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Changes in cytosolic Mg^{2+} levels can regulate the activity of the plasma membrane H^+ -ATPase in maize

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Running head: ATP inhibition of maize plasma membrane H^+ -ATPase

Plant plasma membrane (PM) H^+ -ATPase, a major consumer of cellular ATP, is driven by the magnesium ATP complex which may dissociate at low cytosolic Mg^{2+} activity. We investigated whether hydrolytic activity of PM H^+ -ATPase is inhibited at ATP concentrations exceeding the Mg^{2+} concentration. Activity in isolated maize plasma membranes was measured at pH 6.5 in the presence of 5 mM Mg^{2+} (high) or 2 mM Mg^{2+} (low), while K^+ was applied at concentrations of 155 mM (high) or 55 mM (low). In all experiments, with membrane vesicles either from roots or leaves, the enzyme activity decreased in the presence of Mg^{2+} -free ATP. At inhibitory ATP concentrations, the activity was not influenced by the K^+ concentration. The activity was restored after increasing the Mg^{2+} concentration. ATP inhibition also occurred at pH 7.5. Kinetic modeling shows that Mg^{2+} -free ATP acted as a competitive inhibitor with a K_i in the range of the K_m . K_i decreased by 75% at low K^+ concentration. K_i was one order of magnitude lower at pH 7.5 compared to pH 6.5. The observed inhibition is consistent with a concept in which down-regulation of the cytosolic Mg^{2+} activity is involved in (phyto)hormonal stress responses.

Key words: P-type ATPases, Mg^{2+} activity, free magnesium, nucleotide-binding, nucleotide-inhibition

INTRODUCTION

Plant plasma membrane (PM) H^+ -ATPase is the “powerhouse” for nutrient uptake, turgor formation and cellular growth [1] pumping protons from the cytosol into the extracellular space (apoplast). Under resting conditions enzyme activity is regulated by an autoinhibitory domain [1, 2] and probably by reduced coupling efficiency [3]. The crucial physiological role of down-regulation is evident from fusicoccin effects. This phytotoxin binds to 14-3-3 proteins, inactivates the autoinhibitory domain and causes a persistent activation of PM H^+ -ATPase, leading to abnormal apoplastic acidification and stomatal opening [4].

The enzyme is driven by the Mg-ATP complex. A simple type of down-regulation or inhibition which does not involve protein-protein interaction could arise from an imbalance between the molar concentrations of Mg^{2+} and ATP: ATP in excess of Mg^{2+} inhibits PM H^+ -ATPase of yeast [5] and of *Acetabularia* [6]. ATP is considered to be the most abundant intracellular chelating component of Mg^{2+} in mammalian and plant cells [7, 8]. The dissociation constant for $(Mg-ATP)^{2-}$, the prevailing Mg-ATP species at normal physiological pH, is 28 μ M at 25°C [9]. Taking into account that ATP species with lower Mg^{2+} affinity (mainly $(H-ATP)^{3-}$) and monovalent cations competing with Mg^{2+} for free ATP are also present, an apparent dissociation constant of 71 μ M was determined at pH 7.4 [10]. If the latter value is correct, then the Mg^{2+} activity that could regulate H^+ -ATPase activity would be higher than 30 μ M. Direct measurements of cytosolic Mg^{2+} activity in the plant cytosol are difficult.

Abbreviations used: ABA, abscisic acid; BTP, 1-3-bis[tris-(hydroxymethyl)-methylamino]propane; H^+ -ATPase, proton-exporting ATPase (EC 3.6.3.6); $[(H-ATP)^{3-}]$, concentration of trivalent ATP anion; MES, 2-(*N*-morpholino)ethane sulfonic acid; PEP, phosphoenolpyruvate; PM, plasma membrane; SE, standard error; SD, standard deviation; TRIS; tris-(hydroxymethyl)amino methane

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However, for wheat protoplasts the cytosolic Mg^{2+} activity was estimated from measured adenylate concentrations to be 400 μM in the dark and 200 μM in the light [8]. Poor Mg^{2+} supply from the soil and intracellular processes such as Mg^{2+} sequestration, eg. with phytic acid (*myo*-inositol hexakisphosphate), a common constituent in eukaryotic cells and probably a major fraction of the cellular phosphate pool [11, 12], or Mg^{2+} complexation in chloroplasts [13], may keep or force the cytosolic Mg^{2+} activity to lower levels which then leave a major fraction of ATP uncomplexed. Under such conditions, an inhibition mechanism of PM H^+ -ATPase by Mg^{2+} -free ATP with sufficiently low inhibition constant may play a physiological role. The motivation of this study was to prove ATP inhibition of PM H^+ -ATPase in higher plants and to determine the kinetic parameters of ATP inhibition.

