Progressive Inactivation of Plasma Membrane (Ca^{2+}+Mg^{2+})ATPase by Cd^{2+} in the Absence of ATP and Reversible Inhibition during Catalysis†

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ABSTRACT: The influence of Cd^{2+} ions on plasma membrane (Ca^{2+}+Mg^{2+})ATPase activity from red cells was investigated. When the membranes were preincubated with Cd^{2+} in the absence of ATP, basal (Ca^{2+}+Mg^{2+})ATPase activity (no calmodulin) was slowly and irreversibly inhibited (inactivated) following first-order kinetics ($k/K_i = 0.0057\ \mu M^{-1}\ min^{-1}$ at $[Cd^{2+}] = 0.25-1\ \mu M$). However, preincubation with Cd^{2+} did not affect the degree of stimulation by calmodulin added to the assay medium together with ATP. Inactivation was not released by prolonged exposure of membranes to EGTA prior to catalysis, but it was strongly attenuated when the pH in the preincubation medium was lowered from 7.2 to 6.4. When the reaction was started by supplying membranes simultaneously with Cd^{2+} and ATP (no preincubation), (Ca^{2+}+Mg^{2+})ATPase was inhibited by increasing concentrations of the CdATP complex ($[CdATP]_{50} = 7.2\ \mu M$). In this condition, however, even total inhibition of the pump was almost completely released after addition of enough EGTA to decrease CdATP concentrations to the nanomolar range. These results, taken together, indicate that inactivation of the unphosphorylated enzyme by Cd^{2+} is influenced by dissociation of amino acid residues exhibiting p*K* between 6.0 and 7.0, and that recognition by the pump of the physiological modulator calmodulin is preserved in the preincubated pump molecules which did not undergo inactivation. In addition, they show that the catalytic site is a target for reversible inhibition of the pump by CdATP and that occupancy of the nucleotide binding site prevents inactivation.

It is already known that Cd^{2+} toxicity is in great part due to disruption of cellular Ca^{2+} homeostasis (*1*−*5*) which is fine-tuned under the control of the plasma membrane (Ca^{2+}+Mg^{2+})ATPase (*6*−*8*). It has been proposed that Cd^{2+}, like other heavy metals, induces the oxidation of sulfhydryl groups in many important macromolecular structures (*9*, *10*), including membrane transporters (*11*, *12*). Thus, Cd^{2+}-induced redox cycling and depletion of sulfhydryl groups induce proteins to assume a less functional or inactivated state. With respect to the plasma membrane Ca^{2+} pump, Verbost et al. (*13*) postulated that Cd^{2+} might act as a competitive inhibitor of the pump, by interacting with Ca^{2+} binding sites. More recently, Visser et al. (*14*) reported Cd^{2+} inhibition of the enzyme in the absence and presence of calmodulin (CaM),[1] and presented evidence that no competition occurs between Ca^{2+} and Cd^{2+}.

The observations of Visser et al. (*14*) raise the possibility that Cd^{2+} affects catalytic and/or regulatory domains of the Ca^{2+} pump, aside from the domains involved in ion binding and transport. This inhibition might first involve reversible interactions to give a complex, which then reacts to form a stable product, akin to that formed between group-specific reagents (such as H_2DIDS) and amino acid residues (*15*). It may be that both inactivation and reversible inhibition depend on conformational states of the enzyme, as shown in the inhibition by H_2DIDS of both (Na^++K^+)ATPase (*15*) and plasma membrane (Ca^{2+}+Mg^{2+})ATPase (*16*).

In this paper, we document the time- and concentration-dependent inactivation of the plasma membrane Ca^{2+} pump by Cd^{2+} in the absence of substrate, and the differences found when inhibition occurs before or during ATP hydrolysis in the presence of different nucleotide concentrations. The influence of exogenously added CaM on the enzyme preincubated with Cd^{2+} was also investigated.

