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Inhibition of the sarcoplasmic reticulum (Ca^{2+} + Mg^{2+})-ATPase by Zn (II)

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Micromolar Zn^{2+} concentrations have been found to inhibit the (Ca^{2+} + Mg^{2+})-ATPase of sarcoplasmic reticulum vesicles solubilized with deoxycholate or Triton X-100 or made leaky with A23187 and of the purified enzyme. Kinetic studies on the dependence of this activity upon Zn^{2+} concentrations have been carried out at 25 °C and 37 °C under a variety of experimental conditions, such as absence of Mg^{2+} in the assay medium, different total Ca^{2+}, total Mg^{2+} and total ATP concentrations and at various total membrane protein concentrations. The concentrations of relevant kinetic species (free divalent cations, free ATP and ATP complexes) have been computed for all these assay conditions. As a result, we have found that the inhibition of the (Ca^{2+} + Mg^{2+})-ATPase activity is produced by free Zn^{2+}, with a value of the inhibition constant K_I of 8 ± 2 μM. In addition, Zn^{2+}·ATP can be used as an alternative substrate by this ATPase with a K_M value of 30 μM and with V_{max} of (2.0 ± 0.2) μmol ATP hydrolyzed per min per mg protein at 37 °C. In conclusion, our results suggest the existence of sites in the ATPase distinct to the high-affinity Ca^{2+} binding sites and to the Mg^{2+} subside in the catalytic center, to which binding of Zn^{2+} produces inhibition and a shift of the E_1/E_2 conformational equilibrium.

Papp et al. [1] have reported the presence of traces (1–2 nmol/mg protein) of several divalent transition metal ions in sarcoplasmic reticulum membrane preparations, Zn^{2+} among them. In addition, micromolar concentrations of Zn^{2+} were found to alter the reactivity of -SH groups of these membranes. In this regard it is to be noted that Zn^{2+} combines strongly with sulfur compounds [1,2]. Because most of the -SH groups of these membranes belong to the (Ca^{2+} + Mg^{2+})-ATPase, this result led to the suggestion that upon binding of Zn^{2+} to the membranes the conformation of the (Ca^{2+} + Mg^{2+})-ATPase is altered. The interaction of micromolar Zn^{2+} with sarcoplasmic reticulum membranes was previously shown by Madeira and Carvalho [3], who reported an apparent binding constant of 1.8–5 μM, that Zn^{2+} efficiently impairs Ca^{2+} binding to these membranes and that binding of Zn^{2+} to these membranes release H^+ (1.0–1.5 H^+ per bound Zn^{2+}). All these results suggest the possibility of a regulatory role of Zn^{2+} on functions carried out by the sarcoplasmic reticulum. However, this possibility has been neither rigorously demonstrated nor excluded up to date. The well-known effects of chelating agents, such as EGTA or EDTA, on the sarcoplasmic reticulum (Ca^{2+} + Mg^{2+})-ATPase activity and structure [4–7] may well be related to the removal of Zn^{2+} from these membranes, as suggested by Papp et al. [1]. In particular, the large effect of prolonged exposure of sarcoplasmic reticulum vesicles to EDTA or EGTA on the permeability properties of these membranes [7] suggests occurrence of gross structural changes upon binding of Zn^{2+} to the membranes, see above. In this regard, it is to be noted that although Zn^{2+} is considered to be a relatively non toxic element [8], industrial exposure by inhalation to zinc fumes is characterized by severe disorders of muscle physiology [9].

On the other hand, Zn^{2+} has been shown to be a potent inhibitor of several plasma membrane ATPases, e.g. Mg^{2+}-ATPase from the yeast *Candida albicans* [10], Mg^{2+}-ATPase from *Saccharomyces pombe* [11], bacterial H^+-ATPase [12] and erythrocyte Ca^{2+}-ATPase [13,14], whereas reported to be ineffective to others, such as the (Ca^{2+} + Mg^{2+})-ATPase of rat liver plasma membrane [15]. In this paper, we report that free Zn^{2+} is also a

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; Tes, 2-([2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino)ethanesulfonic acid; ATPase, adenosine triphosphatase; SDS, sodium dodecylsulfate.