Previous kinetic studies of PM H^+ -ATPase from oat roots have demonstrated that ATP hydrolysis drops to 7% when Mg^{2+} is omitted from the assay [14]. With Mg^{2+} in excess of ATP the kinetics were hyperbolic and a K_m for Mg-ATP of 0.3 mM was derived from double-reciprocal plots. Our investigation follows the consequences of a moderate decrease of Mg^{2+} concentration down to a level of 30% of the ATP concentration, but still several-fold higher than the K_m in roots of oat and maize [15]. We show the non-hyperbolic kinetics of maize H^+ -ATPase in plasma membrane vesicles [15, 16, 17] under various ionic conditions. Because of non-hyperbolic kinetics, non-linear regression analysis based on mechanistic models [18, 19] was employed to determine K_m values and constants of ATP inhibition. The discussion of physiological relevance includes a specification of important regulatory processes which may be accompanied by a decrease of cytosolic Mg^{2+} activity.

EXPERIMENTAL

Plant cultivation

Maize seeds (*Zea mays* L. cv. Pioneer 3906) were germinated in the dark at 20°C for 10 d. Seedlings were transferred to nutrient solution [20] which was half-strength for 2 d, then full-strength and replaced every 3 d. Cultivar Pioneer was cultivated in the growth chamber for 16 d at a light intensity of 130 W m⁻² (Master HPI-T Plus 400W, Philips, Netherlands) at 50% rel. humidity, with a day/night cycle of 16 h/8 h at 21°C/16°C. For the experiment on discrimination between inhibition mechanisms *Zea mays* L. cv. AMADEO (KWS, Kleinwanzleben, Germany) was grown in hydroponics for 14 d at 26°C/18°C.

Plasma membrane isolation

Plasma membrane vesicles from the lower 10 cm of roots and from young leaves were prepared by aqueous two-phase partitioning [15, 16, 20]. Shoots were cut above the fourth leaf and immediately placed in a beaker with the cut end immersed in cold water. The lower segment of 10 cm length was cut and the mid-rib was peeled top-down and discarded. Remaining blade tissue was homogenized in the presence of 250 mM KJ in order to remove peripheral membrane proteins [21, 22, 23]. Potassium iodide was not used for preparation of root vesicles [15]. Phase partitioning was performed with a polymer concentration of 6.2% for roots and 6.1% for leaves [20]. Vesicle aliquots were stored in liquid N₂. Protein was quantified according to Bradford [24]. The purity of vesicle preparation was confirmed using specific ATPase inhibitors [15, 25].

Kinetic assays

In order to suppress hydrolytic activity originating from traces of unspecific phosphatases, all assays were performed in the presence of 1 mM Na₂MoO₄. Interference from ATP hydrolysis originating from other membranes was eliminated with two complementary methods: (a) In the vanadate method, the decrease in ATPase activity upon addition of 300 μM Na₃VO₄ was taken as PM ATPase activity [15, 23, 25]. This method has the advantage that unphysiologically high KNO₃ concentrations and azide are avoided. However, since experimental errors of two hydrolytic assays contribute to final experimental error, experimental variation is inherently larger. Moreover, a higher amount of vesicle sample is required. (b) In the cocktail method, ATPase activity in the presence of 1 mM Na₂MoO₄, 1 mM NaN₃ and 100 mM KNO₃ was considered as PM ATPase activity. Since there was no NO₃⁻-sensitive activity in leaf vesicles, KNO₃ was not required in kinetic assays of leaves.

