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Cadmium ion is a non-competitive inhibitor of red cell Ca^{2+}-ATPase activity

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In the presence as well as in the absence of calmodulin, Cd^{2+} inhibits the human erythrocyte plasma membrane Ca^{2+}-ATPase activity non-competitively with $K_i = 2$ nM, whereas ATP-dependent Ca^{2+}-transport across the red cell membrane was found to be inhibited competitively by Cd^{2+} (Verbost, P.M., Flik, G., Pang, P.K.T., Lock, R.A.C. and Wendelaar Bonga, S.E. (1989) J. Biol. Chem. 264, 5613–5615). In this study it will be argued that Cd^{2+} also inhibits Ca^{2+}-transport non-competitively, and that the discrepancy with previous conclusions most probably relies on use of an incorrect computer program that calculates the free concentrations of Ca^{2+} and Cd^{2+} at the experimental conditions applied for measurement of Ca^{2+} uptake.

Introduction

Ca^{2+} plays an important role as second messenger in a variety of cellular functions. Therefore, the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) has to be kept within narrow limits (0.1–1 μM), whereas the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) is about 1 mM [1–3]. The intracellular Ca^{2+} homeostasis is maintained by a delicate balance between several Ca^{2+} transport systems. Ca^{2+} can enter the cell through a number of differently activated channels in the plasma membrane [4–6], and it can be stored in intracellular organelles such as the endoplasmic reticulum, possibly the 'calciosome' [7], and the mitochondria. Ca^{2+} is pumped out of the cell by the plasma membrane Ca^{2+}-ATPase, against a steep concentration gradient [8]. Some cells also possess a Na^+/Ca^{2+} exchange system in the plasma membrane. The study of Ca^{2+} transport across the plasma membrane of most cells is hampered by the fact that it is difficult to isolate plasma membranes without retaining intracellular membranes containing Ca^{2+} transport systems.

Ca^{2+}-ATPase of human erythrocyte plasma membrane is probably the most extensively studied Ca^{2+}-ATPase [8–19], mainly because the erythrocyte plasma membrane is easily attainable in large quantities, without contamination by intracellular membranes. Also, the human erythrocyte lacks a Na^+/Ca^{2+} exchange system [15]. It is easy to prepare inside-out vesicles of erythrocyte plasma membrane; this preparation allows one to study the activity of the Ca^{2+}-ATPase-mediated Ca^{2+}-uptake into these vesicles in media of controlled intracellular ionic composition.

Cd^{2+} is known to have severe toxic effects on cells of animal origin as well as on yeast cells [20,21–24]. Cd^{2+}, as well as a number of other xenobiotics, appears to raise $[Ca^{2+}]_i$ [20]. This could be due to an increase in Ca^{2+} influx or to a decrease in Ca^{2+} efflux. As it is known that Cd^{2+} blocks Ca^{2+} channels in the plasma membrane [25–28], an increase in Ca^{2+} conductance is unlikely. Another possibility for increased Ca^{2+} influx is an aspecific increase in plasma membrane permeability by Cd^{2+}. This has been found in yeast, where Cd^{2+} permeabilizes the plasma membrane from the cytoplasmic side [24,29,30]. The second option is a decrease in Ca^{2+}-ATPase mediated Ca^{2+} efflux. Verbost et al. [31] found for erythrocyte inside-out vesicles (IOVs) a competitive inhibition of Ca^{2+} uptake by Cd^{2+}. However, as mentioned above, in yeast Cd^{2+} has been found to aspecifically permeabi-

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Abbreviations: ATP, adenosine triphosphate; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; $[Ca^{2+}]_o$, extracellular free Ca^{2+} concentration; IOV, inside-out vesicle; ROV, rightside-out vesicle; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEDTA, *N*-(2-hydroxyethyl)ethylenediamine-*N*,*N*′,*N*′-triacetic acid; NTA, nitrilotriacetic acid; NADH, β-nicotinamide-adenine dinucleotide, reduced; PEP, phospho*enol*pyruvate; LDH, lactate dehydrogenase; PK, pyruvate kinase.

lize the plasma membrane, whereas Ca^{2+} protects the membrane against the deleterious effects of Cd^{2+} [24]. If this also occurs with the membrane of IOVs, Cd^{2+} may even without directly interacting with the Ca^{2+}-ATPase inhibit Ca^{2+} uptake into the vesicles. Moreover, the inhibition will then also be of a competitive type, i.e., it will diminish on raising the Ca^{2+} concentration, but in this case because of the Ca^{2+}-dependent protection against membrane damage by Cd^{2+}, and not because of a competition between Ca^{2+} and Cd^{2+} for binding to the Ca^{2+} binding site of the Ca^{2+}-ATPase.

Obviously one should be very careful in interpreting data on effects of Cd^{2+} on Ca^{2+} transport into IOVs in terms of a competition between the two divalent cations for a site on the enzyme. As vectorial transport of Ca^{2+} by the red cell plasma-membrane Ca^{2+}-ATPase is strictly coupled to ATP hydrolysis [3] we have now examined the interaction of Cd^{2+} with the red cell Ca^{2+} pump more directly by characterizing the mode of its inhibition of the Ca^{2+}-ATPase activity.

