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# The plasma membrane $Ca^{2+}$ pump catalyzes the hydrolysis of ATP at low rate in the absence of $Ca^{2+}$

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ABSTRACT

The plasma membrane $Ca^{2+}$ ATPase catalyzed the hydrolysis of ATP in the presence of millimolar concentrations of EGTA and no added $Ca^{2+}$ at a rate near 1.5% of that attained at saturating concentrations of $Ca^{2+}$. Like the Ca-dependent ATPase, the Ca-independent activity was lower when the enzyme was autoinhibited, and increased when the enzyme was activated by acidic lipids or partial proteolysis. The ATP concentration dependence of the $Ca^{2+}$-independent ATPase was consistent with ATP binding to the low affinity modulatory site. In this condition a small amount of hydroxylamine-sensitive phosphoenzyme was formed and rapidly decayed when chased with cold ATP. We propose that the $Ca^{2+}$-independent ATP hydrolysis reflects the well known phosphatase activity which is maximal in the absence of $Ca^{2+}$ and is catalyzed by $E_2$-like forms of the enzyme. In agreement with this idea pNPP, a classic phosphatase substrate was a very effective inhibitor of the ATP hydrolysis.



## Introduction

The $Ca^{2+}$ transporter from plasma membrane (PMCA)[1] is a member of the family of P-type ATPase family of energy transducers [1–3]. The PMCA are autoinhibited pumps and for maximal activity they need to be activated by the binding of calmodulin, acidic lipids or by the removal of the autoinhibitory domain from the molecule.

The normal functioning of the PMCA involves coupling the transport of $Ca^{2+}$ out of the cell with ATP hydrolysis. As for other P-ATPases, the currently accepted reaction cycle of the PMCA supports the existence of two distinct conformational states; $E_1$ endowed with kinase activity can react with micromolar concentrations of ATP to form a high-energy phosphoenzyme $E_1P$, and $E_2$ that can be phosphorylated by inorganic phosphate to give a low-energy phosphoenzyme $E_2P$. The $E_2$ form of the enzyme has also been associated with the ability of the enzyme to catalyze the hydrolysis of phosphate esters like pNPP uncoupled from ion transport ("phosphatase activity"). We have recently shown [4] that in the PMCA the pNPPase activity is maximal in the absence of $Ca^{2+}$, and $Ca^{2+}$ inhibits this activity by binding to $E_1$ with high affinity ($K_{0.5}$ = 0.5–20 μM) and displacing the $E_1$–$E_2$ equilibrium towards $E_1$.

On the basis of the currently accepted model of the reaction cycle for the hydrolysis of ATP, the PMCA should exhibit an absolute requirement for $Ca^{2+}$. However, several studies have reported the ability of other P-ATPases to utilize ATP in the virtual absence of the transported ions [5–7]. More recently, it was reported that the $Cu^{2+}$-transporting ATPase CopA from *Thermotoga maritima* does not require Cu for the formation of a phosphoenzyme from ATP [8,9].

We found that a purified PMCA preparation of high specific activity was able to hydrolyze ATP at a very low rate in the absence of $Ca^{2+}$, this activity was mediated by the formation of a phosphoenzyme intermediate, and like the "Ca-dependent" ATPase activity, the PMCA's "$Ca^{2+}$-independent" ATPase increased when the autoinhibition was relieved by acidic lipids or partial proteolysis.

## Materials and methods

### *Source of materials*

$CaCl_2$ solutions were prepared from AnalaR $CaCO_3$ (BDH Chemicals Ltd., Poole, Dorset, UK). [γ-$^{32}$P]ATP was from New England Nuclear. Polyoxyethylene 10 lauryl ether ($C_{12}E_{10}$), L-α-phosphatidylcholine (PC), type XVI from fresh egg yolk, brain extract lipids (BE) type I Folch fraction I from bovine brain containing approximately 10% phosphatidylinositol, 50% phosphatidylserine, calmodulin-agarose, trypsin, ATP (disodium salt, vanadium-free), sodium dodecyl sulfate, and other reagents were from Sigma.