ATPase hydrolytic activity was determined according to Yan *et al.* (15) at 30°C with 3 µg vesicle protein in 0.5 mL of 30 mM BTP-MES buffer with 0.02% (w/v) Brij 58 (Sigma), 2 or 5 mM MgSO₄ and K salts. Experiments at 155 mM K⁺ were performed with 50 mM KCl + 100 mM KNO₃ + 5 mM K-PEP (root, some leaf experiments) or with 150 mM KCl + 5 mM K-PEP in a leaf experiment for discriminating between inhibitory ATP species. ATP was supplied as Na₂-ATP or diTRIS-ATP (both from Sigma), adjusted with BTP to the assay pH. At maximum activity the increase in the concentration of inorganic phosphate throughout the incubation time did not exceed 3% of the ATP concentration. Unless otherwise stated, formation of ADP and decrease of ATP was prevented by an ATP-regenerating system [26], which included 5 units of pyruvate kinase (Fluka) and 5 mM K-PEP (Fluka) or 5 mM Na₃-PEP (Serva). With the proton/cation exchanger gramicidin for preventing H⁺ gradient across the plasma membrane, ATPase hydrolytic activity of root vesicles was 0.284 µmol mg⁻¹ min⁻¹ compared to 0.271 without gramicidin (n = 3). Unless otherwise indicated, ATPase assays were performed without gramicidin.

All enzyme assays were performed with two chemical parallels. Three or four vesicle preparations from parallel plant cultivations (biological replicates) were used to determine mean values of ATPase activity and standard errors (SE).

Normalisation of ATPase activities

The normalisation procedure applied for some data sets as indicated eliminates differences between replicates, if for all ATP concentrations activities are higher (or lower) by a constant factor. For example, such differences occur when enzyme concentrations slightly differ between stored vesicle “aliquots” for replicates. For each replicate normalised activities were determined as follows: (a) calculating the sum of activities over the whole range of ATP concentrations for each replicate (individual sum), (b) determining the mean sum of all replicates, (c) multiplying the activity values of a replicate with the ratio between the mean sum and the individual sum of that replicate.

Non-linear regression analysis with DynaFit

The principle of the analysis is to define a mechanistic model from which DynaFit derives a mathematical model, i.e. a set of simultaneous rate equations including the unknown rate constants [27] (Supplementary Annex 1). The mathematical model is then solved with numerical methods using iterative procedures. Mean values of non-normalised activity data were subjected to DynaFit regression analysis from which rate constants and their standard deviations were obtained. Regression curves are shown in all figures. If the legend does not specify the inhibition mechanism which was applied for curve fitting, curves did not differ for competitive, uncompetitive and non-competitive inhibition. The fit of different mechanistic models was compared (model discrimination) using the Akaike information criterion [28]. The most plausible model is one with the highest Akaike weight (Max = 1). The probability of one model in comparison to another model was calculated according to Mannervik [29].

DynaFit analysis was based on concentration values in µM and rate constants per s⁻¹ (Supplementary Annex 1). Enzyme concentration (E) was set to 0.1 nM, assuming that 1 mg plasma membrane protein contains 1% H⁺-ATPases with a relative molecular mass of 100000. The start value for fitting the catalytic rate constant (turnover number, k_r) was 3500 min⁻¹ ≈ 58 s⁻¹. Koland and Hammes [5] determined a k_r of 59 µmol min⁻¹ mg⁻¹ for reconstituted yeast PM H⁺-ATPase. One mg of a protein with a relative molecular mass of 100000 corresponds to 10 nmol. Thus, this rate constant can be expressed as 5900 min⁻¹ ≈ 100 s⁻¹. Using a higher enzyme concentration and correspondingly lower k_r start value did not change calculated values of K_m and K_i. Activity data were processed with the dynamic method based on the simulation of pre-steady state dynamics of the biochemical system [27]. In contrast to the rapid-equilibrium method this method is also valid when equilibration between enzyme and enzyme-substrate complex is slow compared to catalytic turnover. Equilibration time was set to 3 s (Supplementary Annex 1, section [progress]).

Discrimination between ATP species

In order to investigate whether a single ATP species, e.g. (K-ATP)³⁻, is more inhibitory compared to others, inhibition in the presence of 155 mM K⁺ was compared to inhibition occurring at 55 mM K⁺

(K_d for $(K-ATP)^{3-} = 68 \text{ mM}$; [9]). Non-linear regression analysis was performed considering three cases of inhibition which differed with respect to the assumed inhibitory ATP species: either $(K-ATP)^{3-}$, $(H-ATP)^{3-}$ or ATP^{4-} (Supplementary Annex 2). For discriminating between these ATP species the set of rate equations was extended by two equilibrium reactions for ATP^{4-} association with H^+ and K^+ [9].