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potent inhibitor of the $(Ca^{2+} + Mg^{2+})$-ATPase of sarcoplasmic reticulum membranes. It can be predicted that gaining a better knowledge of the effects of Zn^{2+} in sarcoplasmic reticulum membranes will be helpful to define the basic enzymology and structural characteristics of Zn^{2+} binding sites on ATPases, for the sarcoplasmic reticulum membrane is one of the membrane systems best characterized both at a biochemical and at a biophysical level. The $(Ca^{2+} + Mg^{2+})$-ATPase cyclically interconverts between conformations E_1 and E_2 [16] and, therefore, a shift of the E_1/E_2 conformational equilibrium is of potential regulatory relevance. Under defined experimental conditions fluorescein isothiocyanate has been shown to label the ATP binding site of the sarcoplasmic reticulum $(Ca^{2+} + Mg^{2+})$-ATPase [17–19]. It has also been extensively shown that the fluorescence of fluorescein covalently bound to the ATPase can be used to quantify the equilibrium between the E_1 and E_2 conformations of this enzyme [20–23], even though the extent of fluorescence change is relatively small (approx. 7–10% of total fluorescein fluorescence). In addition, this approach has become one of the most used to monitor the conformational state of the $(Ca^{2+} + Mg^{2+})$-ATPase. Thus, the putative effect of Zn^{2+} upon the conformational state of the $(Ca^{2+} + Mg^{2+})$-ATPase of sarcoplasmic reticulum has been studied using fluorescein-labeled ATPase.

In this paper we report kinetic studies carried out to obtain the mechanism and basic kinetic parameters of the inhibition of the sarcoplasmic reticulum $(Ca^{2+} + Mg^{2+})$-ATPase by Zn^{2+}, with particular attention to the putative shift of the E_1/E_2 equilibrium upon Zn^{2+} binding to the sarcoplasmic reticulum membrane.

Materials and Methods

Sarcoplasmic reticulum has been purified from rabbit (New Zealand White) hind leg muscle as indicated elsewhere [24]. $(Ca^{2+} + Mg^{2+})$-ATPase was purified from sarcoplasmic reticulum by affinity chromatography through Affi-Gel Blue as described by Gafni and Boyer [25]. The purity of the $(Ca^{2+} + Mg^{2+})$-ATPase was checked by SDS-gel electrophoresis (7.5% acrylamide) and found to be more than 95% pure from the absorbance at 550 nm of Coomassie blue-stained gels. Protein concentration was measured following the method of Lowry et al. [26], using bovine serum albumin as standard. The $(Ca^{2+} + Mg^{2+})$-ATPase activity has been determined by measuring the production of inorganic phosphate, following the method of Fiske and SubbarRow [27]. The linearity of the dependence of ATP hydrolysis upon time after starting the reaction has been checked in all the experimental conditions reported in this study. The standard assay conditions were as follows: 0.1 M Tes, 0.1 M KCl, 5 mM $MgCl_2$, 2.5 mM ATP and 10 μM Ca^{2+} (pH = 7.4) at 37 °C. The Ca^{2+}-independent ATPase activity was measured in the presence of 3.4 mM EGTA, and only those preparations showing a value of this activity lower than 10% of total uncoupled $(Ca^{2+} + Mg^{2+})$-ATPase activity were used in this study. The Ca^{2+}-independent Mg^{2+}-ATPase activity of our preparations of purified $(Ca^{2+} + Mg^{2+})$-ATPase was found to be negligible. 'Leakiness' of sarcoplasmic reticulum vesicles was assessed by measuring the $(Ca^{2+} + Mg^{2+})$-ATPase activity in the presence and in the absence of A23187 (0.04 mg per mg protein). Only those preparations showing a 3–4-fold stimulation of the $(Ca^{2+} + Mg^{2+})$-ATPase activity at 20–22 °C upon addition of A23187 have been used in this study. To prevent oxidation, solutions of Zn^{2+} were prepared immediately before use. Ca^{2+} uptake has been measured at 25 °C with $^{45}Ca^{2+}$ by Millipore filtration, using GSWP02500 Millipore filters. The assay medium used for Ca^{2+} uptake had the following composition: 0.1 M KCl, 0.1 mM $CaCl_2$, 5 mM $MgCl_2$, 2.5 mM ATP and 0.1 M Tes (pH = 7.4). The filters were washed twice with 10 ml of a cold (4 °C) solution containing: 150 mM KCl and 0.4 mM La^{3+}, then dissolved with ethyleneglycol monomethyl ether and counted.

The labeling of the enzyme with fluorescein isothiocyanate was done by incubation of both reactants in dark during 30 min at room temperature, at the molar ratio of 5 mol of fluorescein isothiocyanate per mol of protein monomer at a pH = 7.4, as described in Ref. 24. Fluorescein isothiocyanate was added from a freshly made dimethyl formamide solution and unreacted fluorescein isothiocyanate was removed by passage through a Sephadex G-50 chromatography column (8 cm length $\times$ 1 cm diameter). Using an absorption coefficient of 80000 $M^{-1} \cdot cm^{-1}$ for fluorescein bound to the sarcoplasmic reticulum, the extent of labeling determined after passage through the Sephadex G-50 column was determined as in Froud and Lee [23] and typically ranged from 0.9 to 1 mol fluorescein per mol of $(1.1 \cdot 10^5$ dalton) protein unit of sarcoplasmic reticulum. Fluorescence measurements were carried out at 37 °C with a spectrofluorimeter Hitachi-Perkin Elmer, model 650-40, using excitation and emission wavelengths of 475 and 515 nm, respectively.

