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Inhibition of plasma membrane Ca^{2+}-ATPase by CrATP. LaATP but not CrATP stabilizes the Ca^{2+}-occluded state

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Abstract

The bidentate complex of ATP with Cr^{3+}, CrATP, is a nucleotide analog that is known to inhibit the sarcoplasmic reticulum Ca^{2+}-ATPase and the Na^+,K^+-ATPase, so that these enzymes accumulate in a conformation with the transported ion (Ca^{2+} and Na^+, respectively) occluded from the medium. Here, it is shown that CrATP is also an effective and irreversible inhibitor of the plasma membrane Ca^{2+}-ATPase. The complex inhibited with similar efficiency the Ca^{2+}-dependent ATPase and the phosphatase activities as well as the enzyme phosphorylation by ATP. The inhibition proceeded slowly ($T_{1/2}$=30 min at 37 °C) with a K_i=28±9 μM. The inclusion of ATP, ADP or AMPPNP in the inhibition medium effectively protected the enzyme against the inhibition, whereas ITP, which is not a PMCA substrate, did not. The rate of inhibition was strongly dependent on the presence of Mg^{2+} but unaltered when Ca^{2+} was replaced by EGTA. In spite of the similarities with the inhibition of other P-ATPases, no apparent Ca^{2+} occlusion was detected concurrent with the inhibition by CrATP. In contrast, inhibition by the complex of La^{3+} with ATP, LaATP, induced the accumulation of phosphoenzyme with a simultaneous occlusion of Ca^{2+} at a ratio close to 1.5 mol/mol of phosphoenzyme. The results suggest that the transport of Ca^{2+} promoted by the plasma membrane Ca^{2+}-ATPase goes through an enzymatic phospho-intermediate that maintains Ca^{2+} ions occluded from the media. This intermediate is stabilized by LaATP but not by CrATP.

Keywords: PMCA; Erythrocyte; Chromium; CrATP; Lanthanum; Occlusion; Calcium

1. Introduction

The plasma membrane Ca^{2+}-ATPase (PMCA) is a P-type ATPase present in all eukaryotic cells and is ultimately responsible for fine-tuning the internal Ca^{2+} concentrations needed for cell survival [1–3]. In mammals, this enzyme is found as four major isoforms (PMCA1–4), which are differentially expressed in tissues [2,4]. PMCA shows a high stringency for ATP as the energy donor for Ca^{2+} transport and shuttles one Ca^{2+} ion to the extracellular side for each hydrolysis cycle [5,6]. On the basis of kinetic data and by analogy with the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA), a simplified transport scheme has been proposed (Fig. 1). During the catalytic cycle, the enzyme alternates between two conformers, E_1 and E_2 [7–12]. E_1 has a high affinity for Ca^{2+} and is readily phosphorylated by ATP, while E_2 has a low affinity for Ca^{2+} and can be phosphorylated by Pi. The addition of calmodulin, phosphatidylserine, dimethylsulfoxide up to 10%, or controlled tryptic cleavage dislocates internal self-inhibitory peptides and increases the maximal velocity of ATP hydrolysis, as well as the affinity of the enzyme for Ca^{2+} (see [2,13] for reviews). The amino-acid sequence of the enzyme has been used for modeling secondary and tertiary structures and for

Abbreviations: SERCA, sarcoplasmic reticulum Ca^{2+}-ATPase; pNPP, *p*-nitrophenyl phosphate; PMCA, plasma membrane Ca^{2+}-ATPase; EGTA, ethylene glycol bis (β-aminoethyl ether),*N*,*N*,*N*′,*N*′-tetraacetic acid; CrATP, bidentate chromium(III) ATP complex; HEPES, (*N*-[2-Hydroxyethyl] piperazine-*N*′- [2-ethanesulfonic acid]); PMSF, *p*-methyl sulfonylfluoride; DTT, dithiothreitol

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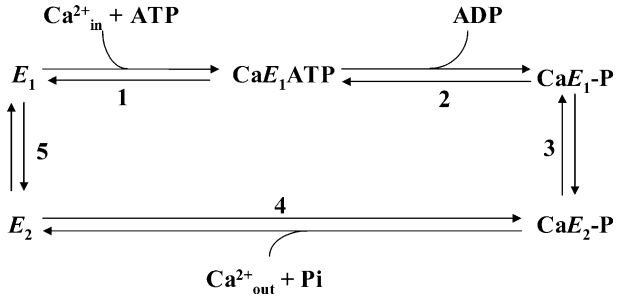

Fig. 1. Transport cycle of the plasma membrane Ca^{2+}-ATPase.

identifying some important residues [7,14]. However, a global perception of the changes that PMCA undergoes during the catalytic cycle is missing due to the difficulty in obtaning the high amounts of protein necessary for structural studies. Most of the details of these changes are inferred from crystallographic studies of SERCA. Nevertheless, in spite of the fact that both enzymes transport Ca^{2+}, there exist important structural, regulatory and kinetic differences between them. Among the most marked differences are: the PMCA is about 30 kDa bigger; in accordance with the different stoichiometry of transport, only one site for Ca^{2+} can be identified in the structure (see Discussion); PMCA does not hydrolyze nucleotides other than ATP [1,15,16]; it is modulated by calmodulin [2,13]; and the complex La^{3+}-ATP favors the accumulation of phosphoenzyme in PMCA [17–21] but inhibits it in SERCA [21–23].