MATERIALS AND METHODS

Materials. Human blood from healthy donors was generously provided by the Centro de Hematologia do Rio de Janeiro. Cadmium sulfate was from Merck (Darmstadt, Germany). CaM from bovine brain, ATP, BSA, DTT, EDTA, EGTA, NADH, P-enolpyruvate, saponin, pyruvate kinase, and L-lactic dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO). Distilled water deionized by the MilliQ system of resins (Millipore Corp., Marlborough, MA) was used in the preparation of all solutions. All other reagents were of the highest grade available. Ionized Ca^{2+} and Cd^{2+} and the concentrations of the complex CdATP were calculated using the computer program CHELATOR (*17*). With the use of this software,

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[1] Abbreviations: BSA, bovine serum albumin; CaM, calmodulin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris-(hydroxymethyl)aminomethane; H_2DIDS, dihydro-4,4′-diisothiocyanatostilbene-2,2′-disulfonate.

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the calculated free Ca^{2+} concentration in the absence of Cd^{2+} was essentially the same as that found using the program described by Sorenson et al. (*18*).

Preparation of Calmodulin-Depleted Ghosts. Ghosts were prepared according to Niggli et al. (*19*), with slight modifications. Blood samples were diluted with 0.2 volume of a cold buffer solution containing 120 mM KCl, 10 mM Tris-HCl (pH 7.4), and 0.5 mM EDTA (buffer A) and centrifuged at 3000 rpm for 10 min in a clinical centrifuge. The plasma and the white cells layered on top of the pellet were carefully removed. The isolated erythrocytes were then washed 3 times with the same solution. Calmodulin-depleted plasma membranes were obtained after incubation of the red cells on ice for 20 min with continuous agitation, using a hypotonic buffer containing 10 mM Tris-HCl (pH 6.7) and 0.5 mM EDTA (buffer B). Membranes were sedimented by centrifugation (20 min at 39900*g*) and washed with the same hypotonic buffer. The process was repeated several times until the supernatant became clear and no more hemoglobin appeared. A third buffer (C) containing 120 mM KCl, 10 mM Hepes-Na (pH 7.2), 0.5 mM $MgCl_2$, and 0.05 mM $CaCl_2$ was then used, and residual hemoglobin appeared in the supernatant, while the pellet became whiter. Washing with this latter solution was repeated 2−3 times until the medium became transparent and the membranes completely white. The ghosts were stored in liquid N_2 until use. The protein concentration was determined according to Lowry et al. (*20*) using BSA as standard.

Permeabilization of Ghosts with Saponin. Ghosts (1.25 mg/mL in buffer C; see above) were permeabilized by treatment with saponin (1.31 mg/mL) for 20 min at room temperature (≈26 °C) under gentle agitation. Except when otherwise indicated, the treated ghosts were immediately diluted to 1 mg/mL in a preincubation medium either with or without $CdSO_4$, with the modifications specified in the corresponding figure legends. In the preincubation media (without ATP or EGTA), [$CdSO_4$] ≈ [free Cd^{2+}].

Measurement of (Ca^{2+}+Mg^{2+})ATPase Activity. Except when otherwise indicated, (Ca^{2+}+Mg^{2+})ATPase activity at high [ATP] was measured in an assay mixture containing 50 mM Hepes−Tris (pH 7.4), 130 mM KCl, 2 mM $MgCl_2$, 2 mM ATP, 0.2 mM EGTA−Tris, and 250 μM $CaCl_2$ (≈18 μM free Ca^{2+}) in the absence or presence of 30 nM CaM. The enzyme activity assay was initiated by addition of saponin-permeabilized ghosts (preincubated or not for different times with or without $CdSO_4$). The reaction was stopped after 1 h at 37 °C with a cold suspension (2 volumes) of activated charcoal. Then the tubes were centrifuged for 20 min at 3000 rpm in a clinical centrifuge, and P_i resulting from ATP hydrolysis was measured using the method of Fiske and Subbarow (*21*). The (Ca^{2+}+Mg^{2+})ATPase activity was the difference between the total ATPase activity (measured in the presence of ≈18 μM free Ca^{2+} with or without 30 nM CaM) and the activity measured in the presence of 0.2 mM EGTA alone. In Figure 4, P_i release was followed in the same reaction medium using the coupled system of enzymes below.