Inhibition of Ca^{2+}-dependent enzyme systems by Cd^{2+} have previously been described; the inhibitory effect has mostly been ascribed to competition with the calcium ion [32–34]. A number of other heavy metals, such as nickel and lead, have also been shown to be inhibitory to the plasma-membrane ATPases of several cell types [34–36]. In at least one other study on metal effects on red cell membrane Ca^{2+}-ATPase, however [37], a series of divalent metal ions, including nickel, lead and Cd^{2+}, appeared to activate the Ca^{2+}-ATPase. Further, it is known that at least some heavy metals have a cytolytic effect [38,39].

Materials and Methods

Preparation of inside-out red cell membrane vesicles (IOVs)

Human red blood cells were isolated from 1-day-old buffy coats obtained from the Red Cross bloodbank at Nijmegen by centrifugation at $2300 \times g$ for 5 min. The pellet of erythrocytes was subsequently washed three times at 4°C with four volumes of a solution containing 150 mM KCl and 20 mM Hepes-Tris (pH 7.4). From these washed erythrocytes calmodulin-deficient IOVs were prepared as described by Sarkadi et al. [13]. Membrane sidedness and the ratio of sealed vesicles were determined by measuring latent acetylcholinesterase and glyceraldehyde-3-phosphate dehydrogenase activities as described by Steck and Kant [40]. IOV content varied between 50 and 80%, while ROV (rightside-out vesicles) content was about 20–30%. Protein content of vesicle samples was determined colorimetrically using a commercial Coomassie blue assay kit (Bio-Rad) with bovine serum albumin as standard. Protein content of IOV preparations varied between 0.56 and 1.80 mg/ml.

Determination of Ca^{2+}-ATPase activity

The Ca^{2+}-ATPase activity was measured spectrophotometrically at 37°C, using a coupled enzyme assay as described by Foder and Scharff [41]. The medium contained 150 mM KCl, 1.5 mM free Mg^{2+}, 0.5 mM EGTA, 0.5 mM HEDTA, 0.5 mM NTA, 3 mM ATP, 0.28 mM NADH, 0.7 mM PEP, 4.2 IU of LDH/ml, 1.4 IU of PK/ml, 2 μg/ml calmodulin (when added), various concentrations of $CaCl_2$ and $CdCl_2$ to achieve the desired free concentrations of these divalent cations and 20 mM Hepes, adjusted to pH 7.4 at 37°C with Tris. The ATPase activity was followed by measuring continuously the difference in absorbance at 366 and 550 nm, using an Aminco DW-2C dual wavelength spectrophotometer. After preincubation of the assay medium (total volume: 2.2 ml) for 30 min at 37°C, the reactions was started by the addition of IOVs (55–110 μg membrane protein) to the medium.

Pilot experiments revealed that total ATPase activity assayed at the above conditions was insensitive to 0.1 mM ouabain (data not shown). ATPase activity was therefore further assayed in the absence of this specific inhibitor of the Na^+/K^+-ATPase [42]. Apparently the low sodium content of the assay media used by us (no sodium salts were added) was sufficient to prevent any Na^+/K^+-ATPase activity. The activity of the Mg^{2+}-ATPase (assayed in the absence of added $CaCl_2$) was subtracted from the total activity assayed in the presence of Ca^{2+}. The resulting difference in ATPase activity is defined as the Ca^{2+}-stimulated, magnesium-dependent ATPase (Ca^{2+}-ATPase). It was ascertained that the coupled enzyme reaction is not affected in the range of Cd^{2+} concentrations used in this study.

Calculation of the concentrations of free divalent cations

Assay media containing desired free concentrations of Ca^{2+}, magnesium and cadmium were prepared by addition of calculated total concentrations of their chloride salts to the media. These total concentrations were calculated by computer according to the method of Van Heeswijk et al. [43] which was also used by Verbost et al. [31] in their study of Cd^{2+}-inhibition of ATP-dependent Ca^{2+} uptake into IOVs. Effects of Cd^{2+} on Ca^{2+}-ATPase activity determined in media prepared according to this method can therefore be directly compared with those of Verbost et al. on Ca^{2+}-pump activity of the enzyme.

During the course of this study it became clear that in assay media prepared according to the above method the actually achieved free ionic metal concentrations may differ appreciably from those desired and that this may give rise to misinterpretation of experimental data in terms of derived kinetic parameters [44]. We therefore conducted our experiments also in assay media in which the free ionic metal concentrations were adjusted by the aid of a newly developed and improved

computer program. This recently published computer program (CHELATOR) [44] differs from the one of Van Heeswijk et al. [43] in that stability constants of metal chelator equilibria are corrected for temperature, pH and ionic strength of the assay media. CHELATOR have been proven to be well suited to accurately control Ca^{2+} concentrations over a wide range. The correlation between calculated and measured free Ca^{2+} concentrations was namely shown to be excellent [44]. Table I lists the corrected stability constants [45] which were used by CHELATOR to calculate total concentrations of chloride salts of the divalent cations that had to be added to the assay media to achieve their desired free concentrations.

Materials

Tris-ATP, EGTA, HEDTA, NTA and ouabain were purchased from Sigma; NADH, PEP, LDH, PK and calmodulin from Boehringer Mannheim.

All reagents were analytical grade, and all solutions were made using twice-distilled water.