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[1] *Abbreviations used:* PMCA, plasma membrane $Ca^{2+}$ pump erythrocytes; pNPP, *p*-nitrophenylphosphate; EGTA, ethylene glycol bis(b-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PC, phosphatidylcholine; BE, lipid extract from bovine brain; EP, phosphoenzyme.

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*Enzyme preparation*

The PMCA was isolated from pig red cells as described previously [10]. Briefly, membranes from pig red cells were resuspended in purification buffer (20 mM MOPS-K, pH 7.4 at 4 °C, 20% glycerol, 130 mM KCl, 1 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride and 100 μM $Ca^{2+}$) and were solubilized with $C_{12}E_{10}$ at 5 mg/ml for 10 min. After removing the non solubilized material by centrifugation at 40,000*g* for 20 min the solubilizate was applied to an agarose-calmodulin column. The column was washed with 10 bed volumes of the same buffer with 0.5 mg/ml of $C_{12}E_{10}$ and the PMCA was eluted in a buffer of the same composition but containing 1 mM EGTA-K instead $CaCl_2$.

*ATPase activity*

Activity was estimated from the release of [$^{32}$P] from [γ-$^{32}$P]ATP at 37 °C [11] in 0.3 ml of ATPase medium containing 3.5 μg of PMCA, 100 mM HEPES-K, pH 7.1 at 37 °C, 100 mM KCl, 4 mM $MgCl_2$, 3 mM [γ-$^{32}$P]ATP, 0.5 mM EGTA-K and enough $Ca_2Cl$ to yield the concentrations indicated in each experiment. Before initiating the reaction the enzyme was reactivated by adding 0.57% $C_{12}E_{10}$ and 0.29% of either L-α-phosphatidylcholine (PC) or a mixture of acidic lipids (BE). The suspension was thoroughly mixed and preincubated for at least 10 min on ice before being added to the ATPase reaction medium. In order to decrease the amount of free $Ca^{2+}$ below 50 nM the concentration of EGTA-K was increased up to 40 mM and the amount of $MgCl_2$ was also increased to keep the concentration of $Mg^{2+}$ fixed at 1.5 mM. The reaction was initiated by the addition of PMCA and terminated by acid denaturation. In order to assure that the total ATP hydrolysis was lower than 20% the reaction time was varied between 5 and 60 min depending on the $Ca^{2+}$ concentration of the media. The term "$Ca^{2+}$-independent" ATPase refers to the ATP hydrolysis measured in a media containing 0.5–10 nM $Ca^{2+}$ minus the ATP hydrolysis in a similar media but without enzyme.

*Free $Ca^{2+}$*

The concentration of free $Ca^{2+}$ was estimated using the computer program Maxchelator Webmaxclite v1.15 (http://www.stanford.edu/~cpatton/maxc.html) considering the pH, temperature, EGTA, ATP, total $Ca^{2+}$, and total $Mg^{2+}$. The ionic strength was varied between 0.27 and 0.30. When no $CaCl_2$ was added to the medium a concentration of total $Ca^{2+}$ of 0.09 mM was estimated to be contributed by the buffers and salts and used for the calculations.

*Electrophoresis analysis of purified PMCA*

SDS electrophoresis was carried out as described previously [12] Proteins were electrophoresed on a 7.5% acrylamide gel according to Laemmli [13] and revealed by staining with Coomassie blue.

*Proteolytic digestion*

One hundred micrograms of PMCA reactivated by adding 0.57% $C_{12}E_{10}$ and 0.29% of PC was added to the standard reaction media containing 100 mM HEPES-K, pH 7.1 at 37 °C, 100 mM KCl, 4 mM $MgCl_2$, and 10.5 mM EGTA-K. The proteolysis reaction was initiated by adding 10 μg of trypsin, and at different times aliquots of the proteolysis media containing 5 μg of PMCA were removed and either precipitated with 10% of trichloroacetic acid and processed for SDS–PAGE, or supplemented with 3 mM [γ-$^{32}$P]ATP and 6 μg of aprotinin, incubated for 30 min at 37 °C and the activity estimated from the amount of [$^{32}$P] released from [γ-$^{32}$P]ATP.

