Archives of Biochemistry and Biophysics 495 (2010) 62–66

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

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

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ARTICLE INFO

Article history:
Received 21 September 2009
and in revised form 16 December 2009
Available online 24 December 2009

Keywords:
Ca^{2+} pump
Ca^{2+} ATPase
Plasma membrane
PMCA
P-ATPase

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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doi:10.1016/j.abb.2009.12.021

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+}

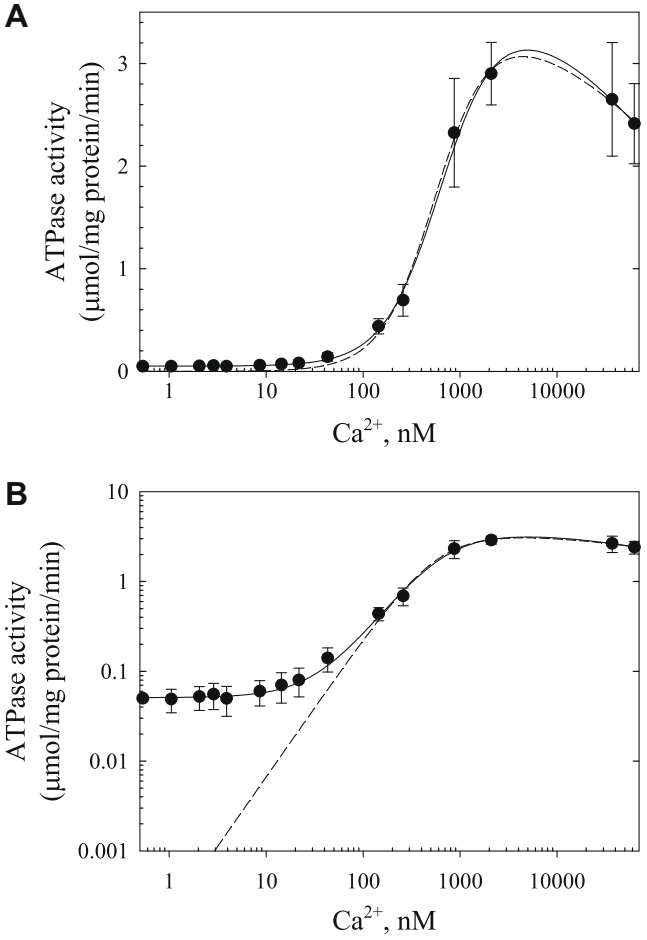

Fig. 1. ATPase activity of the PMCA as a function of the Ca^{2+} concentration. The ATP hydrolysis was measured as indicated in Materials and methods. The same data are represented in (A) and in (B). In (B) the ATPase activity is plotted in log scale. The continuous line represent the best fit to the 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+}.

and, in agreement with previous studies, high concentrations of Ca^{2+} where inhibitory. On the other hand, as the concentration of Ca^{2+} was progressively reduced by adding increasing amounts of EGTA, the ATPase activity decreased until reaching a value of about 1.5% of the maximal activity and did not became zero even at the lowest concentration of Ca^{2+} tested. In spite of the small magnitude of the activity at very low Ca^{2+}, the log representation (Fig. 1 B) makes it clear that the experimental data could not be fitted by using an equation predicting zero activity at zero Ca^{2+}. Thus, the ATPase activity measured at extremely low concentrations of Ca^{2+} could not be accounted for by the Ca^{2+} dependency observed at higher Ca^{2+} concentrations. Moreover, the fact that at high concentrations of EGTA the ATPase activity seemed to reach a plateau suggests that it was not longer Ca^{2+} dependent.