Results

The effect of Cd^{2+} on the Ca^{2+}-ATPase activity determined in media in which the free ionic metal concentrations were adjusted according to Verbost et al. [31] is shown in Fig. 1. The data can be described to a good approximation by a single Michaelis-Menten

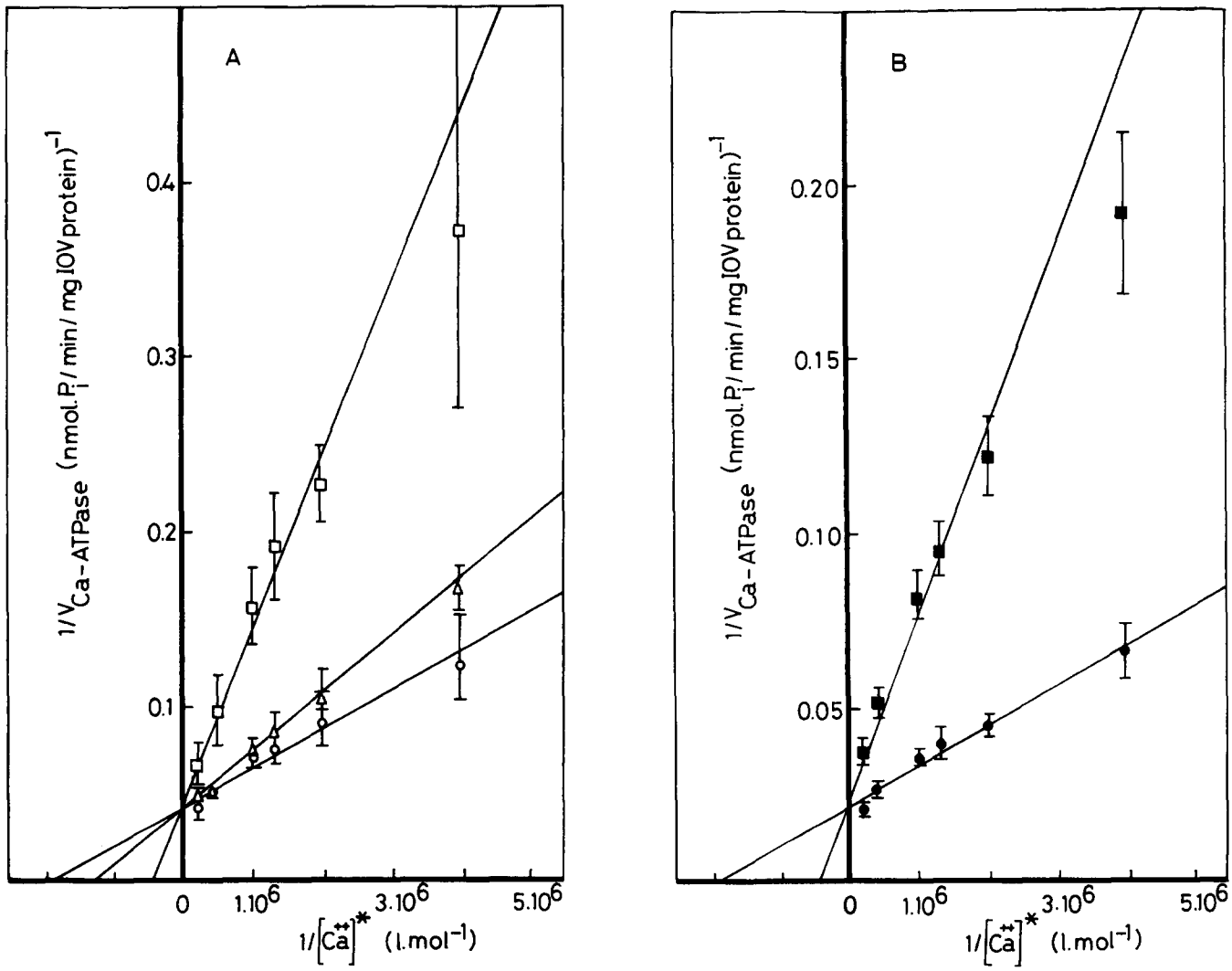

Fig. 1. Lineweaver-Burk plots of the Ca^{2+}-ATPase activity, assayed in media in which ionic concentrations of Ca and Cd (denoted by an asteriks) were adjusted according to Verbost et al. [31] (see Materials and Methods). (A) Data obtained in media without added calmodulin at desired free Cd^{2+} concentrations of 0 (○), 3 (△) and 6 nM (□). (B) Data obtained in the presence of 2 μg/ml calmodulin. (●) Control; (■) at a desired free Cd^{2+} concentration of 6 nM. Each point represents the mean of eight different experiments (each run in triplicate) and the bar represents the standard error of the mean.

relation and represented by a straight line in a Lineweaver-Burk plot according to Eqn. 1.

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_{Ca}}{V_{max}} \cdot \frac{1}{[Ca^{2+}]} \quad (1)$$

where K_{Ca} is the dissociation constant for Ca^{2+}, V_{max} the maximum rate of ATPase activity and $[Ca^{2+}]$ the free Ca^{2+} concentration. In Fig. 1A data obtained with calmodulin-depleted IOVs are shown. The inhibition of the Ca^{2+}-ATPase by Cd^{2+} can within experimental error be ascribed to a competition of Cd^{2+} with Ca^{2+} for binding to the high-affinity Ca^{2+} transport site of the enzyme. The lines drawn through the data points were calculated by means of Eqn. 2 describing the Ca^{2+}-concentration dependence of the ATPase with Cd^{2+} as a competitive inhibitor. They are the best overall fits to the data and calculated by taking V_{max} = 25 nmol P_i/mg IOV protein per min, $K_{Ca} = 0.5$ μM and $K_i = 1.4$ nM. As found for Ca^{2+}-uptake, the presence of calmodulin increased the V_{max} of the ATPase approx. 2-fold without affecting the affinity of Ca^{2+} for the enzyme, nor the mode of inhibition by Cd^{2+} (Fig. 1B).