Unless otherwise indicated, inhibition constants refer to the sum of all Mg^{2+} -free ATP species. If inhibition is attributed to a single ATP species, $K_i(1)$ of this species will be according to the relationship:

$$(1) K_i(1) / K_i(A) = c(1) / c(A),$$

where $K_i(A)$ is the K_i assuming that all Mg^{2+} -free ATP acts inhibitory, $c(1)$ is the concentration of the ATP species and $c(A)$ is the concentration sum of all Mg^{2+} -free ATP (tested in Supplementary Annex 3).

RESULTS

H^+ -ATPase kinetics in root membranes

ATP was applied up to a concentration of 5 mM in the presence of 5 mM Mg^{2+} . The kinetics showed a broad maximum between 1 and 3 mM with a slight decline at highest ATP concentrations (Figure 1A). After normalisation of data, standard errors at 1 to 2 mM ATP were reduced (Figure 1A, lower error bars compared to upper), but the decrease of activity at 5 mM ATP compared to 2 mM ATP remained insignificant (t-test). Four kinetic models were analysed with DynaFit with respect to their fit to the experimental data: three models of inhibition by Mg^{2+} -free ATP (competitive, uncompetitive and non-competitive) and the Michaelis-Menten model (Supplementary Annex 1). The probability of any of the inhibition models compared to the Michaelis-Menten model was 99.9%. Regression curves were identical for all models of inhibition (Figure 1A). Akaike weights were 0.332 for each of the inhibition models (Supplementary Annex 4). In a second experiment the range of ATP concentrations was extended to 13 mM. At this concentration activity decreased to 57% of maximum activity (Figure 1B, non-normalised data in Supplementary Annex 5). Again, competitive, uncompetitive and non-competitive mechanisms all yielded the same fits of regression curves and identical Akaike weights of 0.333. Thus, it was not possible to derive the mechanism of inhibition. For this purpose a third experiment was conducted in which ATPase kinetics was measured at fixed concentration of the inhibitor, i.e. of Mg^{2+} -free ATP (Figure 1C, thick line). In the case of competitive inhibition the relative decrease of activity caused by the inhibitor should become smaller with increasing concentration of the substrate, i.e. of Mg -ATP. Activity without inhibition was measured in the presence of 3 mM Mg^{2+} in excess of total ATP (Figure 1C, thin line). With increasing concentration of Mg -ATP relative inhibition continuously decreased from 85% at 0.1 mM Mg -ATP to 23 % at 7 mM Mg -ATP. Indeed model discrimination with DynaFit yielded the highest possible Akaike weight of 1 for competitive inhibition and Akaike weights of 0 for uncompetitive and non-competitive inhibition (DynaFit script and output for model discrimination in Supplementary Annex 6). The probability of competitive inhibition in comparison with each of the other mechanisms was 100%.

The shapes of the activity curves slightly differed at low ATP concentrations as indicated by the arrows in Figures 1A and 1B. In the first experiment (Figure 1A) maximum activity was virtually attained at 1 mM ATP, while in the second experiment (Figure 1B) activity at 1 mM ATP clearly remained below maximum activity. The first experiment was performed at a K^+ concentration of 55 mM (vanadate method, see Experimental Procedures *Kinetic assays*), while the second experiment was conducted at 155 mM K^+ (cocktail method with additional 100 mM KNO_3).

H^+ -ATPase kinetics in leaf membranes

PM ATPase activity was measured with the cocktail method. Compared to roots a higher ATP concentration of 4.5 mM was required to reach maximum activity of PM ATPase (Figure 2A, non-normalised data in Supplementary Annex 5). ATPase activity significantly decreased by 33% at 8.9 mM ATP. At 13.4 mM ATP activity was only 47% of the observed maximum activity. Thus, activity was the same at 13.4 mM ATP and 0.6 mM ATP.