The simplest explanation of this activity would be the presence of a contaminating Ca^{2+}-independent ATPase in the PMCA preparation. Fig. 2 shows the electrophoretic pattern of the purified PMCA from pig erythrocyte membranes. Only one mayor band with the expected migration of the PMCA was detected when 0.4 μg of protein was loaded. When the amount of protein loaded in each lane was increased, a band corresponding to a PMCA fragment of apparent Mr of about 90 kDa become visible. A similar fragment was previously shown to be recognized by PMCA specific antibodies and suggested to arise by the activity of endogenous proteases [12]. Because the C-terminal autoinhibitory region is cleaved, the 90 kDa fragment in association with the N-terminal 35 kDa fragment has a full Ca^{2+}-ATPase activity.

A characteristic feature of the PMCA is its regulation by autoinhibitory domains which maintain the enzyme in a state of low activity. Thus, the PMCA achieves its maximal Ca^{2+}-ATPase activity by interaction with Ca^{2+}-calmodulin, acidic lipids or by the removal of the autoinhibitory domain from the PMCA molecule. In order to assess the effect of lipids on the PMCA "Ca^{2+}-independent" ATPase the ATP hydrolytic activity of the enzyme supplemented with either with phosphatidylcholine (PC) or a lipid extract rich in acidic lipids from bovine brain (BE) was measured in a reaction media containing about 2 nM Ca^{2+}. In these conditions when the enzyme was supplemented with PC the ATPase activity was 22 nmol Pi/mg protein/min while it increased to 80 nmol Pi/mg protein/min when a mixture of acidic lipids was used instead of PC. Thus the acidic lipids had a stimulatory effect on the "Ca^{2+}-independent" ATPase activity of the PMCA of similar magnitude to that observed in the presence of Ca^{2+}.

Results in Fig. 3A show the effect of proteolysis on the activity of the PMCA supplemented with PC at 2 nM Ca^{2+}. As the digestion progressed, the Ca^{2+}-independent ATPase increased reaching a maximal value at 7.5 min, and then decreasing with further proteolysis. The increase in the "Ca^{2+}-independent" ATPase occurred in parallel with the appearance of a fragment with an apparent Mr of 90 kDa (Fig. 3B), in agreement with the idea that stimulation was caused by the removal of the autoinhibitory sequence. The stimulation of the "Ca^{2+}-independent" ATPase activity by acidic lipids and proteolysis suggest that this activity, like the Ca^{2+}-dependent activity is lower when the enzyme is autoinhibited and argues in favor of the idea that both activities originate from the PMCA protein.

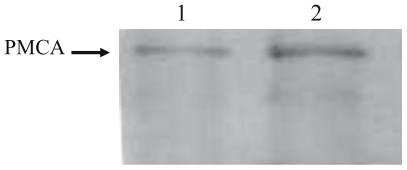

Fig. 2. Electrophoretic analysis of PMCA. Sodium dodecyl sulfate-gel electrophoresis (7.5%) was performed as described in Materials and methods and stained with Coomassie brilliant blue. 0.4 or 0.8 μg of purified PMCA was applied in lanes 1 and 2, respectively.