The assays at low [ATP] were carried out in media containing 50 mM Hepes−Tris (pH 7.4), 130 mM KCl, 422 μM $MgCl_2$, 10 μM ATP, 0.2 mM EGTA−Tris, 3 mM P-enolpyruvate, 0.12 mM NADH, 7 units/mL pyruvate kinase, 10 units/mL L-lactic dehydrogenase, and enough $CaCl_2$ to give a final concentration of ≈18 μM free Ca^{2+}. No CaM was used in these experiments. The reaction was initiated by addition of saponin-permeabilized ghosts to the assay medium (no preincubation). The enzyme activity was followed spectrophotometrically at 340 nm for 15 min (E_{340} = 6.22 × 10^3 M^{-1} cm^{-1}) (*22*).

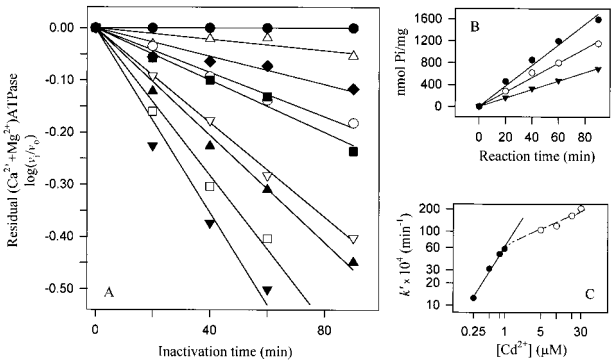

FIGURE 1: Time course of inactivation of (Ca^{2+}+Mg^{2+})ATPase in the presence of different Cd^{2+} concentrations. (A) Saponin-treated ghosts were preincubated with buffer C for different times shown on the abscissa (see Materials and Methods) in the presence of the following Cd^{2+} concentrations (in μM): 0 (●), 0.25 (△), 0.5 (◆), 0.8 (○), 1 (■), 5 (▽), 10 (▲), 20 (□), and 30 (▼). (Ca^{2+}+Mg^{2+})-ATPase activity was assayed for 1 h in the reaction medium described under Materials and Methods (2 mM ATP) supplied with BSA (0.5 mg/mL) and DTT (10 mM) to remove medium Cd^{2+}. Data points are averages of 5−6 experiments performed with different preparations. The straight lines were adjusted to the experimental points by linear regression using eq 1 (see text). The ratio k/K_i (0.0057 μM^{-1} min^{-1}) was calculated from the slopes of the lines in the [Cd^{2+}] range 0.25−1 μM. (B) Time course of ATP hydrolysis catalyzed by (Ca^{2+}+Mg^{2+})ATPase preincubated for 1 h in the absence (●) or in the presence of 0.8 (○) or 30 μM Cd^{2+} (▼). Other conditions as in panel A. (C) Plot of the apparent first-order rate constants of inactivation (k') versus [Cd^{2+}] between 0.25 and 1 μM (●) and 5−30 μM Cd^{2+} (○), calculated from the slopes of the lines shown in (A). The continuous line was calculated by linear regression; the dashed line was drawn by hand.

Data Analysis. All assays and appropriate blanks were run in duplicate or triplicate. The reported values are means of three to six experiments using different membrane preparations. Standard errors were less than 20% of the average values. For normalized data (in percent), the means were calculated using absolute values and expressed as a percent of the corresponding control without Cd^{2+}.

RESULTS

Inactivation of (Ca^{2+}+Mg^{2+})ATPase by Cd^{2+} in the Absence of ATP. Red cell membranes preincubated with Cd^{2+} in the absence of ATP exhibit inactivation of the (Ca^{2+}+Mg^{2+})ATPase. As shown in Figure 1A, the inactivation is time- and concentration-dependent. Furthermore, the linear correlations between the logarithmic values of residual (Ca^{2+}+Mg^{2+})ATPase activity and preincubation time at different Cd^{2+} concentrations up to 30 μM indicate that inactivation follows first-order kinetics. Cd^{2+} ions were able to interact with the enzyme only during the preincubation period, since BSA, DTT, and EGTA were present in the assay medium and the time course of hydrolytic activity was linear throughout (Figure 1B).