In SERCA ATPase, the phosphorylation by ATP renders both Ca^{2+} ions unable to exchange with the free Ca^{2+} of the medium [24–29]. This process of occlusion (the term "occluded ion" refers to a state in which the ions are unable to reach the aqueous media on either side of the membrane) has also been observed with the Na^+ ion in the Na,K-ATPase [30–33]. Occlusion seems to occur in the E_1P conformation (Fig. 1) and it is proposed as a requirement for the mechanism of ion translocation across the membrane coupled to the ATP hydrolysis. Several important residues that form the Ca^{2+} binding site I of SERCA do not exist in the amino-acid sequence of PMCA. Mutation of some of these residues in SERCA renders enzymes that are unable of occlude any calcium. Those observations raise the question if PMCA would be able to occlude calcium. Up to now, the occlusion of Ca^{2+} has not been demonstrated in this enzyme.

The substrate analog CrATP (bidentate chromium(III) ATP complex) has been described as an inhibitor of Na,K-ATPase [34–37] and SERCA [38–44]. The inhibition of these enzymes goes together with the occlusion of Na^+ and Ca^{2+}, respectively. The mixture of La^{3+}-ATP, which forms the complex LaATP, inhibits PMCA by slowing the rate of transformation of E_1P into E_2P (step 3 in Fig. 1) and therefore accumulating E_1P [17,19,20], which is proposed to be the conformation that occludes calcium. In order to validate the assumption that the occlusion occurs in the PMCA, we have studied the CrATP complex as an inhibitor of the PMCA and attempted to determine whether CrATP or LaATP complexes are able to stabilize the occlusion of Ca^{2+} within the enzyme.

2. Experimental procedures

2.1. Reagents

[^{32}P]Pi was obtained from Instituto de Pesquisas Energéticas e Nucleares (São Paulo, SP, Brazil). The enzymes used for the radioactive labelling of ATP were purchased from Boehringer Mannheim. [^{45}Ca]Ca^{2+} was obtained from DuPont (Boston, MA, USA). Sepharose-CaM was purchased from Pharmacia Biotech (Sweden). Other reagents were purchased from Sigma (St. Louis, MO, USA).

2.2. Erythrocyte plasma membranes

Calmodulin-depleted ghost membranes from pig erythrocytes ("ghosts") were prepared as described in [45]. Briefly, blood was spun down for 10 min at 4 °C and 5000×*g*. The supernatant and the coat of white cells were gently removed by aspiration and the pellet (erythrocytes) resuspended in 20 mM Tris–HCl, pH 7.4, 130 mM KCl and 0.6 mg ml^{-1} PMSF. The suspension was washed twice with the same solution by centrifugation as above. The pellet was isotonically lysed by freezing at −70 °C and thawing at room temperature. Then, the lysed cells were washed (3×) for 10 min at 7000×*g* with 5 mM HEPES, pH 7.4, 1 mM EDTA and 0.6 mg ml^{-1} PMSF. Finally, the pellet was resuspended in a solution containing 10 mM Tris–HCl, pH 7.4, 130 mM KCl, 0.5 mM $MgCl_2$ and 0.05 mM $CaCl_2$ and centrifuged (2×) at 700×*g* for 10 min, resuspended in the same solution and stored under liquid nitrogen. The protein concentration was determined according to Lowry et al. [46].

2.3. Purification of Ca^{2+}-ATPase

Purified plasma membrane Ca^{2+}-ATPase (PMCA) was prepared as described by Caroni et al. [47], modified by Pasa et al. [48]. The Ca^{2+}-ATPase was solubilized from pig erythrocyte membranes by stirring for 10 min at 4 °C with polydocanol at 1 mg mg^{-1} protein in a medium containing 1 mM $MgCl_2$, 0.5 mM KCl, 0.6 M sucrose, 40 μM $CaCl_2$, 2 mM DTT, 2 μg ml^{-1} PMSF and 20 mM HEPES, pH 7.4. This suspension was centrifuged at 47,000×*g* for 30 min and the supernatant was applied on a calmodulin-sepharose affinity column. The column was washed with 20 vol. of the same solution but with 0.05 mg ml^{-1} polydocanol and eluted with a solution containing 20 mM HEPES, 0.6 M sucrose, 0.5 mM KCl, 0.05 mg ml^{-1} polydocanol, 3 mM $MgCl_2$, 2 mM EGTA, 50 μM $CaCl_2$, 2 mM DTT and 0.5 mg ml^{-1} mg/ml phosphatidylcholine. Enzyme concentration was determined according to Peterson [49]. Purified enzyme

displays a specific-calmodulin-activated ATP hydrolysis that varies from 1 to 2 μmol Pi mg^{-1} min^{-1} at 37 °C, under standard conditions (see below).

2.4. CrATP synthesis

CrATP was synthesized from the sodium salt of ATP and $CrCl_3$ as described by DePamphilis and Cleland [50]. The bidentate isomer was isolated through an cation-exchange column (Dowex 50-H^+ resin) and the product confirmed and quantified ($\varepsilon_{610\ nm}$=20 • M^{-1} cm^{-1}) by the absorption spectrum between 400 and 800 nm [50].