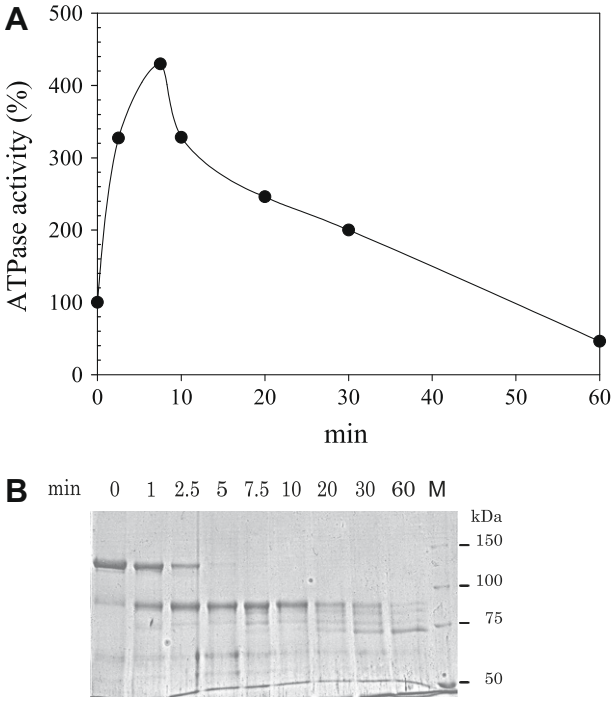

Fig. 3. Effect of the digestion with trypsin of PMCA on the "Ca^{2+}-independent" ATPase. (A) PMCA supplemented with PC was digested with trypsin (w/w PMCA:trypsin 10:1) for different times as indicated in Material and methods, and the ATPase activity was measured. The estimated concentration of Ca^{2+} was 2 nM. (B) Electrophoretic analysis of PMCA fragments generated by digestion with trypsin. Aliquots of the proteolytic reaction containing 5 μg of PMCA treated with trypsin as in (A), were separated in a 9% SDS–PAGE and stained with Coomassie brilliant blue.

We then studied the ATP dependence of the Ca^{2+}-independent ATPase activity (Fig. 4). Within the range of ATP concentrations used, the activity increased with increasing concentrations of ATP along an hyperbolic curve and attained half maximal activity at about 100 μM ATP, a value close to the K_m of the low affinity site for ATP [10].

An interesting question is whether the low ATPase activity observed in the presence of high concentrations of EGTA involved the formation of a phosphoenzyme. As shown in Fig. 5A, when

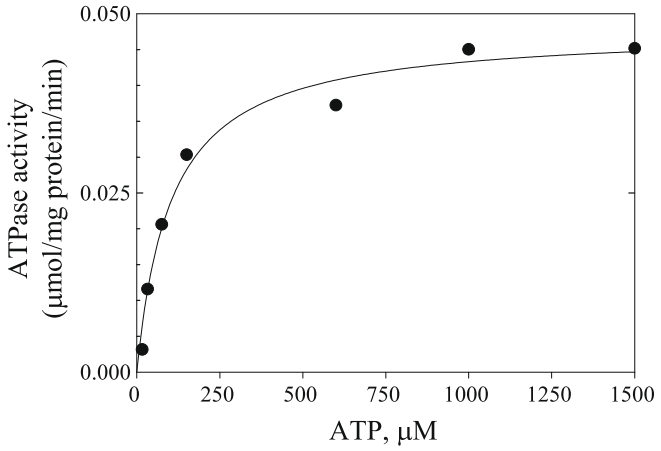

Fig. 4. ATP dependency of the "Ca^{2+}-independent" ATPase. The ATPase activity was measured as indicated in Material and methods. The reaction medium contained 10.5 mM EGTA and no added $CaCl_2$. PMCA was supplemented with BE lipids. ATP was added as an equimolar mixture with $MgCl_2$.