Computation of free and complex species concentrations. The computer program used in this study is that described by Perrin and Sayce [28], with slight modifications needed to run it on a IBM PC microcomputer. The species routinely analyzed were: free ATP, free Ca^{2+}, free Mg^{2+}, free Zn^{2+}, $Ca^{2+} \cdot ATP$, $Mg^{2+} \cdot ATP$ and $Zn^{2+} \cdot ATP$. The following association constants (K_a) at pH = 7.5 have been used [29,30]: log K_a $(Ca^{2+} \cdot ATP) = 3.93$; log K_a $(Mg^{2+} \cdot ATP) = 4.61$ and log K_a $(Zn^{2+} \cdot ATP) = 4.85$.

Chemicals. Bovine serum albumin, sucrose, phospho*enol*pyruvate, fluorescein isothiocyanate, ATP, NADH, deoxycholate, phenylmethylsulfonyl fluoride, EGTA, Tes and Sephadex G-50 were purchased from

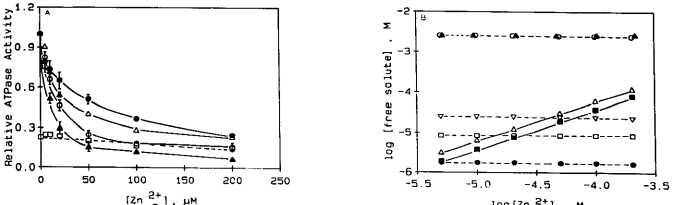

Fig. 1. Panel A. Dependence of the total Mg^{2+}-ATPase activity of sarcoplasmic reticulum upon total Zn^{2+} concentration (Zn^{2+}_T) at 37°C. The assay mixture contained 5 mM $MgCl_2$, 2.5 mM ATP, 10 μM Ca^{2+}, 100 mM KCl and 100 mM Tes (pH = 7.4). The reaction was started with the addition of 20–30 μg protein per ml. Sarcoplasmic reticulum vesicles were made leaky with A23187 (0.04 mg per mg protein) (●——●) and solubilized with deoxycholate (0.5 mg per mg protein) (Δ——Δ) or Triton X-100 (0.24 mM) (○——○). The measured Ca^{2+}-independent Mg^{2+}-ATPase activity of the sarcoplasmic reticulum preparations used in this study was 0.3 ± 0.1 and 0.9 ± 0.1 μmol ATP hydrolyzed per min per mg protein at 22 and 37°C, respectively. The data obtained with sealed sarcoplasmic reticulum vesicles (□- - - - - -□) and purified enzyme (▲——▲) are also included. The Ca^{2+}-dependent Mg^{2+}-ATPase activity of solubilized membranes and of the purified enzyme ranged between 8 and 12 μmol ATP hydrolyzed per min per mg protein in different preparations, and this value was taken as the unity. The relative ATPase activity of sealed sarcoplasmic reticulum vesicles is referred to an average value of 10 μmol ATP hydrolyzed per min per mg protein. Panel B. Computed free species concentrations. The concentrations of kinetically relevant species in the assay mixture of Panel A were computed as indicated in the Materials and Methods. Different symbols stand for: ○- - - - - -○, Mg^{2+}·ATP; ●- - - - - -●, Ca^{2+}·ATP; Δ——Δ, Zn^{2+}·ATP; ▽— — — —▽, free ATP; □— — —□, free Ca^{2+}; ▲— — — —▲, free Mg^{2+}; and ■——■, free Zn^{2+}.

Sigma. Dithiothreitol, pyruvate kinase, lactate dehydrogenase and the ionophore A23187 were obtained from Boehringer Mannheim. Triton X-100 is a trade mark of Rohm & Haas, Co. $^{45}CaCl_2$ was purchased from New England Nuclear. Affi-Gel blue (100–200 mesh) was obtained from Bio-Rad. $LaCl_3$ and $ZnSO_4$ were obtained from Merck. Ethyleneglycol monomethyl ether and toluene were purchased from Carlo Erba and other chemicals used were of the highest analytical purity available.

