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Aluminum inhibits the plasma membrane and sarcoplasmic reticulum Ca^{2+}-ATPases by different mechanisms☆

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ABSTRACT

Aluminum (Al^{3+}) is involved in the pathophysiology of neurodegenerative disorders. The mechanisms that have been proposed to explain the action of Al^{3+} toxicity are linked to changes in the cellular calcium homeostasis, placing the transporting calcium pumps as potential targets.

The aim of this work was to study the molecular inhibitory mechanism of Al^{3+} on Ca^{2+}-ATPases such as the plasma membrane and the sarcoplasmic reticulum calcium pumps (PMCA and SERCA, respectively). These P-ATPases transport Ca^{2+} actively from the cytoplasm towards the extracellular medium and to the sarcoplasmic reticulum, respectively. For this purpose, we performed enzymatic measurements of the effect of Al^{3+} on purified preparations of PMCA and SERCA.

Our results show that Al^{3+} is an irreversible inhibitor of PMCA and a slowly-reversible inhibitor of SERCA. The binding of Al^{3+} is affected by Ca^{2+} in SERCA, though not in PMCA. Al^{3+} prevents the phosphorylation of SERCA and, conversely, the dephosphorylation of PMCA. The dephosphorylation time courses of the complex formed by PMCA and Al^{3+} (*E*PAl) in the presence of ADP or ATP show that *E*PAl is composed mainly by the conformer E_2P.

This work shows for the first time a distinct mechanism of Al^{3+} inhibition that involves different intermediates of the reaction cycle of these two Ca^{2+}-ATPases.

Aluminum is one of the most abundant elements in the Earth's crust. The relationship between aluminum exposure and neurodegenerative diseases, including dialysis encephalopathy syndrome, amyotrophic lateral sclerosis, Parkinsonism dementia and Alzheimer's disease has been extensively reported [1]. The oxidation state of aluminum is +3, and its effective ionic radius in sixfold coordination is 0.54 Å. The primary interaction of Al^{3+} with ligands is electrostatic and has a slow dissociation rate [2]. In aqueous solution, aluminum is solvated with water molecules. This form is known as free aluminum ($Al(H_2O)_6^{3+}$ (or simply, Al^{3+}) and is abundant at acidic pH. When the pH rises, aluminum is complexed with OH^- from deprotonation of water molecules ($Al(OH)_n$). Among others, Al^{3+} binds strongly to ATP and phosphate groups and displaces both Mg^{2+} and Ca^{2+} from their enzyme's binding sites [1]. Hence, changes in the Ca^{2+} homeostasis and protein phosphorylation/dephosphorylation would explain aluminum cellular toxicity [2].

Among the most important Ca^{2+} regulatory mechanisms are the Plasma Membrane Calcium ATPase (PMCA) and Sarcoplasmic Reticulum Calcium ATPase (SERCA). These pumps belong to the family of P-ATPases, which share the formation of an acid-stable phosphorylated intermediate as part of their reaction cycle. PMCA and SERCA use the ATP hydrolysis as a source of energy to transport calcium from cytoplasm to the extracellular medium or the reticulum lumen, respectively. PMCA is more regulated than SERCA, and through its interaction with regulatory, targeting and signaling proteins, regulates both global Ca^{2+} homeostasis and spatially defined Ca^{2+} signaling [3].

Detailed structural information about PMCA is currently lacking. Its little abundance (approximately 0.1% of the total protein in the membrane) and the presence of several isoforms hindered efforts to produce suitable crystals for X-ray diffraction. Although PMCA has not

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been crystallized yet, the structures of several reaction intermediates of SERCA have been resolved (*For revision, see* [4]). Its membrane-buried region is made up of 10 membrane spanning helices and is connected to a large cytoplasmic headpiece, which is further separated into three distinct domains, denoted A ("actuator"), P ("phosphorylation"), and N ("nucleotide binding"). However, unlike SERCA, PMCA is highly regulated by calmodulin (CaM), which activates this protein by binding to an auto-inhibitory region [5] and changes the conformation of the pump from an inhibited state to an activated one [5–7].

The current kinetic model for PMCA and SERCA proposes that enzymes exist in two main conformations: E_1 and E_2. After the binding of intracellular Ca^{2+} to high affinity sites, E_1 can be phosphorylated by ATP with formation of the intermediate E_1P. The conformational transition to E_2P leads to the release of Ca^{2+} to the opposite side of the membrane. The dephosphorylation of E_2P to E_2 and a new conformational transition to E_1 allow a new pump cycle [8,9]. Magnesium is necessary for optimal catalysis of PMCA [10] and SERCA [11]. This ion accelerates the $E_2 \rightarrow E_1$ [11,12] and the $E_1P \rightarrow E_2P$ transitions [13]. Several ligands have been used for studying intermediates (*E*P) in these pumps. In PMCA, lanthanum (La^{III}) is known to prevent the Mg^{2+}-dependent transition $E_1P \rightarrow E_2P$, fixing the pump in E_1P [14,15] while vanadate has been used for mimicking the E_2P state [16]. Furthermore, beryllium fluoride (BeFx) [17], magnesium fluoride (MgFx) [18,19] and aluminum fluoride (AlFx) [20] were useful in SERCA to elucidate the mechanism of ATP hydrolysis, because these phosphate analogues bind to the phosphorylation site mimicking the different steps of the E_2P dephosphorylation [21]. In the presence of calcium and ADP, aluminum fluoride stabilizes the E_1P analogue conformation [22].