Free ionic concentrations of Ca^{2+} and Cd^{2+} in the media used in the experiments represented in Fig. 1 were recomputed by means of CHELATOR. Fig. 2A shows that in the controls of Fig. 1 without Cd^{2+} present, free concentrations of Ca^{2+} up to 1 μM have been underestimated. Above 1 μM free Ca^{2+} was consistently overestimated in the media prepared according to Verbost et al. In the presence of added Cd^{2+}, however, free Ca^{2+} concentrations were underestimated in the whole concentration range. Moreover, the free Cd^{2+} concentration, assumed to remain constant, appeared to decrease substantially on raising the Ca^{2+} concentration.

By making use of the recomputed free Ca^{2+} concentrations, the control experiments of Fig. 1 have been replotted. The results are given in Fig. 3. Clearly, a better fit to a single Michaelis-Menten equation is obtained with the recomputed free Ca^{2+} concentrations. The uncorrected data are better described by a curvi-linear relationship in a Lineweaver-Burk plot, while the corrected data can be fitted to a single straight line. The estimated values of the V_{max} and K_{Ca} are 34.3 ± 0.4 nmol P_i/min per mg IOV protein and 1.44 ± 0.03 μM for the control without added calmod-

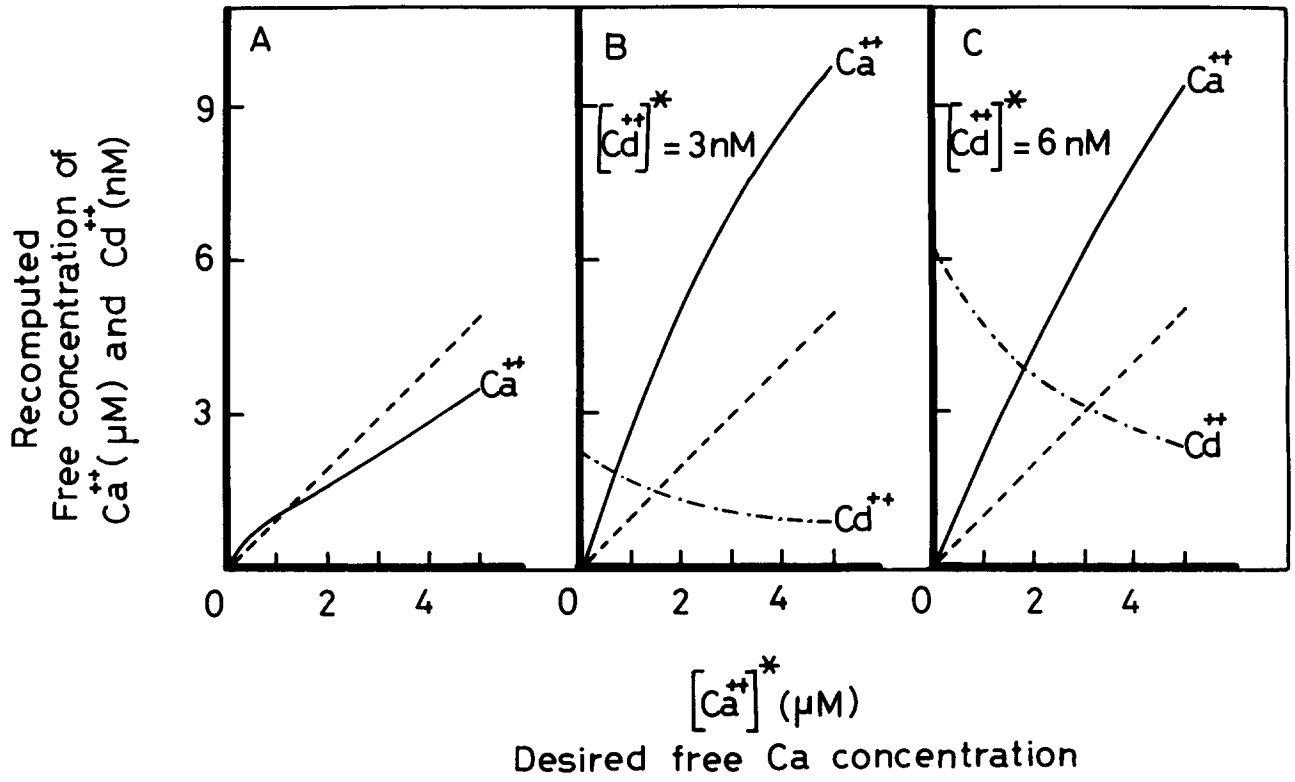

Fig. 2. Recomputation of the free Ca^{2+} and Cd^{2+} concentrations of the assay media in which ionic concentrations have been adjusted according to Verbost et al. [31]. From the total metal concentrations that were added to the media in order to obtain the desired free concentrations (denoted by an asterisk) their free concentrations were recomputed with CHELATOR (see Materials and Methods). These recomputed concentrations are plotted against the desired free Ca^{2+} concentrations for the conditions that the desired free Cd^{2+} concentration is 0 (A), 3 nM (B) and 6 nM (C), respectively. The broken lines through the origin represent the free Ca^{2+} concentration calculated according to Verbost et al. [31].