2.5. [γ-^{32}P]ATP synthesis

[γ-^{32}P]ATP was synthesized as described by Walseth and Johnson [51].

2.6. Ca^{2+}-ATPase activity

The Ca^{2+}-ATPase activity was assayed at 37 °C in a medium containing: 20 mM Tris–HCl (pH 7.4), 100 mM KCl, 2 mM $MgCl_2$, 0.25 mM EGTA, enough $CaCl_2$ to obtain 20 μM free Ca^{2+} and 50 μg ml^{-1} protein (ghost) or 2.5 μg ml^{-1} (purified PMCA), in the presence of 2 μg ml^{-1} calmodulin. Free calcium concentrations were calculated as described by Fabiato and Fabiato [52] using the dissociation constant of the Ca-EGTA complexes reported by Schwartzenbach et al. [53]. The reactions were initiated by the addition of [γ^{32}P] Na-ATP (4×10^5 cpm μmol^{-1}) and stopped after the hydrolysis of 5%–10% of the ATP by the addition of 0.2 vol. of 1 N HCl to the reaction mixture. The reaction product (^{32}Pi) was quantified by the Grubmeyer and Penefsky [54] method.

2.7. pNPPase activity

pNPP hydrolysis at 37 °C was measured in a medium containing: 20 mM Tris–HCl, pH 7.4, 100 mM KCl, 2 mM $MgCl_2$, 1 mM ouabain, 0.25 mM EGTA, enough $CaCl_2$ to give 0.5 μM free Ca^{2+} and 50 μg ml^{-1} erythrocyte membrane. The reaction was initiated by the addition of 3 mM pNPP. After 60 min, the reaction was terminated by the addition of one volume NaOH 0.3 N, followed by two volumes of Milli-Q water. The pNP was estimated spectrophotometrically at 425 nm using an extinction coefficient of 1.1×10^4 M^{-1} cm^{-1}.

2.8. Phosphorylation by [γ^{32}P]ATP

The phosphorylation of PMCA in ghosts (4.1 mg ml^{-1}) was carried out at room temperature for 5 s, in a medium containing 100 mM KCl, 2 mM $MgCl_2$, 20 mM Tris–HCl, pH 7.4, 30 μM $CaCl_2$ and 2 μg ml^{-1} calmodulin. It was started by the addition of 10 μM [γ^{32}P] Na-ATP (6×10^8 cpm μmol^{-1}) and stopped with 10 vol. of cold acid solution (10% (w/v) TCA and 10 mM H_3PO_4). The suspension was centrifuged for 5 min at 2000×*g* and the pellet washed 3 times with the same cold acid solution. After the last centrifugation, the pellet was solubilized in 1% (w/v) SDS. Aliquots of this solution were used to determine protein concentration according to Lowry et al. [46], and radioactivity was measured on a liquid scintillation counter. The phosphorylation of SERCA from sarcoplasmic reticulum vesicles (125 μg ml^{-1}) was carried out at room temperature, for 3 s, in a medium containing 100 mM KCl, 5 mM $MgCl_2$, 20 mM Tris–HCl, pH 7.0, and 5 μM $CaCl_2$. The reaction was started by the addition of 50 μM [γ^{32}P] Na-ATP (6×10^8 cpm μmol^{-1}) and stopped with 20 vol. of cold acid solution (50 mM PCA and 10 mM H_3PO_4). The suspension was filtered trough Millipore filters (HAWP, 0.45 μm) and washed 3 times with 3.5 ml of cold acid solution. The radioactivity on the filters was estimated by liquid scintillation counting.

2.9. $^{45}Ca^{2+}$ occlusion

The occlusion of $^{45}Ca^{2+}$ promoted by CrATP in sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) was measured as described by Visen and Andersen [55]. Sarcoplasmic reticulum vesicles (100 μg ml^{-1}) were incubated with 120 μM CrATP at room temperature, for 4 h, in a medium containing 100 mM NaCl, 80 mM Tris–HCl, pH 7.0, 5 μg/mg of protein of A23187 and 113 μM [^{45}Ca]$CaCl_2$ (4.4×10^4 cpm $pmol^{-1}$). The reaction was stopped with 20 vol. with a cold solution containing 80 mM Tris–HCl, pH 7.0, and 1 mM EGTA. The suspension was filtered in Millipore filters (HAWP, 0.45 μm) and washed three times with 3.5 ml of the same cold solution. The radioactivity on the filters was estimated by liquid scintillation counting.

Occlusion of Ca^{2+} in PMCA was assayed by incubating red cell ghosts (0.6 mg ml^{-1}) at room temperature in 0.25 ml of a medium containing 100 μM CrATP, 20 mM Tris–HCl, pH 7.4, 100 mM KCl, 5 μg A23187/mg protein, and 13 μM [^{45}Ca]$CaCl_2$ (3.8×10^3 cpm $pmol^{-1}$) in the presence or absence of 1 mM $MgCl_2$. After 4 h incubation, the reaction was stopped by the addition of 15 ml ice-cold washing solution (20 mM Tris–HCl, pH 7.4, 100 mM KCl, 30 μM $CaCl_2$). Immediately, the suspensions were vacuum-filtered through a Millipore filter (HAWP; 0.47 μm, 1 in. diameter) and washed three times with 3.5 ml of the same solution. The time interval between stopping the reaction and the end of washing was 8–10 s. The assays of occlusion with LaATP were carried out in the conditions described above for CrATP except that $MgCl_2$ concentration was 0.2 mM and a mixture of 100 μM ATP plus 200 μM $LaCl_3$ was used to start the reaction. For the LaATP assays, the reaction times were 10 s. Considering the high amount of protein necessary for the occlusion assays with ghosts, the suspension resulting from each reaction was split into two and filtered on separate Millipore filters. This procedure decreased the load on each filter and increased considerably

the washing flux. For the ghost assays, the values obtained with ATP instead of CrATP or LaATP were used as blanks and substracted from the values obtained with the substrate analogs. These blanks were usually 10 to 20% of the total occluded Ca^{2+} values obtained with LaATP.