The fact that the enzyme remains inhibited during ATP hydrolysis after removal of medium Cd^{2+} indicates that irreversible inhibition is the final step in the interaction

between Cd^{2+} and the calcium pump (E) when it occurs before catalysis takes place. This might occur according to the minimal Scheme 1:

Scheme 1

$$E + Cd^{2+} \overset{K_i}{\leftrightarrow} E{\cdot}Cd \overset{k}{\rightarrow} {*}E{\cdot}Cd$$

where K_i is the dissociation constant of the E·Cd complex, k is the inactivation rate constant, and *E·Cd is the irreversibly inhibited (Ca^{2+}+Mg^{2+})ATPase (*15*, *23*, *24*). The experimental data support this model since they could be fit, at low [Cd^{2+}] (0.25−1 μM), by the equation:

$$\log (v_i/v_0) = -k[Cd^{2+}]^n t/2.3K_i \qquad (1)$$

where v_i is the activity of the enzyme preincubated with Cd^{2+}, v_0 is the activity of the nonpreincubated enzyme, and t is the time of preincubation. Since the best fits are obtained when n approaches 1, this value can be considered the average order of the reaction. This suggests that only one enzyme site is involved in the process of inactivation in this lower Cd^{2+} concentration range. Figure 1C shows the plots of the apparent rate constants of inactivation (k') as a function of [Cd^{2+}]. The plot is clearly biphasic, suggesting that at elevated Cd^{2+} concentrations (above 1 μM) at least one additional site is involved which reacts with a different rate constant.

The hypothesis that the pump is inactivated (irreversibly inhibited) by Cd^{2+} in the absence of catalysis is reinforced by the following experiment. Preincubated for 1 h in the presence of 0.8 μM Cd^{2+} (which promotes ≈40% inactivation) and preincubated again for another 1 h with sufficient EGTA to lower the free [Cd^{2+}] to a very small value (3.51×10^{-16} M), the enzyme is unable to recover its activity when ATP is added (data not shown).

Inactivation by Cd^{2+} at Different pH Values and Influence of Preincubation with Cd^{2+} on Enzyme Stimulation by CaM. The influence of pH in the inactivation process was investigated. In Figure 2A, the membranes were preincubated for 1 h in the presence of varying concentrations of $CdSO_4$ at two different pH values (7.2 and 6.4). At pH 6.4, inactivation is not detectable at Cd^{2+} concentrations (0.25−1 μM) which promote a pronounced effect at pH 7.2 (inset to Figure 2A; see also Figure 1A), and half-inactivation is attained at around 30 μM Cd^{2+}, a metal concentration in which enzyme activity is almost completely lost at pH 7.2. In addition, Figure 2A (main panel) shows that the inactivation curve is shifted to higher Cd^{2+} concentrations at pH 6.4. This indicates that complete inactivation involves metal binding at two or more distinct enzyme sites, and that these sites have different affinities for Cd^{2+} that vary with pH.

Figure 2A,B also shows the effects of CaM on the enzyme preincubated with Cd^{2+} before assay of (Ca^{2+}+Mg^{2+})ATPase activity. Note that the normalized curves in the absence and in the presence of CaM in the assay medium are superimposable (compare empty and filled symbols in Figure 2A), indicating that the degree of stimulation of (Ca^{2+}+Mg^{2+})-ATPase by CaM remains unmodified even after preincubation of the membranes with high Cd^{2+} concentrations. An example of the stimulation by CaM is shown in Figure 2B, where absolute rates of (Ca^{2+}+Mg^{2+})ATPase activity from membranes preincubated at pH 7.2 are presented. The same degree of stimulation by CaM is observed regardless of whether Cd^{2+} is present in the preincubation medium or not.