Influence of cations on ATP inhibition

ATP in excess of Mg^{2+} is partly protonated and partly bound to K^+ with a K_d of 68 mM [9]. In the search for the inhibitory ATP species it was investigated whether inhibition was modified when $[\text{K}^+]$ was reduced from 155 mM to 55 mM. Figure 2B shows that the activities at high ATP concentrations were not changed by K^+ . However, the decrease of KCl concentration caused an increase of activity at low ATP concentrations (thick line vs. thin line). In this experiment, ATP was supplied as metal-free ATP (diTRIS-ATP), while in preceding experiments $\text{Na}_2\text{-ATP}$ was used. Therefore, the experiment also showed that sodium was not responsible for the inhibition at high $[\text{ATP}]$. Furthermore, the experiment demonstrated that inhibition was not related to high ionic strength imposed by increasing the ATP concentration.

Upon increasing Mg^{2+} concentration from 5 mM to 15 mM, the decrease in ATPase activity at high $[\text{ATP}]$ was prevented (Figure 3A). On the other hand, when Mg^{2+} concentration was reduced to 2 mM, inhibition of PM ATPase already started at lower ATP concentrations (Figure 3B).

In the absence of free Mg^{2+} the surplus of ATP may bind to other complexing agents present in the assay solution. Since complex formation between the divalent buffer cation BTP and ATP and inhibitory action of ATP-BTP could not be ruled out, we used the monovalent TRIS instead of BTP. Figure 4A shows that inhibition also occurred in the absence of BTP. Inhibition was also observed at pH 7.5 (Figure 4B).

Kinetic constants

Kinetic constants were derived from non-linear regression analysis (Table 1). For k_r and K_m only values based on the model of competitive inhibition are shown since these constants were very similar for all models (deviation less than 3%). The rate of ATP hydrolysis of PM $\text{H}^+\text{-ATPase}$ is known to increase with K^+ concentration. Usually, a test range of up to 50 mM K^+ is employed [3]. For our leaf preparations k_r was 33% higher at 155 mM K^+ compared to 55 mM K^+ . However, this increase in K^+ concentration did not change k_r of root preparations (Table 1). K_m for leaf PM ATPase was similar with $\text{Na}_2\text{-ATP}$ and with metal-free ATP ($977 \pm 70 \mu\text{M}$ and $907 \pm 155 \mu\text{M}$; mean $\pm$ SD, $n=3$). When the concentration of Mg^{2+} was decreased from 5 mM to 2 mM in leaf preparations at 155 mM K^+ , the K_m decreased from $907 \pm 155 \mu\text{M}$ ($n=3$) to $340 \pm 77 \mu\text{M}$ ($n=4$). When the concentration of K^+ was decreased from 155 mM to 55 mM, at 5 mM Mg^{2+} , the K_m decreased from $907 \pm 155 \mu\text{M}$ to $128 \pm 30 \mu\text{M}$ in leaf preparations and from $410 \pm 46 \mu\text{M}$ to $203 \pm 28 \mu\text{M}$ in root preparations ($n=3$). For leaves the low K_m at 55 mM K^+ was confirmed by measuring the activity at ATP concentrations of 50, 100, 200, 600, 1000 and 3000 μM using $\text{Na}_2\text{-ATP}$. K_m was $92 \pm 20 \mu\text{M}$ ($n=3$; data not shown).

Inhibition constants shown in Table 1 refer to the sum of Mg^{2+} -free ATP species. As already illustrated (Fig. 1C), K_i values assuming competitive inhibition are most probable. It is evident that in leaves the high K^+ concentration strongly increased K_i (Table 1: Exp. 4 vs. 5). At high K^+ concentration, leaf K_i exceeded root K_i (Exp. 3 vs. 2). However, leaf K_m also exceeded root K_m . It is clear that this change of K_m is also relevant to the degree of inhibition in a mechanism in which substrate and inhibitor compete for the same site at the enzyme: At given concentrations of substrate and inhibitor an increase of K_m without a change in K_i decreases the activity. Thus, for comparing leaves and roots the ratio between K_i and K_m is an additional instructive parameter: At saturating substrate concentration, the competitive mechanism of inhibition allows to relate the ratio between K_i and K_m to the Mg^{2+} -activity at which 50% inhibition by Mg^{2+} -free ATP would occur which will be explained below. For competitive inhibition the relationship between the inhibitor concentration for 50% inhibition $[\text{ATP}]_{50}$ and the substrate concentration $[\text{Mg-ATP}]$ is given by Eqn. (9) of Cortes et al. [30]:

$$(2) [\text{ATP}]_{50} = K_i (1 + [\text{Mg-ATP}]/K_m)$$

Dividing by $[\text{Mg-ATP}]$ yields

$$(3) [\text{ATP}]_{50} / [\text{Mg-ATP}] = K_i (1/[\text{Mg-ATP}] + 1/K_m)$$

At saturating substrate concentrations, $1/[\text{Mg-ATP}]$ becomes negligible on the right side of the equation and

$$(4) [\text{ATP}]_{50} / [\text{Mg-ATP}] \approx K_i / K_m$$

Since the dissociation equilibrium of Mg-ATP is given by

$$(5) [\text{ATP}]_{50} [\text{Mg}]_{50} / [\text{Mg-ATP}] = K_d$$

where all concentrations refer to the free species, it follows that

$$(6) K_i / K_m \approx K_d / [Mg]_{50}$$

Thus, K_i / K_m is inversely related to the free Mg^{2+} concentration at which 50% inhibition by Mg^{2+} -free ATP occurs. A ratio of 1.92 was obtained for root preparations, while ratios were between 0.32 and 1.23 for leaf preparations. The ratio decreased by 50% when ATP was supplied as metal-free ATP instead of Na_2 -ATP. If inhibition were uncompetitive or non-competitive, K_i values would be in the order of 5 to 10 mM at pH 6.5 with 5 mM Mg^{2+} (Table 1).

DISCUSSION

All experiments in this investigation showed that ATP in excess of Mg^{2+} causes a down-regulation of PM ATPase. A first explanation could be that the enzyme is inhibited by kinases. Kinase action is without doubt a crucial mechanism to regulate the activity of PM H^+ -ATPase *in vivo* under a variety of stress conditions such as osmotic stress or salt stress [13, 31].

Consideration of kinase action

Ca^{2+} -dependent kinases [32] can be ruled out, since our assays were performed without Ca^{2+} . The involvement of a PKS5-like kinase [33], which may be present in the vesicle membrane in close vicinity to H^+ -ATPase [34], is very unlikely because of three arguments:

(a) Usually kinase activity also requires Mg^{2+} . Our data demonstrate that inhibition increased while the Mg ATP concentration was constant. This means that the kinase, e.g. PKS5 [33], should utilise a Mg^{2+} -free ATP species as substrate. It is generally accepted that the conserved catalytic domain of protein kinases binds Mg ATP or Mn ATP [35, 36]. Binding of the divalent cation neutralizes the negative charges of the nucleotide, in order to facilitate phosphoryl transfer [35]. Members of the SNF1-related kinase family, to which PKS5 belongs, require Mn^{2+} or Mg^{2+} for phosphorylation. PKS5 is not active in kinase assays without Mn^{2+} or Mg^{2+} [33]. PKS5 activity was stopped by chelating Mg^{2+} or Mn^{2+} in the presence of 12.5 mM EDTA [33]. When attributing H^+ -ATPase inhibition to PKS5-like kinases in the absence of the complexes Mg ATP or Mn ATP, one has to assume a high-affinity binding site of the kinase for the divalent cation. The K_d of the Mg -kinase complex should be smaller than 30 μ M, the K_d of Mg ATP [9], in order to maintain Mg^{2+} binding in the presence of free ATP. This would be unexpected since neither free ATP nor free Mg^{2+} bind well to the conserved catalytic domain of protein kinases [37].

(b) Inhibition by a kinase of the PKS family in our measurements is also unlikely because ATPase activity decreased at high ATP concentrations above 5 mM. Therefore, the kinase should not be saturated with ATP at 5 mM ATP. A K_m value in the millimolar range would be required. Gong *et al.* [36] determined a K_m for ATP of 0.83 μ M for another member of the PKS family (PKS11). Accordingly for PKS5, kinase assays were conducted at 10 μ M ATP [33]. Fuglsang *et al.* [33] performed H^+ -pumping assays with plasma membrane vesicles (Figure 8A in their publication) which for PKS5 support a K_m for ATP below 0.5 mM. The initial rate of H^+ pumping was determined at various ATP concentrations up to 3 mM in vesicles from PKS5-free plant material and after adding PKS5 to the vesicle preparation. Inhibition was already detectable at ATP concentrations of 0.5 mM. Importantly, relative inhibition did not increase at higher ATP concentrations which is consistent with the low K_m reported for PKS11.