Results and Discussion. The dependence of the (Ca^{2+} + Mg^{2+})-ATPase activity of sarcoplasmic reticulum upon the concentration of Zn^{2+} is presented in Fig. 1 (panel A). It can be observed that this cation behaves as a potent inhibitor of this activity, both of solubilized or Ca^{2+} permeable membranes and of purified enzyme. Sealed sarcoplasmic reticulum vesicles, on the contrary, are much less sensitive to inhibition by Zn^{2+}. It is to be noted that it is in these latter conditions where the Ca^{2+}-independent Mg^{2+}-ATPase activity of our membrane preparations is higher with respect to the (Ca^{2+} + Mg^{2+})-ATPase activity, and amounts to approx. 30–40% of the total Mg^{2+}-ATPase activity. Therefore, it follows from these data that the Ca^{2+}-independent Mg^{2+}-ATPase activity of these membranes is not significantly inhibited by total concentrations of Zn^{2+} lower than 50 μM. In addition, the possibility that the inhibition of uncoupled or solubilized sarcoplasmic reticulum membranes by Zn^{2+} could bear a significant contribution of inhibition of the Ca^{2+}-independent Mg^{2+}-ATPase activity can safely be ruled out.

The level of steady-state Ca^{2+} accumulation of our preparations of sarcoplasmic reticulum vesicles has been found to be approx. 80–90 nmol Ca^{2+} per mg protein. We have found only a slight decrease (approx. 20–25%) of Ca^{2+} accumulation within sarcoplasmic reticulum vesicles in the presence of 1 mM Zn^{2+}. Comparison of these results with those given in Fig. 1 shows that the extent of inhibition of the (Ca^{2+} + Mg^{2+})-ATPase by Zn^{2+} is somewhat larger than the observed decrease on net Ca^{2+} uptake. Thus, these results suggest that the rate of passive Ca^{2+} efflux could be altered by this cation as well. Direct attempts to measure this efflux using vesicles preloaded with Ca^{2+} have been unsuccessful so far, because the concentrations of Zn^{2+} that significantly inhibit the (Ca^{2+} + Mg^{2+})-ATPase activity of sealed sarcoplasmic reticulum vesicles (i.e., in the millimolar range) produce a large aggregation (flocculation) of these membranes preloaded with 1 mM $CaCl_2$.

The dependence of the apparent $K_{0.5}$ of inhibition upon the protein concentration between 20 and 90 μg protein per ml has also been studied. Because there is no significant change of the $K_{0.5}$ for protein concentrations up to 90 μg protein per ml (results not shown), the free concentration of Zn^{2+} does not need to be corrected under the experimental conditions used in the Fig. 1. In Panel B of Fig. 1, the computed concentrations of free divalent cations (Ca^{2+}, Mg^{2+} and Zn^{2+}) and of M^{2+}·ATP (free ATP, Mg^{2+}·ATP, Ca^{2+}·ATP and Zn^{2+}·ATP) corresponding to the experimental conditions of the panel A of Fig. 1 are presented. Upon inspection of these data it is evident that the changes of

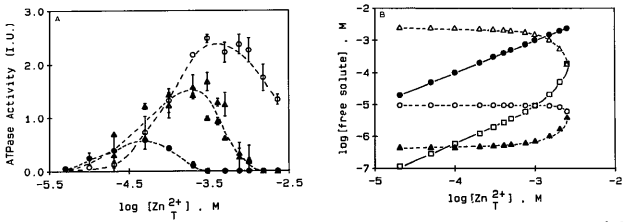

Fig. 2. Panel A. Dependence of Zn^{2+}-ATPase activity of sarcoplasmic reticulum membranes upon the total concentration of Zn^{2+} (Zn^{2+}_T) at different total ATP concentrations: ATP 0.5 mM (●); ATP 2.5 mM (▲, △) and ATP 5 mM (○). Sarcoplasmic reticulum vesicles were made leaky with A23187 (0.04 mg per mg protein) (△, ○, ●) and solubilized with DOC (0.5 mg per mg protein) (▲). The assay mixture did not contain Mg^{2+}. Other experimental conditions as indicated in Fig. 1 (Panel A). I.U. stands for international units, i.e. μmol ATP hydrolyzed per min per mg protein. Panel B. Computed free species concentrations in the assays of Panel A at a total ATP concentration of 2.5 mM. Different symbols stand for: △— — —△, free ATP; ○— — —○, $Ca^{2+}\cdot$ATP; ●———●, $Zn^{2+}\cdot$ATP; ▲— — —▲, free Ca^{2+}; and □———□, free Zn^{2+}.

free Mg^{2+}, free Ca^{2+}, free ATP, $Mg^{2+}\cdot$ATP and $Ca^{2+}\cdot$ATP in the concentration range of Zn^{2+} that inhibits the ATPase activity are negligible. However, two molecular species, namely, free Zn^{2+} and $Zn^{2+}\cdot$ATP, undergo large changes of concentration under these experimental conditions. Therefore, we have attempted to clarify which of them accounts for the observed inhibition.