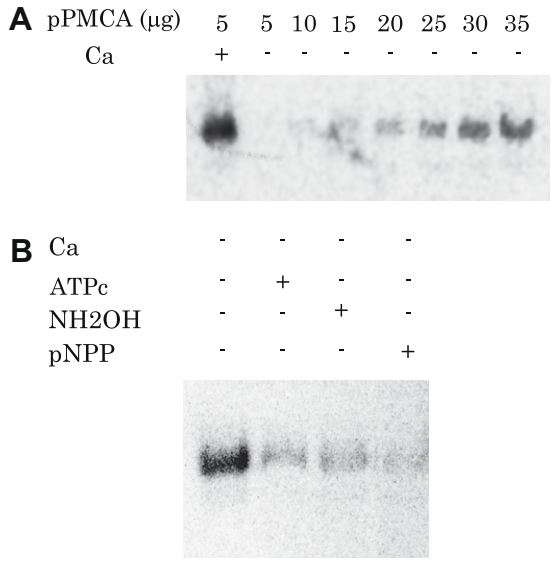

Fig. 5. Formation and decay of the phosphorylated intermediate. (A) The micrograms of PMCA indicated in the figure were phosphorylated at 4 °C for 1 min by 500 μM [γ-^{32}P]ATP and 1.3 mM EGTA as described in Material and methods and submitted to electrophoresis in acidic conditions. (B) Thirty micrograms of PMCA was phosphorylated as in (A) in the absence of Ca^{2+}, and as indicated in the figure *ATPc*, chased for 3 s with 16 mM cold ATP-Mg, *NH_2OH*, the phosphorylation stopped and the phosphoenzyme treated with 150 mM of hydroxylamine for 10 min at 20 °C, *pNPP*, 14 mM pNPP was added during the phosphorylation.

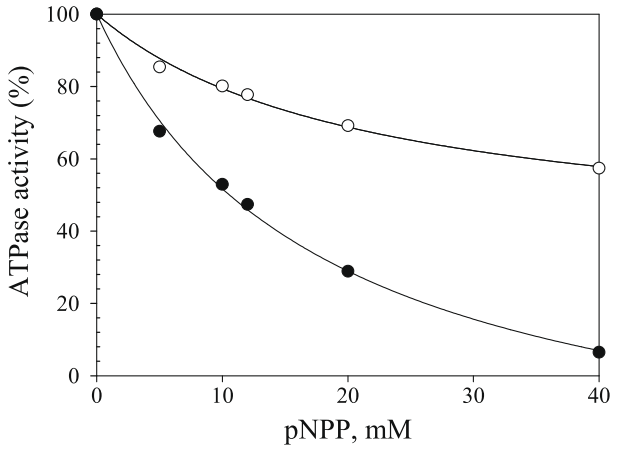

Fig. 6. Effect of pNPP on the ATPase activity of the PMCA in the presence and in the absence of Ca^{2+}. The ATP hydrolysis was measured as indicated in Materials and methods in the presence of either 10 μM (empty circles) or 2 nM Ca^{2+} (filled circles). pNPP was added as a pNPP-Mg in a molar ratio of 40:1. The continuous line represent the best fit to the data given by a decreasing hyperbola $v = v_0 + (100 - v_0)K_{pNPP}/(K_{pNPP} + x)$ with the following parameters, in the presence of 10 μM Ca^{2+} $v_0 = 35\%$, $K_{pNPP} = 21$ mM, and in the presence of 2 nM Ca^{2+} $v_0 = -4\%$, $K_{pNPP} = 18$ mM.

5 μg of PMCA were phosphorylated by 0.5 mM ATP in the presence of 1.3 mM EGTA no phosphoenzyme was detected. However, upon increasing the amount of enzyme, the phosphoenzyme formed was visible. Judging by the intensity of the bands the amount of phosphoenzyme obtained in this condition was about 8% of that formed in the presence of saturating amounts of Ca^{2+}. Results in Fig. 5B show that the phosphoenzyme formed in the presence of 1.3 mM EGTA rapidly decreased when chased with cold ATP. Moreover, the phosphoenzyme was sensitive to the treatment with hydroxylamine suggesting that it involved the formation of an acylphosphate. Interestingly, the addition of 14 mM pNPP together with ATP during the phosphorylation nearly abolished the formation of phosphoenzyme from ATP.

The effect of pNPP was investigated in more detail by measuring the effect of increasing concentrations of pNPP on the Ca^{2+}-dependent and "Ca^{2+}-independent" ATPase activity of the PMCA (Fig. 6). In the presence of 10 μM Ca^{2+}, increasing concentrations of pNPP decreased the ATPase to about 40% along a hyperbolic curve with an apparent K_i of 21 mM. In contrast pNPP fully inhibited the low ATPase activity measured in 2 nM Ca^{2+} with a K_i similar to that observed in the presence of 1 μM Ca^{2+}.