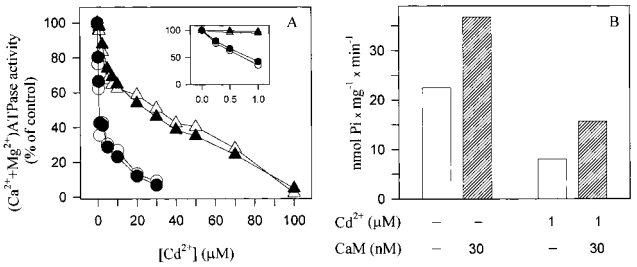

FIGURE 2: Effect of pH on the inactivation of (Ca^{2+}+Mg^{2+})ATPase by 0−100 μM Cd^{2+}. (A) Saponin-treated ghosts were preincubated with buffer C at pH 7.2 (○, ●) or pH 6.4 (△, ▲) for 1 h in the presence of the Cd^{2+} concentrations indicated on the abscissa. Then ATP hydrolysis was assayed for 1 h at pH 7.4 as described under Materials and Methods (2 mM ATP). After addition of the reaction medium containing EGTA and ATP, free Cd^{2+} and CdATP concentrations ranged from 0.08 to 18 pM and from 7.1 pM to 1.6 nM, respectively, depending on the initial Cd^{2+} concentrations in the preincubation medium. Each curve is normalized to its own control value (no Cd^{2+}). The stimulation by CaM after preincubation with or without Cd^{2+} persists; an example is shown in (B). Empty symbols, no CaM in the assay medium; filled symbols, 30 nM CaM in the assay medium. Inset: Data for 0−1 μM [Cd^{2+}] on an expanded concentration base. (B) Stimulation of (Ca^{2+}+Mg^{2+})-ATPase by CaM after 1 h preincubation of the membranes at pH 7.2 in the absence (−) or presence (+) of 1 μM Cd^{2+}. Similar results were obtained at pH 6.4 with 0 and 70 μM Cd^{2+}. In both panels, values are means of 3−4 determinations using different membrane preparations and run in duplicate.

Reversible Inhibition during Catalysis and Comparison with the Degree of Inactivation in the Absence of ATP. The following experiments were designed to investigate the effects of Cd^{2+} in the presence of the physiological ligand ATP. When Cd^{2+} (0.8 μM) and ATP are added simultaneously to the medium (no preincubation), inhibition of the pump is nearly complete (>90%; Figure 3A, empty triangles). However, (Ca^{2+}+Mg^{2+})ATPase is almost completely reactivated after addition of EGTA (filled triangles). This indicates that in this case, in contrast with the experiments of Figure 1, enzyme activity is restored when Cd^{2+} is chelated by EGTA and [CdATP] drops from 69.2 μM to 3 nM. This is shown in another way in Figure 3B. When the enzyme is first preincubated in the presence of 0.8 μM Cd^{2+} for 1 h and then supplied with ATP, the addition of EGTA 30 min later only leads to recovery of the catalytic activity (≈10%; Figure 3B, filled triangles) reversibly inhibited by the CdATP (792 nM) formed in the medium after addition of 2 mM nucleotide (see also CdATP concentration dependence with high [ATP] in Figure 4C below).

Reversible Inhibition by Cd^{2+} during Catalysis at High and Low ATP Concentrations. Since the presence of ATP prevents inactivation, the influence of the physiological ligand ATP on the degree of reversible inhibition by Cd^{2+} was investigated using two different nucleotide concentrations. The experiments shown in Figure 3A were performed with 2 mM ATP. In experiments run in parallel using 2 mM or 10 μM ATP in media containing 0.8 μM free Cd^{2+} (Figure 4A), (Ca^{2+}+Mg^{2+})ATPase is reversibly inhibited by 80% with high [ATP] whereas inhibition is only 10% with low [ATP]. Over a range of free Cd^{2+} concentrations (0−1 μM, Figure 4B), the enzyme is less affected by free Cd^{2+} at