TABLE I

Apparent stability constants for binding of protons and metals to metal-chelating compounds corrected for use at 37°C and an ionic strength of 163 mM

Logarithmic values of the corrected stability constants are given. K_1, K_2 and K_3 represent stability constants for the binding of the ions to the unprotonated, mono- and diprotonated chelator, respectively. K_4 is a stability constant for the reaction $MC \cdot H_2O \rightleftharpoons MC \cdot OH + H$ (MC stands for a metal-chelator complex; electrical charges omitted for clarity).

Ions	K	log K; Chelators			
		ATP	EGTA	HEDTA	NTa
H^+	K_1	6.40	9.22	9.40	9.50
	K_2	3.81	8.65	5.16	2.42
	K_3		2.58		
Ca^{2+}	K_1	3.70	10.34	8.09	6.33
	K_2	1.95	5.10	1.23	
Mg^{2+}	K_1	4.02	5.10	5.69	5.15
	K_2	2.08	3.14	1.27	
Cd^{2+}	K_1	5.38	15.79	13.09	9.43
	K_2	1.67	10.38	2.29	11.92

ulin and 63.5 ± 2.6 nmol P_i/min per mg IOV protein and 1.34 ± 0.11 μM in the presence of 2 μg/ml calmodulin, respectively.

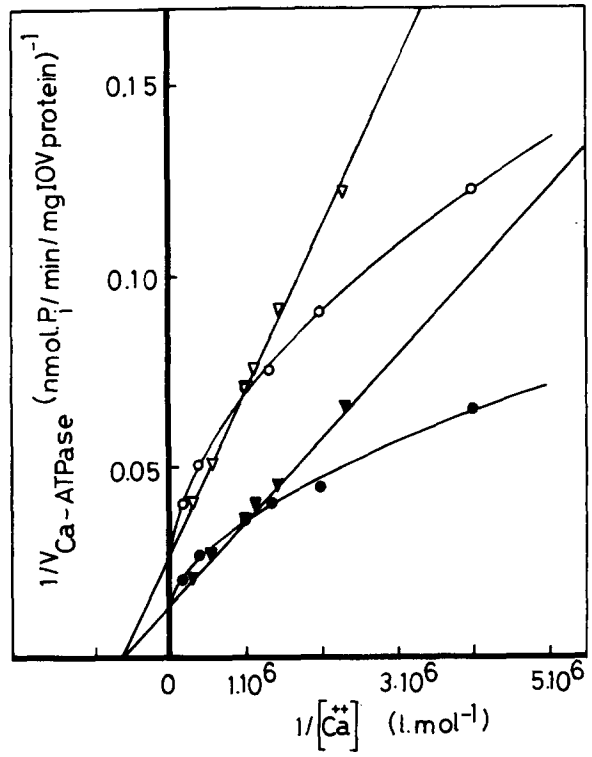

Fig. 3. Effect of recomputing free Ca^{2+} on the concentration dependence of the Ca^{2+}-ATPase activity in the absence of added Cd^{2+}. Lineweaver-Burk plots of control data taken from Fig. 1. Uncorrected data are represented by the circles. The triangles are the data after correction for the error in the free Ca^{2+} concentration represented in Fig. 2A. Open symbols without and closed symbols in the presence of 2 μg/ml added calmodulin.

Because the free Cd^{2+} concentration appears to decrease on raising the Ca^{2+} concentration, replotting of the data of Fig. 1 with recomputed values of free Ca^{2+} in the same way as has been done for the controls is of no use for the evaluation of the type of inhibition of the ATPase by Cd^{2+}. We therefore followed an other strategy for analyzing the experimental data of Fig. 1. If Cd^{2+} is a competitive inhibitor, the concentration dependence of the Ca^{2+}-ATPase activity in the presence of Cd^{2+} should obey the relation presented by Eqn. 2:

$$V = \frac{V_{max} \cdot [Ca^{2+}]}{K_{Ca}\left(1 + \frac{[Cd^{2+}]}{K_i}\right) + [Ca^{2+}]} \quad (2)$$

with $[Cd^{2+}]$ representing the free Cd^{2+} concentration and K_i, the dissociation constant of Cd^{2+} bound to the enzyme.

Eqn. 2 can be rearranged into:

$$X = \left(\frac{V_{max}}{V} - 1\right) \cdot \frac{[Ca^{2+}]}{K_{Ca}} - 1 = [Cd^{2+}]/K_i \quad (3)$$

in which X is an operational parameter that may be calculated from the experimentally determined rate of the Ca^{2+}-ATPase V at the corresponding free Ca^{2+} concentrations recomputed by CHELATOR. The values of V_{max} and K_{Ca} estimated from the recomputed control data of Fig. 3 have been used to calculate X. X is plotted against the corresponding recalculated value of the free Cd^{2+} concentration. Fig. 4A shows that the relation between X and $[Cd^{2+}]$ cannot be represented by a single straight line. Consequently, competitive inhibition of the ATPase activity by Cd^{2+} should be rejected.