Discussion

The results described here indicate that at concentrations of Ca^{2+} low enough to maintain the transport Ca^{2+} sites empty the PMCA exhibits a low ATPase activity. This activity has not been reported previously and in most studies of the PMCA, particularly in those performed using isolated membranes as a source of the enzyme, the ATP hydrolysis in media containing nanomolar concentrations of Ca^{2+} were usually attributed to Ca^{2+}-independent contaminating enzymes. In these cases, the specific activity of the PMCA is estimated subtracting the rate of ATP hydrolysis detected in media containing Ca^{2+} chelators and no added Ca^{2+} from the total hydrolytic activity in the presence of Ca^{2+} [15]. The results presented here indicate that in affinity purified preparations of the PMCA, the ATPase activity at very low concentrations of Ca^{2+} should not be disregarded but instead be considered as a catalytic expression of the enzyme.

We found that the ATPase activity in the absence of Ca^{2+} was stimulated by acidic lipids and by the proteolytic digestion of the autoinhibitory region of the protein, in an effect similar to that they exert on the activity of the PMCA in the presence of Ca^{2+}. However, because of the small magnitude of the Ca^{2+}-independent activity and the fact that PMCA preparations usually contain small amounts of proteolytically activated fragments derived from the action of endogenous proteases, our data cannot exclude the possibility that in the native full-length autoinhibited PMCA this activity is lower or even absent.

The Ca^{2+}-independent ATPase activity that we describe here for the PMCA is interesting from the mechanistic point of view because it may emphasize a general ability of the P-ATPases of utilizing ATP in the absence of the transported ions.

We have considered the possibility that a low ATPase activity may arise from fluctuations of the enzyme through a full E_1–E_2 cycle in the absence of Ca^{2+}. However, recent studies of SERCA suggest that although E_1 can be attained at high pH in the absence of Ca^{2+}, the binding of Ca^{2+} is still required for a catalytic competent E_1Ca_2 [16].

Alternatively, we propose that the low level of ATP hydrolysis we detected in the absence of Ca^{2+} is a manifestation of the phosphatase activity catalyzed by E_2-like forms of the enzyme. It has been known for a long time that, like other P-ATPases, the PMCA can hydrolyze non nucleotidic phosphate esters like pNPP [3]. This activity termed "phosphatase activity" is maximal in the absence of Ca^{2+} [4]. Consistently with this idea we found that, like the pNPPase activity [4], the Ca^{2+}-independent ATP hydrolysis, increased when the PMCA was activated by acidic lipids or proteolysis. Noteworthy, the concentration of ATP for half maximal ATPase in the absence of Ca^{2+} was close to the K_m for the low affinity site for ATP [10]. Moreover, pNPP has been proposed to be hydrolyzed in the low affinity site for ATP [17]. Consistently with this proposal, we found that pNPP totally inhibited the ATPase in the absence of Ca^{2+}. Altogether, these results suggest that the binding of ATP with low affinity not only produces the well know turnover activation, but in the absence of the transported ion this ATP may itself be slowly hydrolyzed.

The crystal structures of SERCA show that the γ-phosphate from ATP becomes optimally positioned for phosphoryl transfer only in the Ca_2E_1-ATP form [18]. Nevertheless, our results suggest that in the PMCA phosphoryl transfer may occur in E_2-ATP albeit with much lower efficiency. This property may not be unique of the PMCA. Indeed, recent studies show that phosphorylation from ATP in the CopA from *T. maritima* is maximal in the absence of the transported ion and the phosphoenzyme produced shows a proteolytic pattern similar to that of E_2P [8,9].

Acknowledgments

The authors thank Dr. Luis Bredeston for critical reading and helpful comments. This work was supported by Grant B09 from Universidad de Buenos Aires, CONICET PIP 112-200801-02022 and ANPCyT Préstamo BID PICT 15-25965. H.P.A. is an established investigator of Consejo de Investigaciones Científicas y Técnicas (CONICET).

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