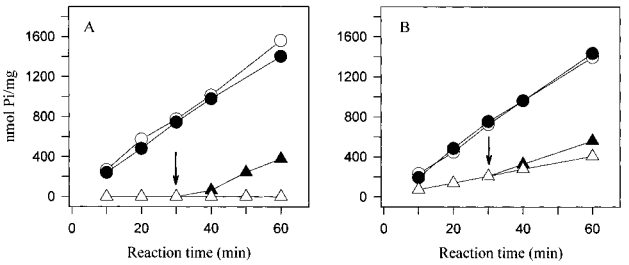

FIGURE 3: Reversible inhibition in the presence of Cd^{2+} and ATP. (A) Nonpreincubated membranes were diluted (0.2 mg/mL) in a reaction medium containing 50 mM Hepes−Tris (pH 7.4), 130 mM KCl, 2 mM $MgCl_2$, and 2 mM ATP in the absence (○, △) or presence (●) of 0.2 mM EGTA, and in the absence (○, ●) or presence (△) of 0.8 μM Cd^{2+} (70 μM $CdSO_4$). The different media contained appropriate $CaCl_2$ concentrations (44.4 μM, ○; 250 μM, ●; 50 μM, △) to give a constant free [Ca^{2+}] (≈18 μM). At the time indicated by the arrow, one tube of the Cd^{2+}-containing assays was supplied with CaEGTA buffer (180 μM $CaCl_2$, 0.2 mM EGTA; final concentrations) to reduce the free Cd^{2+} concentration to 34 pM (▲) at constant free [Ca^{2+}]. (B) Enzyme preincubated during 1 h with buffer C in the absence (○, ●) or presence (△) of 0.8 μM Cd^{2+} was supplied with reaction medium containing 50 mM Hepes−Tris (pH 7.4), 130 mM KCl, 2 mM $MgCl_2$, and 2 mM ATP in the absence (○, △) or presence (●) of 0.2 mM EGTA. Free Ca^{2+} concentration in the reaction medium was kept constant at ≈18 μM by adding appropriate $CaCl_2$ concentrations (44.4 μM, ○; 250 μM, ●; 44.4 μM, △). At the time indicated by the arrow, one tube of the Cd^{2+}-containing assays (9 nM free Cd^{2+} after mixing of preincubation and reaction media) was supplied with CaEGTA buffer (242.4 μM $CaCl_2$, 0.2 mM EGTA; final concentrations) to reduce the Cd^{2+} concentration to 0.2 pM (▲) at constant free [Ca^{2+}]. In both panels, values are means of 4 experiments using different membrane preparations and run in duplicate.

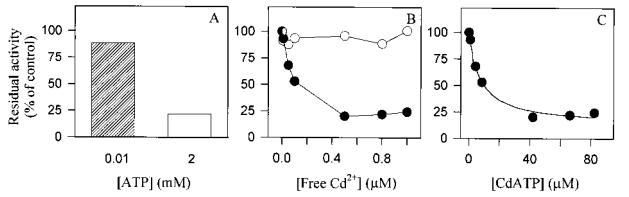

FIGURE 4: Influence of ATP concentration on reversible inhibition of (Ca^{2+}+Mg^{2+})ATPase by cadmium. (A) Nonpreincubated enzyme was assayed in the presence of 0.8 μM free Cd^{2+} and the total ATP concentrations shown on the abscissa. (B) Nonpreincubated enzyme was assayed in the presence of the free Cd^{2+} concentrations shown on the abscissa and either 10 μM (○) or 2 mM total ATP (●). The lines were drawn by eye. Other assay conditions in (A) and (B) as described under Materials and Methods. (C) Dependence of reversible inhibition on CdATP concentrations. The data from panel B (●) are plotted against the corresponding CdATP concentrations calculated as described under Materials and Methods. The smooth curve was calculated by nonlinear regression using eq 2 (see text). Using the reciprocal form of eq 2 in the CdATP range of 0−42 μM, a straight line ($r = 0.999$) was found (not shown). In all panels, values are means of 3 experiments using different membrane preparations and run in duplicate.

a micromolar concentration of the nucleotide (compare empty and filled circles). Finally, Figure 4C indicates that reversible inhibition of the (Ca^{2+}+Mg^{2+})ATPase can be described by a function that considers the complex CdATP as the true inhibitor:

$$v_i = v_r + (v_0 - v_r)/\{1 + ([CdATP]/[CdATP]_{50})\} \quad (2)$$

where v_i is (Ca^{2+}+Mg^{2+})ATPase activity in the presence of inhibitor, v_0 is the activity measured in the absence of CdATP, $[CdATP]_{50}$ (7.2 μM) is the concentration for half-maximal inhibition, and v_r is the asymptote of the activity that is insensitive to CdATP.

DISCUSSION

Different mechanisms of inhibition of the plasma membrane (Ca^{2+}+Mg^{2+})ATPase by Cd^{2+} have been described (*4*, *13*, *14*, *25*), yet the influence of the functional state of the pump as well as the influence of the physiological ligand ATP is still unclear. For example, it is uncertain whether Cd^{2+} affects the pump in the same way in the absence and presence of CaM (*14*, *25*), and although in some studies modification of sulfhydryl groups by Cd^{2+} has been invoked (*4*, *13*), kinetic analysis of the Cd^{2+}-induced molecular changes of plasma membrane (Ca^{2+}+Mg^{2+})ATPase is lacking.