In the case of non-competitive inhibition, the concentration dependence of the Ca^{2+}-ATPase should obey the relation given by Eqn. 4.

$$V = \frac{V_{max} \cdot [Ca^{2+}]}{\left(1 + \frac{[Cd^{2+}]}{I_i}\right)(K_{Ca} + [Ca^{2+}])} \quad (4)$$

Upon rearrangement an operational parameter Y can be defined that is linearly related to the free Cd^{2+} concentration.

$$Y = \left[V_{max} \cdot \frac{[Ca^{2+}]}{K_{Ca} + [Ca^{2+}]} \cdot \frac{1}{V}\right] - 1 = [Cd^{2+}]/K_i \quad (5)$$

From the same data used to calculate X, Y is calculated and plotted against corresponding values of the free Cd^{2+} concentration.

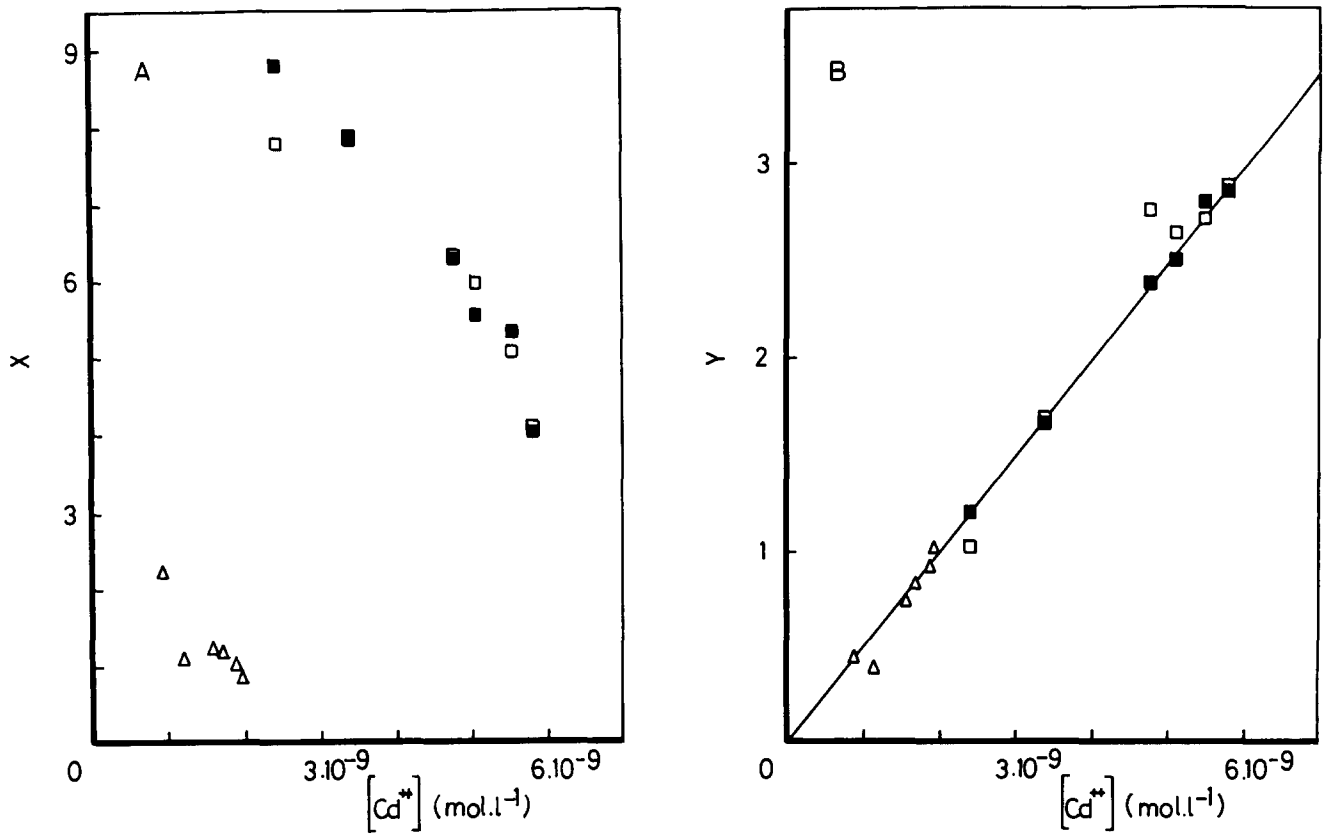

Fig. 4. Reanalysis of the effect of Cd^{2+} on the Ca^{2+}-ATPase activity represented in Fig. 1. The recomputed concentrations of Ca^{2+} and Cd^{2+} of Fig. 2 are used for the analysis. Analysis of the data from Fig. 1 according to a model for competitive inhibition (A) and non-competitive inhibition (B) of the Ca^{2+}-ATPase by Cd^{2+}. The data are plotted according to Eqns. 3 and 5, respectively, using a value for V_{max} of 34.5 nmol P_i/mg IOV-protein per min, and a K_{Ca} value of 1.46 μM. For meaning of symbols, see legend to Fig. 1.

Fig. 4B shows that the relation between Y and $[Cd^{2+}]$ can be described by a single straight line. Therefore, non-competitive inhibition of the Ca^{2+}-ATPase by Cd^{2+} is accepted as the most probable interaction. From the slope of the line a K_i for Cd^{2+} of 2 nM is calculated.