3. Results

Preincubation of red cell ghosts with 50 μM CrATP inhibited the Ca^{2+}-dependent ATPase activity (Fig. 2). This effect was irreversible, since extensive washing of the ghosts was unsuccessful in releasing the enzyme inhibition (Fig. 3). The inhibition was slow ($T_{1/2}$=20 to 30 min), as shown for the inhibition of other ion transport ATPases by CrATP [34–44]. Interestingly, inhibition was independent of the presence (50 μM) or absence of Ca^{2+} in the preincubation medium (Table 1). This suggests that phosphorylation of the Ca^{2+}-ATPase by the ATP analog was not necessary for enzyme inhibition. However, the omission of $MgCl_2$ during preincubation caused a pronounced reduction in inhibition.

Purified CrATP is stable in the experimental conditions used here, but we cannot completely exclude the presence of some free Cr^{3+} in the solutions. When 50 μM $CrCl_3$ was used, only a marginal inhibition was observed (Fig. 2), suggesting that any contamination by Cr^{3+} would have a negligible effect on the measured activities.

The complex CrATP is considered to be an analog of MgATP for several P-ATPases, causing irreversible inhibition by binding to the substrate site. This also seems to be the case for the PMCA inhibition since ATP, ADP or AMPPNP, when present in the preincubation medium,

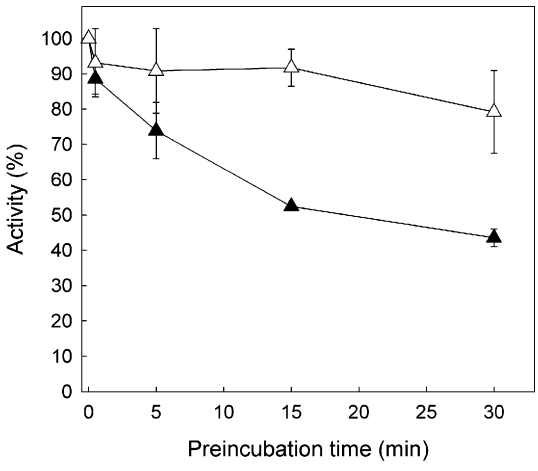

Fig. 2. Effect of $CrCl_3$ and CrATP on the Ca^{2+}-ATPase activity of PMCA. Ghosts (1.0 mg ml^{-1}) were preincubated with (△) 50 μM $CrCl_3$ or (▲) 50 μM CrATP at 37 °C in a medium containing 100 mM KCl, 2 mM $MgCl_2$, 20 mM Tris–HCl, pH 7.4, and 30 μM $CaCl_2$. At different times, 25 μL aliquots were withdrawn to assay Ca^{2+}-ATPase activity in the presence of 1 mM [γ^{32}P]ATP as described in "Experimental procedures" (values are means±S.D. of 3 determinations). Ca^{2+}-ATPase activity in the absence of inhibitors was 2.08±0.06 μml mg^{-1} h^{-1}.

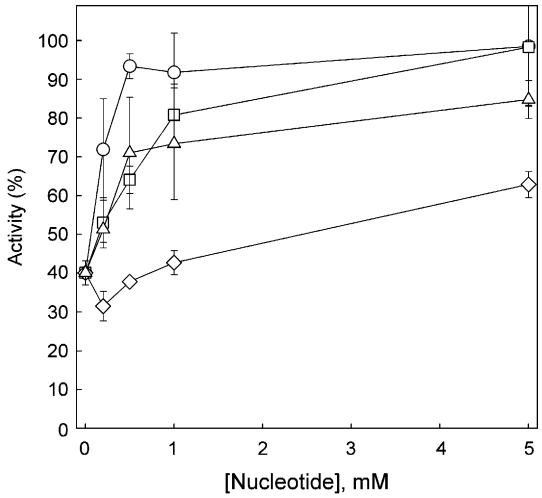

Fig. 3. Protection of nucleotides against inhibition of PMCA by CrATP. Ghosts (1.0 mg/ml) were preincubated with 50 μM CrATP in a medium containing 100 mM KCl, 2 mM $MgCl_2$, 20 mM Tris–HCl, pH 7.4, and 30 μM $CaCl_2$ for 30 min at 37 °C, in the presence of (○) ATP, (□) ADP, (△) AMP-PNP or (◇) ITP. After preincubation, ghosts were washed (×3) by centrifugation (14,000×*g*—5 min) with a medium containing 100 mM KCl, 2 mM $MgCl_2$ and 20 mM Tris–HCl (pH 7.4) to remove excess CrATP and nucleotides; then Ca^{2+}-ATPase activity was assayed in the presence of 1 mM ATP, as described under "Experimental procedures" (values are means±S.D. of 3 determinations). Ca^{2+}-ATPase activity in the absence of inhibitors was 1.96±0.04 μml mg^{-1} h^{-1}.

protected the enzyme from inhibition (Fig. 3). ATP was the best protector, while ITP, which is not a substrate of PMCA, was ineffective.