*Phosphorylation and dephosphorylation*

Various amounts of purified ATPase were incubated at 4 °C in a reaction media containing 0.05% of BE lipids, 0.15% $C_{12}E_{10}$, 16 mM MOPS-K, pH 7.4 at 4 °C, 15% glycerol, 100 mM KCl, 2.8 mM $MgCl_2$, 1.3 mM EGTA with or without $CaCl_2$ to give a concentration of 100 μM free $Ca^{2+}$. The reaction was started by the addition of 500 μM [γ-$^{32}$P]ATP and was stopped after 1 min with 10% ice-cold trichloroacetic acid. The denatured proteins were collected by centrifugation at 20,000*g* for 10 min, washed once with 5% trichloroacetic acid and 150 mM $NaH_2PO_4$ and once more with distilled water. The precipitated protein was suspended in sample buffer and separated by acidic SDS–PAGE. The gels were dried and the radioactivity detected using a Storm Molecular Image System. The dephosphorylation was initiated by the addition of 16 mM of cold ATP-Mg and continued for 3 s on ice before the addition of 10% ice-cold trichloroacetic acid. The treatment of the phosphoenzyme with hydroxylamine was performed as described previously [14].

## Results

The ATPase activity of the purified PMCA supplemented with acidic lipids was determined in an extended range of $Ca^{2+}$ concentrations (Fig. 1). Maximal activity was achieved at about 1 μM $Ca^{2+}$

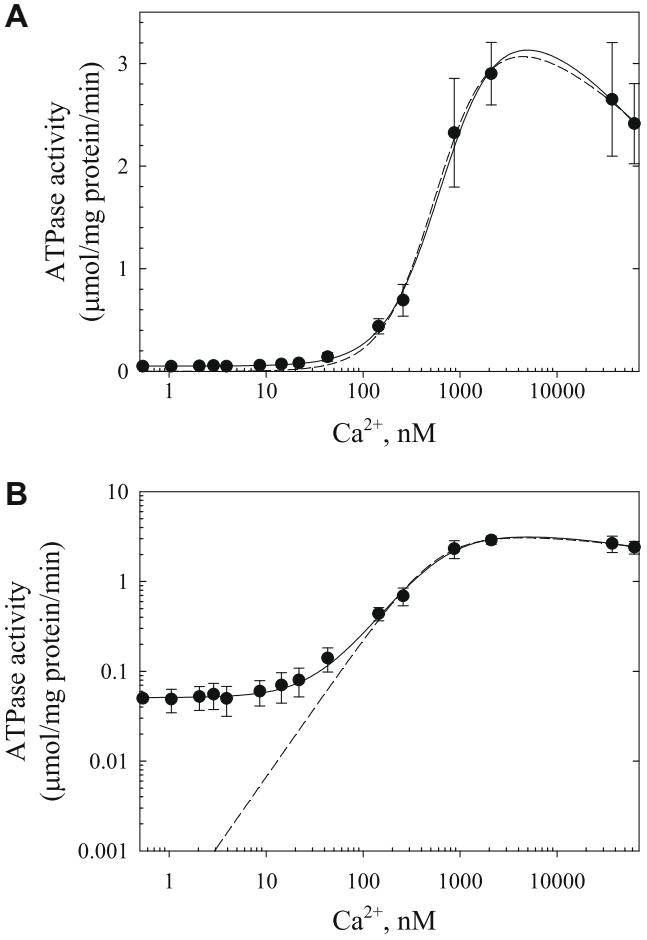


**Fig. 1.** ATPase activity of the PMCA as a function of the $Ca^{2+}$ concentration. The ATP hydrolysis was measured as indicated in Materials and methods. The same data are represented in (A) and in (B). In (B) the ATPase activity is plotted in log scale. The continuous line represent the best fit to the data given by the a modified Hill equation $v = v_0 + (V \cdot x^n/(K_{ca}^n + x^n + x^2/K_i)$ with the following parameters $v_0$ = 0.05 μmol/mg/min, $V$ = 3.7 μmol/mg/min, $K_{ca}$ = 663 nM, $K_i$ = 644 nM, $n$ = 1.4. The dashed line represents the best fit to the same data given by a similar equation but predicting zero activity in the absence of $Ca^{2+}$.