According to outcomes of calculations with CHELATOR it would be possible to prepare assay media in which free Cd^{2+} concentrations remain constant on raising that of Ca^{2+}. In these media the non-competitive character of Cd^{2+}-inhibition should be clearly demonstrated by Lineweaver-Burk plots of the Ca^{2+}-concentration dependence of the Ca^{2+}-ATPase, i.e., the intercepts of the lines with the abscissa should not be affected by Cd^{2+}, while those with the ordinate should increase, reflecting the decrease in V_{max} of the Ca^{2+}-ATPase on raising free Cd^{2+}. Fig. 5 shows that within experimental error the data obtained in media in which free Ca^{2+} and Cd^{2+} concentrations have been adjusted by means of CHELATOR fulfill that demand, both in the absence and presence of calmodulin. The lines drawn through the data points have been calculated for non-competitive inhibition of the Ca^{2+}-ATPase by using the value of 2 nM for the K_i of Cd^{2+} estimated from the data in Fig. 4B.

Discussion

The present study shows that Cd^{2+} is a non-competitive inhibitor of the red cell plasma membrane Ca^{2+}-ATPase activity and stresses the importance of a correct calculation of metal ion concentrations of media in which Ca^{2+}-dependent enzymes are assayed.

In media used by Verbost et al. [31] in their study of the effect of Cd^{2+} on Ca^{2+} uptake into IOVs, free ionic concentrations have been calculated and adjusted by the aid of a computer program in which stability constants are not corrected for temperature and ionic strength, and pH is interpreted as the negative logarithm of the H^+ concentration instead of the H^+ activity. As shown already before by Schoenmakers et al. [44] in such media free concentrations of Ca^{2+} may differ appreciably from those calculated. In this study we have calculated that this not only applies to calcium ions but to Cd^{2+} as well, as is shown in Fig. 2. Our present results show that because of that not only kinetic parameters of Ca^{2+}-dependent enzymes may be misevaluated, but also the mode of inhibition of Cd^{2+}.

Using media in which free Ca^{2+} and Cd^{2+} are not correctly calculated, Verbost et al. found that the inhibition of Ca^{2+} uptake into IOVs by Cd^{2+} may be