The same rate of inhibition was observed in ghosts and in purified PMCA (Fig. 4), suggesting that the presence of other proteins or even the phospholipids of the membranes did not interfere with the mechanism of inhibition. Furthermore, in both preparations, the rate of inhibition by CrATP was almost the same irrespective of the ATP concentration (2 μM or 1 mM) used in the assay of ATPase activity. The small difference in the inhibition observed in these two assay conditions was probably due to the fact that 2 μM ATP was not enough to stop the action of residual CrATP present in the assay medium. Considering the dilution of the preincubation medium into the medium used

Table 1
Effect of ion composition on inhibition by CrATP

Preincubation condition	Activity (%)
Without CrATP	100
50 μM $CaCl_2$+2 mM $MgCl_2$	40.8±6.9
50 μM $CaCl_2$+2 mM $MgCl_2$+1 μg/ml CaM	40.7±5.0
0.3 mM EGTA+2 mM $MgCl_2$	47.1±5.6
EDTA 0.1 mM, without $MgCl_2$	79.8±1.8

Ghosts (1 mg ml^{-1}) were preincubated with 200 μM CrATP at 37 °C in the medium containing 100 mM KCl, 20 mM Tris–HCl, pH 7.4, and the indicated additions. After 30 min, aliquots were taken for Ca^{2+}-ATPase measurements as in Fig. 2. The composition of the media used for the ATPase measurements was adjusted to compensate for the ionic composition of the preincubation medium (values are means±S.D. of 4 determinations).

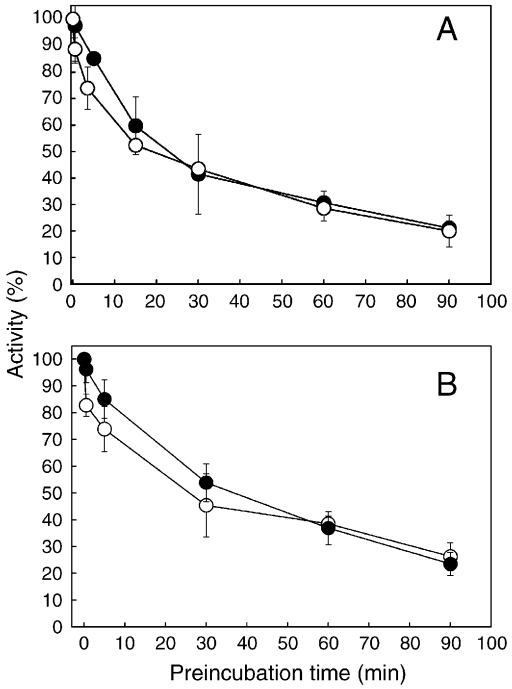

Fig. 4. Time dependence of the inhibition of PMCA by CrATP. Ghosts (1.0 mg/ml) (A) or purified PMCA (50 μg/ml) (B) were preincubated with 50 μM CrATP at 37 °C in a medium containing 100 mM KCl, 2 mM $MgCl_2$, 20 mM Tris–HCl, pH 7.4, and 30 μM $CaCl_2$. At different times, 25 μL aliquots were withdrawn to assay Ca^{2+}-ATPase activity in the presence of (○) 2 μM ATP or (●) 1 mM ATP as described under "Experimental procedures" (values are means±S.D. of 3 determinations). Ca^{2+}-ATPase activities in ghost in the absence of inhibitors were 0.35±0.03 and 2.12±0.04 μml mg^{-1} h^{-1} at 2 μM and 1 mM ATP, respectively. The activities for the affinity purified enzyme were 15.8±0.7 and 293±29 μmol mg^{-1} h^{-1} at 2 μM and 2 mM ATP, respectively.

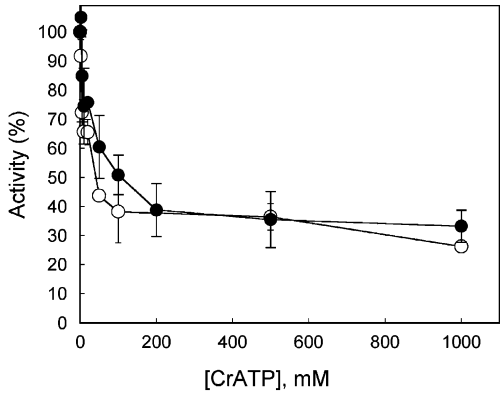

Fig. 5. CrATP concentration dependence of Ca^{2+}-ATPase inhibition. Ghosts (1.0 mg/ml) were preincubated with CrATP for 30 min at 37 °C in a medium containing 100 mM KCl, 2 mM $MgCl_2$, 20 mM Tris–HCl, pH 7.4, and 30 μM $CaCl_2$. After preincubation, ghosts were washed (×3) by centrifugation (14,000×*g*—5 min) with a medium containing 100 mM KCl, 2 mM $MgCl_2$ and 20 mM Tris–HCl (pH 7.4) to remove excess CrATP, and then Ca^{2+}-ATPase activity was assayed in the presence of 1 mM (●) or 2 μM (○) ATP as described under "Experimental procedures" (values are means±S.D. of 3 determinations). Ca^{2+}-ATPase activities in ghost in the absence of inhibitors were 0.30±0.03 and 1.91±0.05 μml mg^{-1} h^{-1} at 2 μM and 1 mM ATP, respectively.

for ATPase measurements, the CrATP concentration in this media was no more than 2.5 μM, enough for some slow inhibition.