To address this point we have carried out titrations of the ATPase with Zn^{2+} in the absence of Mg^{2+}. The results obtained at different ATP concentrations are presented in Fig. 2 (Panel A). The curves obtained are biphasic. The concentrations of kinetically relevant species have been computed in these experimental conditions as well, and the results obtained for a ATP concentration of 2.5 mM are plotted in Fig. 2 (Panel B). The analysis of these data shows that the activation of the ATPase produced by lower Zn^{2+} concentrations is likely related to the use of $Zn^{2+}\cdot$ATP as substrate, for $Ca^{2+}\cdot$ATP, the other putative substrate does not increase in this concentration range. In addition, the inhibition afforded by higher Zn^{2+} concentrations is likely due to the raise of free Zn^{2+} concentration above 1 μM, for neither free Ca^{2+}, nor free ATP significantly change (the concentration of Ca^{2+} even increases) in the concentration range of total Zn^{2+} that largely inhibit the ATPase activity. Let us note that the major changes of computed free ATP and $Ca^{2+}\cdot$ATP concentrations occur once the ATPase activity is almost completely inhibited.

Because at the onset of the inhibition of the ATPase by Zn^{2+} in the experiments shown in Fig. 2 the concentration of $Zn^{2+}\cdot$ATP is still rising, the V_{max} using $Zn^{2+}\cdot$ATP as a substrate cannot be directly estimated only from these data. Therefore, we have carried out a

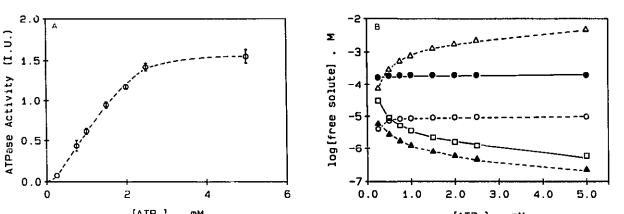

Fig. 3. Panel A. Dependence upon total ATP concentration (ATP_T) of Zn^{2+}-ATPase activity at a fixed concentration of total Zn^{2+} (200 μM). Sarcoplasmic reticulum vesicles (10–15 mg protein per ml) were solubilized by a 15 min incubation with deoxycholate (0.5 mg per mg protein). The assay mixture contained 10 μM Ca^{2+}, 100 mM KCl, 100 mM Tes (pH = 7.4), 200 μM Zn^{2+}, 20–26 μg protein per ml and ATP added at the concentrations indicated. Assay temperature 37 °C. Panel B. Computed free species concentrations in the assay mixture. Different symbols stand for: △— — —△, free ATP; ○— — —○, $Ca^{2+}\cdot$ATP; ●———●, $Zn^{2+}\cdot$ATP; ▲— — —▲, free Ca^{2+}; and □———□; free Zn^{2+}.

TABLE I

Computed free species concentrations

The assay mixture contained 10 μM Ca^{2+}, 100 mM KCl, 100 mM Tes (pH = 7.4) and ATP, Mg^{2+} and Zn^{2+} were added at the concentrations indicated in the table. The maximum change in concentration of Mg^{2+}·ATP is 5.84%, 2.43% and 1.20% for the 1, 2.5 and 5 mM total ATP series, respectively.

Added species			Computed free species						
ATP (mM)	Mg^{2+} (mM)	Zn^{2+} (μM)	Mg^{2+}·ATP (mM)	Ca^{2+}·ATP (μM)	Zn^{2+}·ATP (μM)	ATP (μM)	Mg^{2+} (mM)	Ca^{2+} (μM)	Zn^{2+} (μM)
1	2	0	0.975	1.66	0	23.4	1.02	8.34	0
		5.0	0.972	1.65	3.11	23.2	1.03	8.35	1.89
		10.0	0.969	1.64	6.20	23.1	1.03	8.36	3.80
		20.0	0.963	1.63	12.4	22.8	1.04	8.37	7.65
		50.0	0.946	1.58	30.5	22.0	1.05	8.42	19.5
		75.0	0.932	1.54	45.2	21.4	1.07	8.46	29.8
		100.0	0.918	1.51	59.6	20.8	1.08	8.49	40.4
2.5	2	0	2.470	1.70	0	24.0	2.53	8.30	0
		5.0	2.470	1.70	3.15	24.0	2.53	8.30	1.85
		10.0	2.470	1.69	6.29	23.9	2.53	8.31	3.71
		20.0	2.460	1.69	12.6	23.8	2.54	8.31	7.45
		50.0	2.440	1.66	31.2	23.5	2.56	8.34	18.8
		100.0	2.410	1.63	61.9	22.9	2.59	8.37	38.1
5	10	0	4.970	1.71	0	24.3	5.03	8.29	0
		5.0	4.970	1.71	3.16	24.3	5.03	8.29	1.84
		10.0	4.970	1.71	6.32	24.2	5.03	8.29	3.68
		20.0	4.960	1.71	12.6	24.2	5.04	8.29	7.38
		50.0	4.940	1.70	31.5	24.0	5.06	8.30	18.5
		100.0	4.910	1.68	62.7	23.7	5.09	8.32	37.3