The data presented in this study support a model (Scheme 1) in which interaction of the Ca^{2+} pump with Cd^{2+} in the absence of ATP leads to progressive and irreversible enzyme inactivation (Figure 1), after reversible binding at one high-affinity Cd^{2+} binding site and then at a second, low-affinity site (Figure 2A). The affinity of the high-affinity site for Cd^{2+} is decreased by an order of magnitude when inactivation is carried out at pH 6.4 instead of pH 7.2. Since protonation of sulfhydryl groups does not change in this narrow pH interval (*26*), it may be that other functional groups that dissociate at this pH value, such as histidyl groups, contribute to Cd^{2+} binding and enzyme inactivation.

In one study (*25*), relaxation of a Cd^{2+}-inhibited state of the red cell (Ca^{2+}+Mg^{2+})ATPase was observed when EGTA was added shortly before initiation of the enzyme reaction with ATP and $CaCl_2$. Inactivation, however, occurs when the enzyme is preincubated with Cd^{2+} for longer periods (Figure 1A), and inhibition of preincubated enzyme is not released even after a long period in the presence of all physiological ligands, when repeated cycles of catalysis have occurred (Figure 1B). The observations of Åkerman et al. (*25*) and those from the present study indicate that the conversion of E·Cd to the irreversibly inhibited form *E·Cd (Scheme 1) may be a complex transition in which some initial steps are slow and still reversible.

Both irreversible and reversible inhibition of ion-transporting ATPases by compounds that interact with amino acid side chains in specific functional domains (*15*, *24*) have been found. It has also been observed that physiological ligands (*15*) as well as medium pH (*16*) modify the responsiveness of ATPases to these types of organic compounds, indicating that the irreversible step proposed in Scheme 1 (E·Cd → *E·Cd) involves conformational changes that are modified (or blocked) when specific ligands are bound.

The observation that ATP prevents inactivation by Cd^{2+} even at a high concentration of the metal (Figure 3A) indicates that the irreversible E·Cd → *E·Cd transition does not occur when nucleotide is bound to the pump. However, the complex CdATP at the catalytic site brings about reversible inhibition during catalysis (Figure 3A). This view is supported by the observation that inhibition is stronger with high [ATP] at the same free Cd^{2+} concentrations (Figure 4A,B) and that it can be described by a function in which the inhibitor is the CdATP complex (eq 2; Figure 4C). It is therefore likely that competition occurs between CdATP and MgATP at the nucleotide binding site, and not between Ca^{2+}

and Cd^{2+} for binding to the cation transport domains (*13*). Decreasing the ATP concentration and increasing the $MgCl_2$ concentration attenuate inhibition of the $(Na^+ + K^+)$ATPase by Cd^{2+} (*27*). Moreover, interactions of Cd^{2+} at the catalytic site of ATPases may affect coupling between ATP hydrolysis and ion transport, as revealed by the Cd^{2+} sensitivity of ATP-driven ion fluxes in epithelial cells (*28*).

Cadmium ions can replace Ca^{2+} in activation of CaM (*29*), probably due to their similarities in charge and ionic radius (*30*); binding of Cd^{2+} to CaM has been implicated in the inhibition of $(Ca^{2+}+Mg^{2+})$ATPase by Cd^{2+} (*25*). As shown in Figure 2, the response of the preincubated enzyme to CaM is not modified, suggesting that interaction of the auto-inhibitory CaM-binding domain with its pump sites (*31*) is preserved in the pump molecules which are not irreversibly affected by Cd^{2+}. This may mean that metal binding produces its irreversible effect by acting on a very specific enzyme domain, such as the active site. This suggestion is reinforced by the experiments of Figure 3A, which indicate that binding of the CdATP complex prevents the effect of free Cd^{2+}. The Cd^{2+} target might be specific amino acid side chains at or near the catalytic site, although there is also the possibility of long-range effects of ATP binding (*32*). The fact that low concentrations of CdATP ($[CdATP]_{50}$ = 7.2 μM) inhibit the $(Ca^{2+}+Mg^{2+})$ATPase in the presence of a physiological concentration of nucleotide (2 mM) might explain the increase in cytosolic Ca^{2+} observed in cells exposed to micromolar Cd^{2+} (*1*, *4*, *5*).

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