The apparent affinity of Ca^{2+}-ATPase for CrATP was quite high since 21 μM was enough for 50% of the maximal inhibition observed after 30 min incubation (Fig. 5). Almost the same affinity was observed when ATPase assays were done with 2 μM ATP (Fig. 5, open circles). The similarity of inhibition at high and low ATP concentrations suggests that the regulatory site of ATP was not occupied by CrATP. If this site were occupied, two classes of catalytically competent enzymes (i.e. with the catalytic site free) would be observed, one with the regulatory site free (capable of full activation) and another with the regulatory site blocked, fully active at 2 μM ATP but not at 1 mM ATP. In this case, the relative inhibition would be greater at 1 mM than at 2 μM ATP. This behavior was not observed (Figs. 5 and 6). The inhibition was constant throughout the range of ATP concentrations used; in other words, the degree of activation by ATP at the regulatory site was the same in both the control and inhibited enzyme. The Km values for ATP at catalytic and regulatory sites were the same in the presence and absence of CrATP, as expected for an irreversible inhibitor that blocks all enzymatic activity once it is bound.

In support of the mechanism proposed above (irreversible binding to the catalytic site), the preincubation of ghosts with CrATP inhibited in parallel not only the Ca^{2+}-dependent ATPase but also the phosphatase activity and the Ca^{2+}-dependent phosphorylation by ATP (Fig. 7).

A singular characteristic of the inhibition of SR Ca^{2+}-ATPase and the Na,K-ATPase by CrATP is the trapping of the enzymes in a conformation where the transported ion,

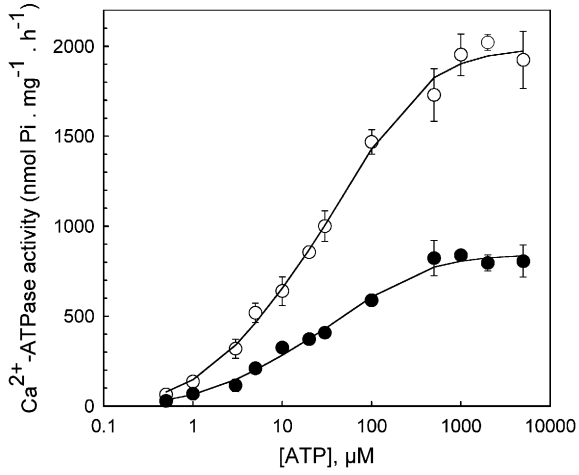

Fig. 6. ATP dependence of the Ca^{2+}-ATPase activity of PMCA inhibited by CrATP. Ghosts (6.0 mg/ml) were preincubated with (●) or without (○) 30 μM CrATP for 15 min at 37 °C as described in Fig. 5. Preincubation was stopped with 5 mM Mg ATP. Ghosts were washed (3×) in saline medium, and Ca^{2+}-ATPase activity was assayed as described under "Experimental procedures" (values are means±S.D. of 3 determinations).

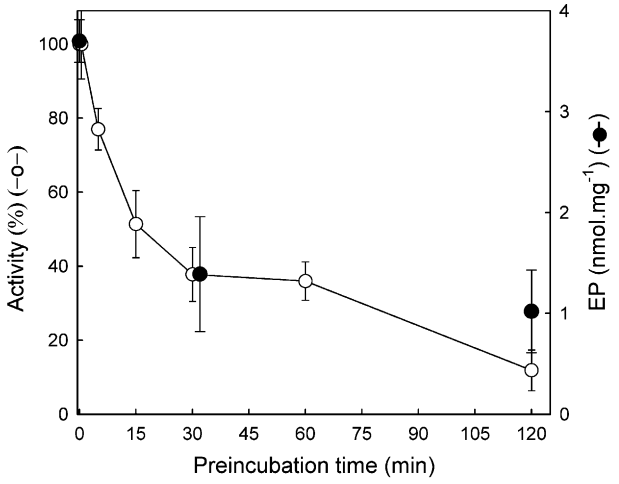

Fig. 7. Inhibition by CrATP of pNPPase activity and phosphorylation by ATP. Ghosts (10 mg ml^{-1}) were preincubated with 200 μM CrATP at 37 °C as described in Fig. 1, then washed in saline medium and resuspended in the same medium. pNPPase activity (○) and phosphorylation by 10 μM [γ^{32}P]ATP (●) were assayed as described under "Experimental procedures" (values are means ± S.D. of 3 determinations). In the absence of CrATP, the pNPPase activity was 106 ± 11 nmol of Pi mg^{-1} h^{-1} and the phosphoenzyme level was 3.6 ± 0.2 pmol mg^{-1}.