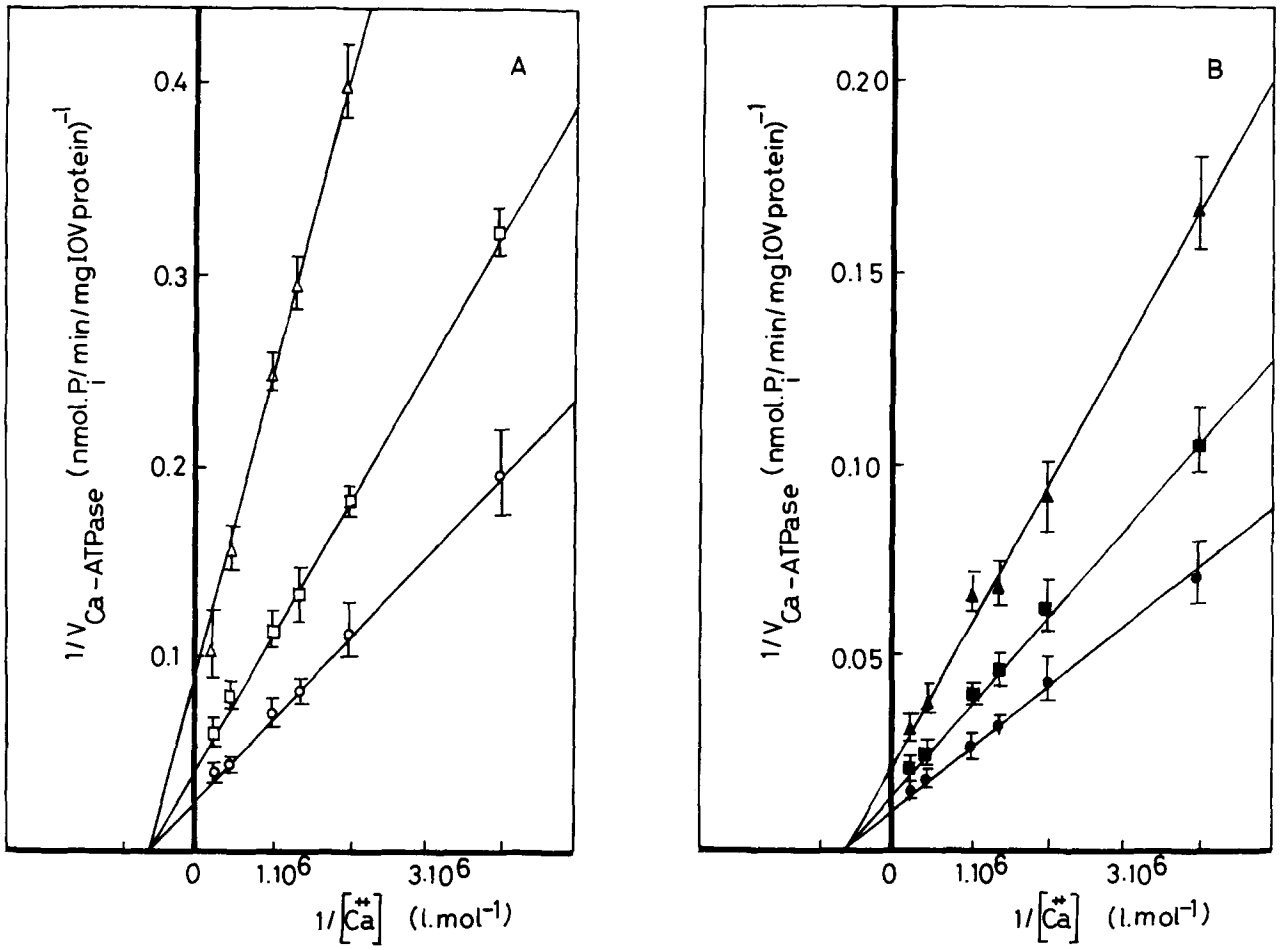

Fig. 5. Lineweaver-Burk plots of the Ca^{2+}-ATPase activity at of 0 (○), 1 (□) and 5 nM free Cd^{2+} (△). ATPase activities were assayed in media in which ionic concentrations were adjusted with CHELATOR according to Schoenmakers et al. [44]. (A) Without calmodulin (open symbols). (B) In the presence of 2 μg/ml added calmodulin (closed symbols). Each point represents the mean of five different experiments (each run in triplicate) and the bar represents the standard error of the mean.

ascribed to competition of Ca^{2+} and Cd^{2+} for binding to the transport site of the Ca^{2+} pump. Our finding that also the Ca^{2+}-ATPase activity, measured in these media, is apparently competitively inhibited by Cd^{2+} (Fig. 1) is in agreement with the notion that Ca^{2+} transport and ATP hydrolysis by the red cell Ca^{2+} pump are strictly coupled processes [3] and it also shows that the previously reported competitive inhibition of Ca^{2+} uptake into IOVs by Cd^{2+} can not be ascribed to a permeabilization of the membrane of the IOVs by Cd^{2+}. In such a case, namely, one would not expect that also the ATPase activity of the pump will be inhibited competitively by Cd^{2+} with the same K_i as found in Ca^{2+} uptake. Not being aware of the fact that free Ca^{2+} and Cd^{2+} concentrations are not properly calculated in these experiments one would easily accept that Cd^{2+} is a competitive inhibitor of the red cell Ca^{2+} pump, which, however, is not true.

Schoenmakers et al. [44] have shown that errors in the calculation of free Ca^{2+} may strongly influence the apparent kinetic behaviour of Ca^{2+}-dependent processes. For example the Ca^{2+}-concentration dependence of Na^+/Ca^{2+} exchange activity in isolated basolateral plasma membrane vesicles from intestinal cells of fish, which was measured by Flik et al. [46] in media prepared according to Verbost et al. could only be described adequately by a double Michaelis-Menten relationship with K_{Ca} values of 181 nM and 3.3 μM, respectively. When the experiment was performed using Ca^{2+} concentrations calculated with CHELATOR the data obeyed a single Michaelis-Menten relationship with a K_{Ca} of 1.21 μM [45]. Also the concentration dependence of the Ca^{2+}-ATPase is affected in a same way, as is shown in Fig. 3. The uncorrected data of the controls which have been fitted to single straight lines are in fact curvi-linear in shape, while after correction a much improved fit to straight lines is obtained. Due to the correction of the free Ca^{2+} concentrations, the value of K_{Ca} increases from 0.5 to 1.4 μM and V_{max} values become approx. 40% higher.

The recalculation of free metal concentrations in the media prepared according Verbost et al. revealed

that the free concentration of Cd^{2+} decreases appreciably on raising that of Ca^{2+} (Figs. 2B and 2C). This means that, in the range of Ca^{2+} concentrations used, the inhibition of the Ca^{2+}-ATPase by Cd^{2+} as shown in Fig. 1 may be appreciably underestimated at the higher free Ca^{2+} concentrations. Already intuitively one would expect that then the inhibition should be of a non-competitive type. Using the recomputed free Ca^{2+} and Cd^{2+} concentrations, we have analyzed the data of Fig. 1 according to models in which Cd^{2+} is either a competitive or a non-competitive inhibitor of the Ca^{2+}-ATPase. Comparison of Figs. 4A and 4B clearly shows that the experimental data can be fitted only to the model in which Cd^{2+} acts as a non-competitive inhibitor. The line drawn through the data points of Fig. 4B has been calculated by linear regression and from the slope of this line a K_i value for non-competitive inhibition of the Ca^{2+}-ATPase by Cd^{2+} of 2 nM is estimated. In order to confirm the outcomes of the above analysis we have determined the effect of Cd^{2+} on the Ca^{2+}-ATPase activity in media in which ionic concentrations of Ca^{2+} and Cd^{2+} were adjusted by making use of CHELATOR, i.e., under conditions that free Cd^{2+} concentrations remain constant upon raising the free Ca^{2+} concentration. The results of these experiments confirm that the inhibition of the Ca^{2+}-ATPase by Cd^{2+} is of the non-competitive type (Fig. 5). It is therefore most likely, that Cd^{2+} inhibits Ca^{2+} uptake into IOVs also non-competitively and that the competitive type of inhibition observed by Verbost et al. underlies an incorrect calculation of the free concentrations of Ca^{2+} and Cd^{2+} in the assay media they used. This conclusion differs from the outcomes of several studies dealing with the effect of Cd^{2+} on Ca^{2+}-dependent enzyme systems [32–34]. The inhibitory effect that was found in those cases appeared to be competitive. Åkerman et al. [39], however, also found that Cd^{2+} inhibits red cell Ca^{2+}-ATPase non-competitively, but only at micromolar concentrations of Cd^{2+}, whereas we found that cadmium ions inhibit the Ca^{2+}-ATPase activity in the nanomolar range. We have no explanation for this discrepancy in results.

Acknowledgements

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