In this work, we study the effect of aluminum on PMCA purified from human erythrocytes -about 90% PMCA4 and 10% PMCA1 [23]- and SERCA isolated from skeletal muscle -mostly SERCA1 [24]-. Our results show that Al^{3+} can bind quickly and strongly to both pumps inhibiting the Ca^{2+}-ATPase activity. This binding is affected by Ca^{2+} in SERCA, but not in PMCA. In SERCA, Al^{3+} prevents the phosphorylation, while in PMCA it prevents the dephosphorylation of the pump. Our results suggest that, in the presence of calcium and ATP, Al^{3+} fixes PMCA in an E_2P analogue conformation. This is the first evidence of an inhibitor that stabilizes an E_2P analogue conformation with the phosphate covalently bound.

1. Materials and methods

1.1. Reagents

All chemicals used in this work were of analytical grade and purchased mostly from Sigma. Recently drawn human blood for the isolation of PMCA was obtained from the Hematology Section of Fundación Fundosol (Argentina). Blood donation in Argentina is voluntary, and therefore the donor provides informed consent for the donation of blood and for the subsequent legitimate use of the blood by the transfusion service.

1.2. Purification of PMCA from human erythrocytes

PMCA was isolated from calmodulin-depleted erythrocyte membranes by the calmodulin-affinity chromatography procedure [25]. Protein concentration after purification was about 15 μg/ml. No phospholipids were added at any step along the purification procedure. The purification procedure described preserves transport activity and maintains the kinetic properties and regulatory characteristics of the enzyme in its native condition [25].

1.3. SERCA preparation

SERCA was directly solubilized with $C_{12}E_{10}$ (0.5%) from sarcoplasmic reticulum membranes prepared from rabbit skeletal muscle as described in Mangialavori et al. [26].

1.4. Measurement of Ca^{2+}-ATPase activity

Ca^{2+}-ATPase activity was measured by following the release of inorganic phosphate from ATP as described previously by Fiske and Subbarrow [27] or by measuring the [^{32}P]Pi released from [γ^{32}P]ATP as described by Richards et al. [28]. In each condition, the quantity of Pi released in the absence of calcium was subtracted from the Pi released in its presence. The reaction medium contained 120 mM KCl, 30 mM MOPS-K (pH 7.2 at 37 °C), 120 μM $C_{12}E_{10}$, 35 μM DMPC and 30 μM EGTA. Optimal Ca^{2+}-ATPase activities of PMCA and SERCA in the mentioned medium were determined in the presence of 1.8 mM free Mg^{2+} and 80 μM free Ca^{2+} or 1 mM free Mg^{2+} and 10 μM free Ca^{2+}, respectively. When indicated, an aqueous five-fold concentration solution of $AlCl_3$ was added to the reaction medium (named pre-incubation medium hereafter) containing PMCA or SERCA and was allowed to equilibrate for 5 min. The addition of $AlCl_3$ solutions does not change the pH of the pre-incubation medium. When necessary we dissolved $AlCl_3$ in an acidic pH and employed HEPES-acetate. The reaction was started by the addition of ATP-Mg^{2+} (final concentration of 2 mM for the non- radioactive assay and 30 μM for the radioactive one). The experimental set-up was adjusted to ensure that PMCA and SERCA (10 nM) initial velocity conditions were met. Measurements were carried out at 37 °C (non-radioactive assay) or 25 °C (radioactive assay).

For the time course experiments, the $AlCl_3$ the $AlCl_3$ solutions were added to the pre-incubation medium containing PMCA or SERCA and optimal concentrations of free Mg^{2+} and Ca^{2+} for each pump. After pre-incubation, Ca^{2+}-ATPase activity was started with 2 mM ATP-Mg. Due to the higher affinity of Al^{3+} for ATP, all the remaining aluminum binds to ATP, leaving free aluminum in the reaction medium [29].

1.5. Determination of phosphorylated intermediates

The phosphorylated intermediates (*E*P) were measured as the amount of acid-stable ^{32}P incorporated into the enzyme, according to the method described by Echarte et al. [30]. The phosphorylation was measured at 25 °C in the same medium in which the Ca^{2+}-ATPase activities of PMCA and SERCA were determined. For PMCA, a condition in the presence of 100 μM $LaCl_3$ was included as control of the maximal amount of *E*P intermediate. The reaction was started by the addition of [γ-^{32}P]ATP under vigorous stirring, and after 30 s, it was stopped with an ice-cold solution of TCA (10% (w/v) final concentration). The tubes were centrifuged at 10,000 rpm for 5 min at 4 °C. The samples were then washed once with 7% TCA, 150 mM H_3PO_4 and once with double-distilled water and processed for SDS-PAGE. For this purpose, the pellets were dissolved in a medium containing 150 mM Tris-HCl (pH 6.5 at 14 °C), 5% SDS, 5% DTT, 10% glycerol, and bromophenol blue (sample buffer). Electrophoresis was performed at pH 6.5 (14 °C) in a 7.5% polyacrylamide gel. The reservoir buffer was 0.1 M sodium phosphate, pH 6.3, with 0.1% SDS. Gels were stained, dried and exposed to a Storage Phospho-Screen (*Amersham Biosciences*). Un-saturated autoradiograms were scanned with *Storm Molecular Image System* and stained gels were scanned with an *HP Scanjet G2410*. Analysis of the images was performed with *GelPro Analyzer*. *E*P quantification was achieved as described by Echarte et al. [30].

1.6. Dephosphorylation procedure

Phosphorylation of PMCA was carried out at 4 °C in the absence or in the presence of 25 μM $AlCl_3$. The reaction was started by the addition of 30 μM [γ-^{32}P]ATP under vigorous stirring. After 3 min, phosphorylation was stopped by the addition of 1 mM ADP or 0.5 mM ATP and enough DMPC, $C_{12}E_{10}$, ATP, and Mg^{2+} to maintain the phosphorylation conditions. Dephosphorylation was carried out at 4 °C under stirring during the time periods described in the figures and stopped manually