Ca^{2+} and Na^{+}, respectively, is occluded [32,38–42,55]. The occlusion is characterized by the retention of the ions after extensive washing of the enzyme. In contrast, the PMCA seems not to occlude significant Ca^{2+} after the CrATP treatment (Table 2). The theoretical maximal for the amount of occluded Ca^{2+} can be estimated from the maximal values of phosphorylation levels ($7.2 \pm 0.23 \times 10^{-3}$ nmol/mg) formed from ATP in the enzyme not treated with CrATP and taking into account a stoichiometry of one Ca^{2+} transported per ATP hydrolyzed. The experimental values were about 10% of that value. In contrast, La-ATP, an ATP analog that stabilizes the enzyme in the phosphorylated conformation, proved to be very efficient in the occlusion. The values in this case was about 50% higher than the maximal expected. A possible explanation for this is that some Ca^{2+} is occluded by the enzyme in a non-phosphorylated state. Values of phosphoenzyme higher than the reported here have been quoted in the literature [20], so it is possible that our values represent an underestimation of the amount of active enzyme. It is important to mention that the occlusion of Ca^{2+} in SERCA obtained in the inhibition with CrATP occurs without phosphorylation of the enzyme [39,40]. It is conceivable that part of the occluded Ca^{2+} observed in this work is not associated to a phosphorylated enzyme but to an "La-ATP-enzyme" complex.

4. Discussion

The main results of this work show that the CrATP is an efficient irreversible inhibitor of the plasma membrane Ca^{2+}-ATPase, as in other P-ATPases, but it does not lead to the occlusion of Ca^{2+}. In contrast, LaATP, which stabilizes a phosphorylated form of the enzyme (E_1PCa in Fig. 1), keeps the Ca^{2+} bound and inaccessible from the medium, indicating that Ca^{2+} occlusion must occur during the Ca^{2+} transport by the PMCA Ca^{2+}-ATPase.

The β,γ-bidentate CrATP is a stable complex of ATP with Cr^{3+}. The ionic radius of Cr^{3+} (0.58 Å) is close to that of Mg^{2+} (0.65 Å), which permits Cr^{3+} to form covalent bonds with the oxygen atoms of phosphates β and γ. Four other coordination bonds are formed with water molecules. The complex CrATP can be considered similar to the MgATP complex, which is the true substrate of P-ATPases. The CrATP reacted slowly and irreversibly with the plasma membrane Ca^{2+}-ATPase, which is indicative of the formation of coordination bonds with amino-acid residues of the enzyme. These residues appear to belong to the ATP catalytic site. The principal evidence that supports this idea is the protective effect of ATP, ADP and AMPPNP, together with the low performance of ITP, which is not a substrate of PMCA. Accordingly, the other enzymatic activities of the PMCA, phosphatase and ATP phosphorylation, were inhibited with the same efficiency as ATP hydrolysis (Fig. 7).

All the transport P-ATPases studied so far have two Km vales for ATP [56–58]. One reflects the occupation of the catalytic site, with a high affinity for ATP, in the range of a few μM. A second, in the order of 0.1 to 0.3 mM, is associated with a notable increase in ATPase activity. This activation is commonly referred as "occupation of the regulatory ATP site", though there is no structural evidence for the existence of a second nucleotide site. When analyzed on the hypothesis of two sites for ATP, CrATP inhibition of PMCA seems to be caused by binding to the ATP catalytic site alone. If a regulatory site were also affected, a lower

Table 2
CrATP or LaATP-induced Ca^{2+} occlusion in SERCA and PMCA

Condition	Measured Ca^{2+} occluded (nmol/mg)
SERCA + CrATP	2.11 ± 0.20 (102)
PMCA + CrATP (−$MgCl_2$)	$0.81 \pm 0.07 \times 10^{-3}$ (11)
PMCA + CrATP (+$MgCl_2$)	N D (0)
PMCA + LaATP	$11.30 \pm 0.70 \times 10^{-3}$ (156)

The values of Ca^{2+} occlusion were obtained by incubating sarcoplasmic reticulum vesicles or ghosts with CrATP or LaATP, respectively, in the medium containing ^{45}Ca–Ca^{2+}, as described in detail in "Experimental procedures". In parenthesis are the percent of occlusion levels in relation to the theoretical levels calculated from the maximal phosphoenzyme levels obtained for each enzyme and considering a stoichiometry of 2 Ca^{2+} per phosphorylation site in SERCA and 1 Ca^{2+} in PMCA. The maximal phosphoenzyme level for SERCA was 1.05 ± 0.02 nmol mg^{-1}. It was attained with 50 μM [γ^{32}P]ATP as substrate as described in "Experimental procedures". The maximal level of phosphoenzyme for PMCA in ghost was $7.2 \pm 0.23 \times 10^{-3}$ nmol mg^{-1}. It was attained as described in [20] using a mixture of 10 μM [γ^{32}P]ATP plus 100 μM $LaCl_3$ to start the reaction (5 s at 37 °C) and in the presence of 0.05 mM $CaCl_2$, 0.5 mM $MgCl_2$, 20 mM Tris–HCl, pH 7.4, and 100 mM KCl. (ND, nondetected; values are means ± S.D. of 3 determinations).

degree of activation at high ATP concentrations should be observed. The results of Figs. 3–5 show that this is not the case. If analyzed under the hypothesis of only one ATP site, the activation at high concentrations of ATP would reflect the acceleration of the cycle by the occupancy of the same site by ATP after ADP lives. In this case, our results can be interpreted as an irreversible binding of CrATP to this site, impairing the functioning of the enzyme.

