase activity with a concurrent decrease of the ATPase activity

Table 1: Kinetic Parameters for pNPP Hydrolysis by the Erythrocyte Membrane Ca^{2+}-ATPase (Ghosts) in the Presence of 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic Acid[a]

	with 1 μM free Ca^{2+}				without Ca^{2+}	
	control	+DIDS	CaM	CaM + DIDS	control	+DIDS
V_{max}	3.6 ± 0.6	11.2 ± 1.7	4.4 ± 0.5	11.4 ± 0.9	2.6 ± 0.6	18.6 ± 3.9
K_m	4.8 ± 1.4	10.1 ± 2.6	3.8 ± 0.4	7.0 ± 0.8	9.3 ± 0.7	15.5 ± 1.1

[a] The values for K_m and V_{max} were generated by the best fit to the experimental points by using a nonlinear regression and considering a simple Michaelian response to pNPP concentration. The data are presented as mean ± SE of experiments with three different preparations. V_{max} is expressed in nanomoles per milligram per minute, K_m in millimolar.

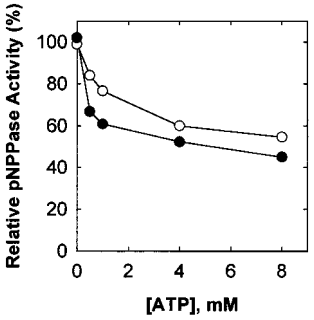


FIGURE 6: Inhibition of the hydrolysis of pNPP by ATP in the presence of DIDS. The effect of different concentrations of ATP was measured at pH 7.4 in medium composed of 120 mM KCl, 1 mM ouabain, 5 mM free Mg^{2+}, 1 mM EGTA with no addition of $CaCl_2$, 3 mM pNPP, and 0.2 mg/mL ghost protein, either in the presence (●) or absence (○) of 5 μM DIDS. The data are from a typical experiment. Similar results were obtained with different preparations. The activities were approximately 1.8 and 4.3 nmol mg^{-1} min^{-1} for the control and for the activated pNPPase, respectively.

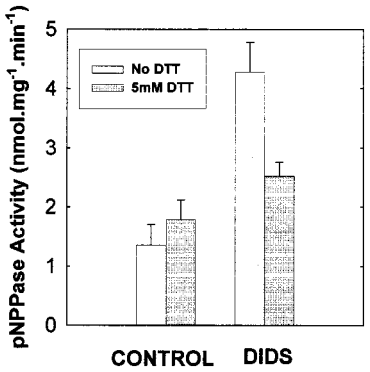
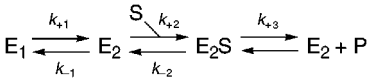


FIGURE 7: Effect of the addition of DTT to ghosts preincubated with DIDS. After pretreatment and washing out free DIDS as in Figure 4, ghosts were resuspended in medium containing (dark gray) or lacking (light gray) 5 mM DTT. The pNPPase activity was measured at pH 7.4 as in Figure 4. Values shown are mean ± SE ($n = 7$).

(Figure 1). At DIDS concentrations higher than 5−10 μM, the enzyme becomes strongly inhibited (Figure 1B), presumably by binding to a second site.

Hydrolysis of pNPP in the presence of DIDS could be performed by an E_2-like conformation, since the increase in Ca^{2+} concentration as well as medium alkalinization (which drives the equilibrium toward E_1) impair the pNPPase activity (Figures 3 and 4). In the case of erythrocyte isoenzymes, if we consider that two enzymatic forms are in equilibrium, and only the conformer E_2 is active in pNPP hydrolysis, then



On solving the rate equations for the steady state, we obtain a Michaelis−Menten equation with V_{max} independent of k_{+1} and k_{-1}, and

$$K_m = [(k_{-2} + k_{+3})/k_2](1 + k_{-1}/k_{+1}) \quad (1)$$

If DIDS only displaced the equilibrium toward E_2 (i.e., decreased the ratio k_{-1}/k_{+1}) the modified enzyme should have the same V_{max} and a lower K_m. On the contrary, Figure 5 shows that DIDS causes a large increase in the V_{max} and a significant increase in the K_m. An alternative explanation could be that DIDS changes the equilibrium in promoting a new enzyme conformation, hereby denoted E_2* for the sake of simplicity. This conformation can bind both ATP and pNPP but it hydrolyzes only pNPP (Figures 1 and 6), and this hydrolysis now occurs with a higher rate than that observed for unmodified E_2. The increase observed in the K_m for pNPP after DIDS modification would reflect the increase in the catalytic constant (k_{+3} in eq 1) rather than in the k_{-1}/k_{+1} ratio, or/and a possible binding of pNPP to both E_1 and E_2* states with similar affinities. As the molecular mechanisms that determine the reactivity of PMCA toward its substrates are still not clear, DIDS appears to be a very interesting tool to study the enzyme reactivity and its mechanism of catalysis.

At least four different genes have been found that code for the plasma membrane Ca^{2+}-ATPase (*50*). The number of isoenzymes is greatly increased by alternate splicing occurring at two or three sites in the pump (*51*−*55*). Plasma membrane Ca^{2+}-ATPases 1 and 4 accumulate in kidneys and erythrocytes, are ubiquitous, and are differentially distributed among the tissues (*5*, *56*−*59*). Small amounts of isoforms 2 and 3 are also found on kidney tissues (*59*). Using a preparation from basolateral membranes of kidney proximal tubules, which is not activated by CaM at micromolar Ca^{2+} concentrations (*60*), Guilherme et al. (*31*) found that DIDS binds reversibly to protonated lysines at plasma membrane Ca^{2+}-ATPase and dislocates the equilibrium toward E_2. Our data with the erythrocyte isozymes may raise the possibility of using DIDS to differentiate residues involved in catalysis and to differentiate plasma membrane Ca^{2+}-ATPase isoforms on a chemical basis.

Two different classes of residues, lysine and cysteine, have been proposed as the target for DIDS in plasma membrane Ca^{2+}-ATPases and a number of other P-type ATPases (*27*, *31*, *61*). Our data point to cysteine as the probable target for DIDS in the pig erythrocyte Ca^{2+}-ATPase. The fact that the activation of the pNPPase activity (and inhibition of the ATPase activity, not shown) are reversed by the addition of DTT (Figure 7) seems to correlate to reaction of the isothiocyanate groups of DIDS with cysteine residues of the PMCA. There is substantial evidence that isothiocyanates

may react more readily with sulfhydryl groups than with amino groups (*48*, *49*, *62*, *63*). Dithiocarbamic acid, the product formed by reaction of isothiocyanates with thiol groups, is relatively unstable and easily cleaved by thiol reducing agents or even mildly alkaline conditions (*48*, *49*, *62*, *63*), thus rendering identification of these groups very difficult with the usual chemical methods. Furthermore, we observed that the effects of preincubating erythrocytes with DIDS in the pH range 6.0−8.0, followed by assessment of the pNPPase activity at the same pH of preincubation, resulted in essentially the same effect as that observed when preincubation was done at pH 7.4 and activities were measured at different pH values (not shown). Therefore, protonation or deprotonation of amino groups does not seem to be important for the reaction of DIDS with the erythrocyte Ca^{2+}-ATPase, thus further supporting the hypothesis of modification of cysteine residues by the isothiocyanate groups of DIDS.

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