titration of the ATPase activity with ATP in the absence of Mg^{2+} and the results obtained are presented in Fig. 3 (Panel A). The computed concentrations of kinetically relevant species in these experimental conditions are presented in the Panel B of Fig. 3. These data show that the concentration of Zn^{2+}·ATP only slightly changes above a total ATP concentration of 0.25 mM, whereas that of Ca^{2+}·ATP only show a slight change in the concentration range of ATP studied herein. Therefore, the increase of activity observed in Fig. 3 (panel A) as the total ATP concentration raises appears to be related to the concomitant decrease of free Zn^{2+} concentration, because the parallel increase of free ATP and the decrease of free Ca^{2+} concentrations would, at most, have some inhibitory effects on the ATPase, see, for example, Refs. 23, 31 and 32. From the results presented in Figs. 2 and 3 the values of the free Zn^{2+} concentrations needed to inhibit 50% of the maximum ATPase activity ($K_{0.5}$) can be directly obtained in different experimental conditions. These values of $K_{0.5}$ range between 2 and 5 μM. A good agreement between $K_{0.5}$ of inhibition obtained from different kinetic series is, thus, observed. Hill plots of the data of the inhibitory phase of Fig. 2 and of the data of Fig. 3 versus free Zn^{2+} concentration (not shown) yields slopes of approx. 2.0 ± 0.2, thus, indicating the existence of positive cooperativity in the inhibition process. Additionally, these data show that Mg^{2+}, up to 5 mM, has not as significant influence on the binding of Zn^{2+} to the ATPase. Therefore, Zn^{2+} does not appear to compete under these experimental conditions for the kinetically relevant Mg^{2+} binding sites in this enzyme.

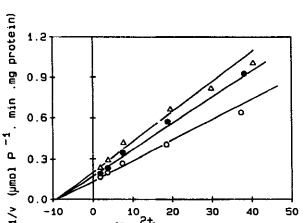

Fig. 4. Dixon plot of inhibition by free Zn^{2+} of the ($Ca^{2+} + Mg^{2+}$)-ATPase activity. Sarcoplasmic reticulum vesicles (10–15 mg protein per ml) were solubilized upon 15 min incubation with deoxycholate (0.5 mg per mg protein). The assay mixture contained 10 μM Ca^{2+}; 100 mM KCl; 100 mM Tes (pH = 7.4) and ATP, Mg^{2+} and Zn^{2+} were added as indicated in Table I. Symbols: △——△, ATP = 1 mM, Mg^{2+} = 2 mM; ●——●, ATP = 2.5 mM, Mg^{2+} = 5 mM; and ○——○, ATP = 5 mM, Mg^{2+} = 10 mM. The reaction was started with the addition of 20–27 μg of protein per ml and was stopped after 4 min as indicated in the Materials and Methods. Assay temperature: 37 °C.

Having in mind the value of $K_{0.5}$ of inhibition by free Zn^{2+} referred to above and that the peak of the bell-shaped curves of Fig. 2 (panel A) is reached at free Zn^{2+} concentrations close to 0.6 μM (see Fig. 2, panel B, and results not shown) we can estimate a value of V_{max} of 2.0 $\pm$ 0.2 μmol/min per mg protein for the ATPase using Zn^{2+}-ATP as substrate at 37°C. In addition, Hill plots (not shown) of the data of Fig. 2 versus log [$Zn^{2+} \cdot$ ATP], calculated as indicated in the methods at the ionic conditions of every experimental data, yields slopes of approx. 1.8 $\pm$ 0.3, thus, showing the presence of positive cooperativity in the saturation by the substrate. This observation is in contrast to the apparent lack of positive cooperativity in the dependence of the ATPase activity using $Mg^{2+} \cdot$ ATP as the substrate [33]. From the abscissae intercept of the Hill plots of these data we have estimated a value for the apparent K_M of $Zn^{2+} \cdot$ ATP of 30 μM. This value is only about 4–10-fold higher than the value of K_M reported for $Mg^{2+} \cdot$ ATP as the substrate [31].