The existence of a second Mg^{2+} site was suggested for the SERCA based on crystallographic [28,60] and kinetic studies [55,61]. One Mg^{2+} coordinates with the Asp351 side chain and the γ-phosphate, for the in-line attack, and a second seems to stabilize the ADP conformation. The fact that free Mg^{2+} was needed for a better inhibition of PMCA by CrATP suggests that there exists a site for Mg^{2+} independent of the Mg^{2+} complexed with ATP. Considering that the same dependence of inhibition by CrATP on Mg^{2+} has also been related in SERCA [55] and Na,K-ATPase [59], the requirement of a second Mg^{2+} ion in the mechanism of catalysis may be a common fact. This second Mg^{2+} would be involved in the transfer of phosphate to the enzyme [28].

During active transport, the Ca^{2+}-ATPase binds a calcium ion on one side of the membrane, translocates it across the membrane, and releases it at the other side. This process is driven by the hydrolysis of ATP, which promotes the necessary conformational changes in the protein structure (Fig. 1). This can only be achieved by a device in which the calcium ion has access to either side of the membrane. The possibility that the ion in the enzyme has access to both sides simultaneously is not plausible, since this would allow calcium to leak across the barrier. Alternatively, either a single conformational change closes the site of entrance and opens the pathway to the exit at the opposite side of the membrane, or the translocation is realized in two steps, where the first closes the entrance and then a second step opens the exit gate. In this case, between the two steps, the enzyme has both gates closed and the ion is trapped (occluded) between them. This last mechanism has been shown clearly in the Na,K-ATPase [30–33] and in the Ca^{2+}-ATPase of sarcoplasmic reticulum [24–29]. The occlusion in these systems has been characterized either with the use of CrATP [32,38–42,55] or under restricted experimental conditions. The results of this work suggest that occlusion also occurs in the PMCA.

From the kinetic studies, it is suggested that in the normal cycle, the occlusion occurs only after the phosphate transfer, producing E_1P[Ca]. The occlusion by CrATP would be theoretically possible because of the formation of a very stable CrATP-E complex similar to the transitional state of the phosphorylation reaction. It is not clear why the CrATP does not occlude Ca^{2+}. The amino acids involved in the binding of ATP and in the catalysis in SERCA are fully conserved in the PMCA. It is possible that the transitional state formed by CrATP in PMCA fixes a protein conformation with the entrance gate for calcium not completely closed. An alternative would be that the PMCA does not occlude calcium during the catalytic cycle. However, this does not seem to be the case, since the LaATP complex, which forms a stable E_1P phosphoenzyme, allows the occlusion of Ca^{2+} (La-E_1P[Ca] conformation). Whether this occlusion exists in the normal cycle is not possible to assess but considering the results from other transport systems, we speculate that the occlusion seem to be an obligatory step of all these ion transport ATPases. In this regard, LaATP may become a useful tool for future studies of the structure–function relationships of the plasma membrane Ca^{2+}-ATPase.

Data from the inhibition of SERCA clearly show that during occlusion of calcium, there is no phosphorylation by CrATP in this enzyme [39,40]. In PMCA, the inhibition occurs even in the absence of Ca^{2+}, suggesting that phosphorylation is unnecessary for the inhibition of this enzyme. It was not possible to determine whether the γ-phosphate of CrATP was transferred to Asp475 in the active site, as it is with MgATP. The high specific activity of [γ-^{32}P]CrATP needed for such experiments made them impractical.

The sequence of PMCA has extensive homology with SERCA. In the membrane domain, however, only M1–M6 align with SERCA ATPase [14]. Neither M7–M8 transmembrane portions nor the small connecting loops show any homology. In addition, PMCA exhibits a long C-terminal sequence, not present in the SERCA enzyme, which participates in the regulation by calmodulin, acidic phospholipids, selective proteolysis and phosphorylation by kinases. The amino-acid residues that form the coordination cage of site II for Ca^{2+} in SERCA (Val304, Ala305, Ile307, Asn796, Asp800, and Glu309) are fully conserved in the PMCA. On the contrary, most residues that form site I (Asn768, Glu771, V795, Thr799, Asp800 and Glu908) are not conserved, with the exception of Asp800, which is essential for site II, and Asn768. This indicates that site I of SERCA does not exist in PMCA, which is consistent with the measured stoichiometry of one Ca^{2+} transported per ATP hydrolyzed.

Some single mutations of those amino acids, either in site I or II of SERCA ATPase, abolish the occlusion of both Ca^{2+} by CrATP as well as the Ca^{2+}-ATPase activity at Ca^{2+} lower than 100 μM [38,40]. It is surprising that PMCA, which has only two of amino-acid residues of site I, preserves a fully functional site II, including the occlusion. Among the residues critical for the occlusion in the SERCA enzyme, Glu309 is conserved in PMCA (Glu433). This residue, together with the movements of the M1–M2 transmembrane helices, is believed to be part of the closing gate mechanism on the cytoplasmatic side [28,29]. The question of which residues play a role in the occlusion of Ca^{2+} in the plasma membrane Ca^{2+}-ATPase is still open. The use of LaATP in the PMCA Ca^{2+}-ATPase should be a valuable tool for understanding this process.

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