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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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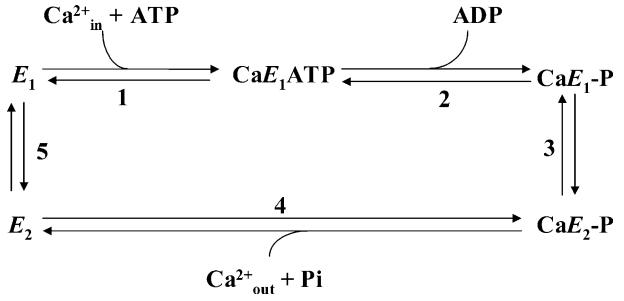

Fig. 1. Transport cycle of the plasma membrane Ca^{2+}-ATPase.

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