That the inhibition of the ATPase is produced by interaction of Zn^{2+} with inhibitory binding sites, and not by binding to a subsite in the catalytic center was further assessed carrying out the titration of the ATPase by Zn^{2+} in the presence of different $Mg^{2+} \cdot$ ATP concentrations. The results obtained are presented in Fig. 4, as a Dixon plot. Table I lists the computed concentrations of kinetically relevant species in these experimental conditions. These data strongly support the existence of an inhibitory binding site for Zn^{2+} in the ATPase, because the inhibition is uncompetitive with the substrate $Mg^{2+} \cdot$ ATP. The value of K_i that can be obtained from this plot, 8 $\pm$ 2 μM, which is in reasonably good agreement to the $K_{0.5}$ values of inhibition by Zn^{2+} given above.

The possibility that Zn^{2+} competes with the low-affinity binding sites for Ca^{2+}, whose saturation inhibits this enzyme, has been considered. We have, thus, carried out titrations of the ATPase activity with high Ca^{2+} concentrations, i.e. from 10 μM to the millimolar range, in the absence and in the presence of concentrations of Zn^{2+} that inhibit this enzyme. The results obtained are presented in Fig. 5. Hill plots of the data (given in the inset of Fig. 5) reveal that there is only a slight effect of Zn^{2+} on the $K_{0.5}$ of inhibition by Ca^{2+} and there is not any significant effect on the cooperativity of this process. Therefore, Zn^{2+} does not appear to inhibit the ATPase by binding to these Ca^{2+} sites.

In addition, the possibility that binding of Zn^{2+} to the $(Ca^{2+} + Mg^{2+})$-ATPase shifts the conformational E_1/E_2 equilibrium has been studied. This possibility is strongly suggested by the positive cooperativity of the inhibition process (see above). Fluorescein labeled $(Ca^{2+} + Mg^{2+})$-ATPase has been shown to monitor the major conformational states of this enzyme [20–23], in particular the E_1 form shows about 7–10% lower intensity of fluorescence than the E_2 form. Table II summarizes the effect of Zn^{2+} on the fluorescence of labelled sarcoplasmic reticulum $(Ca^{2+} + Mg^{2+})$-ATPase. It can be readily observed that Zn^{2+} 50 μM to 100 μM, quenches by approx. 4% the fluorescence of fluorescein, this effect is being little affected by the presence of 5 mM $MgCl_2$ in the buffer (results not shown). At Zn^{2+} concentrations of 200 μM or higher the intensity of fluorescence of fluorescein continuosly decays as a function of time at a rate of approx. 1.45% per min.

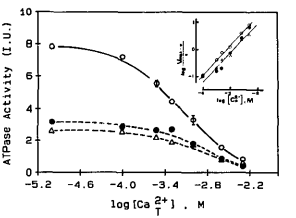

Fig. 5. Dependence upon total Ca^{2+} concentration (Ca^{2+}_t) of the $(Ca^{2+} + Mg^{2+})$-ATPase activity. Sarcoplasmic reticulum vesicles (10–15 mg protein per ml) were solubilized upon 15 min incubation with deoxycholate (0.5 mg per mg protein), in the absence (○———○) and in the presence of 50 μM (●— — —●) and 100 μM (△— — —△) Zn^{2+}. Inset: Hill plots of the data shown in the figure. The assay mixture contained 10 μM Ca^{2+}, 5 mM $MgCl_2$, 2.5 mM ATP, 100 mM KCl, 100 mM Tes (pH = 7.4). The reaction was carried out 37°C and started with the addition of 20–26 μg of protein per ml.

TABLE II

Effect of Zn^{2+} on the fluorescence of fluorescein labelled $(Ca^{2+} + Mg^{2+})$-ATPase

Buffer: 100 mM KCl, 50 μM EGTA and 100 mM Tes (pH = 7.4). For the experiments involving the addition of two ions, these were sequentially added to the cuvette, so that the fluorescence readings were taken after each addition. Data are averages from triplicated measurements. The average variation of $\Delta F/F_0$ between different samples was less than $\pm$1%; F_0 means the fluorescence intensity in the absence of added ligands (control value).

Experimental conditions	($\Delta F/F_0$) (%)
– No addition	0
– 200 μM Ca^{2+}	–4.0
– 200 μM VO_3^-	+2.2
– 50 μM Zn^{2+}	–4.3
– 75 μM Zn^{2+}	–3.1
– 100 μM Zn^{2+}	–2.5
– (50 μM Zn^{2+} + 200 μM Ca^{2+})	–4.9
– (50 μM Zn^{2+} + 200 μM VO_3^-)	–2.9
– (75 μM Zn^{2+} + 200 μM Ca^{2+})	–5.3
– (75 μM Zn^{2+} + 200 μM VO_3^-)	–1.8
– (100 μm Zn^{2+} + 200 μM Ca^{2+})	–4.7
– (100 μM Zn^{2+} + 200 μM VO_3^-)	–1.2
– (200 μM VO_3^- + 200 μM Ca^{2+})	–3.5

Thus, the effect of Zn^{2+} on fluorescein fluorescence cannot be appropriately measured under these experimental conditions. In addition, these results suggest that the enzyme is unstable in the presence of these concentrations of Zn^{2+}. Table II also shows that addition of 200 μM Ca^{2+} after Zn^{2+} (50 to 100 μM) produces a minor effect on fluorescein fluorescence. The effect of Ca^{2+} in the absence of Zn^{2+} produced an effect similar to that reported in the literature by several groups (see the Table II). Vanadate (200 μM) was found to revert, only partially, the effects of this divalent cation on the fluorescence of fluorescein (see also the Table II). The effect of addition of 200 μM vanadate alone in our experimental conditions is included in Table II as well. Taken together these results clearly suggest that Zn^{2+}, in the concentration range that inhibit this enzyme, shifts the (Ca^{2+} + Mg^{2+})-ATPase towards an E_1-alike conformational state.

These results, thus, support the existence of sites in the ATPase, to which binding of Zn^{2+} produces a strong inhibition, in parallel to a conformational shift to an E_1-alike conformation. The possibility that this inhibition and conformational perturbation of the ATPase could be due to an indirect effect attributable to a putative surface potential change resulting upon Zn^{2+} binding to the sarcoplasmic reticulum membranes is unlikely because: (i) the ATP binding site is located far from the lipid/water interface [34] and (ii) there is only a very limited adsorption of this cation on the sarcoplasmic reticulum membrane, as inferred from the negligible effect of protein concentration on the $K_{0.5}$ of inhibition.

On the other hand, the fact that Zn^{2+} shifts the ATPase towards and E_1-alike conformation and the lack of kinetic competition with the substrate or with low-affinity Ca^{2+} binding sites suggest the possibility that Zn^{2+} binds to the sites of high affinity to Ca^{2+} in the E1 conformation, the inhibition then resulting from displacement of Ca^{2+} from these sites. However, this seems to be unlikely on the basis of the lack of effect of micromolar Zn^{2+} on sealed sarcoplasmic reticulum vesicles. Because only upon membrane disruption the inhibitory effect of micromolar Zn^{2+} concentrations is readily evident, it seems reasonably to assume that the inhibitory binding sites to which Zn^{2+} binds are not accessible to the outer medium of sealed sarcoplasmic reticulum vesicles. On the basis of the current knowledge of this enzyme (see, for example, Refs. 31, 32 and 35) there are two different divalent cations binding centers of this type in the ATPase: The Ca^{2+} binding sites in the E_2 conformation (i.e. low-affinity Ca^{2+} binding sites) and occluded Ca^{2+} binding sites. The data presented in this paper allow to exclude the first possibility (see above). Binding to occluded Ca^{2+} binding sites or to a different and more specific inhibitory site can at present account for the kinetic data reported in this paper. In addition, to rationalize the kinetic data reported herein for the inhibition of the ATPase by Zn^{2+} it has to be assumed that upon binding of this cation to the protein the overall rate of $E_1 \rightleftharpoons E_2$ cyclic interconversions during the catalysis must be lowered. Regarding the inhibition of the (Ca^{2+} + Mg^{2+})-ATPase of sealed sarcoplasmic reticulum by higher Zn^{2+} concentrations, it seems that the basic mechanism is largely different, for it is irreversible (i.e., it is not reverted by dialysis of Zn^{2+}) and it leads to precipitation of these membranes, e.g. it likely derives from enzyme denaturation.

In conclusion, free micromolar Zn^{2+} concentrations inhibit the activity of the sarcoplasmic reticulum (Ca^{2+} + Mg^{2+})-ATPase upon binding to sites different to the high-affinity Ca^{2+} binding sites or to the Ca^{2+} binding sites on E_2 conformation and different to the Mg^{2+} subsite in the catalytic center. These sites are accessible to Zn^{2+} on solubilized sarcoplasmic reticulum membranes and on purified (Ca^{2+} + Mg^{2+})-ATPase, but not on tightly coupled sarcoplasmic reticulum in the experimental conditions used in this study. Upon binding of Zn^{2+} to these sites the (Ca^{2+} + Mg^{2+})-ATPase is shifted towards an E_1-alike conformation on the basis of the properties of the fluorescence of fluorescein. In addition, we have found that Zn^{2+}-ATP can be used as a substrate by this ATPase.

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