(c) A third argument against a kinase-based interpretation of our results arises from the use of a chaotropic salt in our leaf vesicle preparation. PKS5 should associate with the plasma membrane *in vivo* and should remain associated with plasma membrane vesicles during vesicle preparation. Fuglsang *et al.* [33] clearly showed that vesicles prepared from *Arabidopsis* expressing the PKS5 gene had lower H^+ -pumping activity compared to vesicles from loss-of-function *pks5* mutants. These experiments prove the presence of inhibitory phosphorylated sites in vesicle preparations. However, they do not prove that phosphorylation occurred during the pumping assay. This may already have occurred before vesicle preparation. Up to now PKS5 was not identified in plasma membrane vesicles of *Arabidopsis*, although other members of this kinase family have been found in lipid rafts of vesicle preparations [34]. The SNF1-like kinase family to which PKS5 belongs does not contain integral membrane proteins [31]. Therefore, PKS5 should have been anchored in the membrane by binding to another molecule with hydrophobic character prior to vesicle preparation; or PKS5 itself should have bound to the membrane surface as a peripheral membrane protein. Anchoring could have been achieved by interaction with a CBL protein, the N-terminal myristoyl group of which may immobilise

PKS kinases at the plasma membrane. Indeed, interaction of PKS5 with a CBL protein was demonstrated *in vivo*. However, the physiological relevance of this interaction is not yet clear since PKS5 was active *in vitro* without CBL association [33]. After association of PKS5 with the plasma membrane this association should be stable enough to withstand our membrane isolation procedure. In contrast to the vesicle preparations from Shahollari *et al.* [34], in which CBL proteins were proven, we used the chaotropic salt KJ, in order to remove peripheral membrane proteins as outlined in our method section. This treatment removes 30-40% of total membrane protein in maize root vesicles [22]. Therefore, our investigation most likely reveals direct effects of Mg^{2+} -free ATP on PM H^+ -ATPase activity.

Interaction of Mg^{2+} -free ATP with H^+ -ATPase

If the Mg^{2+} activity falls below the range of the dissociation constant of the Mg -ATP complex, then Mg^{2+} -free ATP will occur. One possible interaction of Mg^{2+} -free ATP with H^+ -ATPase could be that Mg^{2+} -free ATP competes with the enzyme for Mg^{2+} which is required as a cofactor in P-type ATPases. In fact, for Na^+/K^+ -ATPase the dissociation constant of Mg^{2+} interaction with the $\text{E1ATP}(\text{Na}_3)$ state was identical to the dissociation constant of Mg -ATP [38] which would cause Mg^{2+} withdrawal from the enzyme upon addition of Mg^{2+} -free ATP. This would also occur in the normal functional cycle, in which ATP binds to the E2 or E2P state (see next section), if the dissociation constant of the Mg^{2+} interaction with respect to these enzyme states were similar or even larger compared to the $\text{E1ATP}(\text{Na}_3)$ state. Another mode of inhibition would be competition between Mg -ATP and Mg^{2+} -free ATP for the same enzyme site. Inhibition by Mg^{2+} -free ATP has been observed for many Mg^{2+} dependent ATPases. For yeast mitochondrial F_1 -ATPase Jenkins [39] suggested that excess ATP inhibits by complexing free Mg^{2+} assuming that it is free Mg^{2+} which binds to the enzyme-ATP complex. For crayfish mitochondrial F_1 -ATPase Li and Neufeld [40] explained the kinetic data with a classical model of competitive inhibition by Mg^{2+} -free ATP.

Binding site for Mg^{2+} -free ATP

Our data on inhibition by Mg^{2+} -free ATP are consistent with a model of competitive inhibition (Figure 1C). Competition between Mg -ATP and Mg^{2+} -free ATP for the same binding site may occur in different states of the functional cycle of P-type ATPases. According to the most widely used model of the mechanism of P-type ATPases (Albers-Post model or E1-E2 model) ATP binds to the dephosphorylated E2 state [41]. Indeed, ATP binding to this state dramatically accelerates the $\text{E2} \rightarrow \text{E1}$ transition of other structurally related P-type ATPases, i. e. PM Na^+/K^+ -ATPase and sarcoplasmic reticulum Ca^{2+} -ATPase. However, ATP binding to the E2P phosphoenzyme of Na^+/K^+ -ATPase was recently demonstrated utilising a fluorescent dye which revealed changes of the E2P state at Mg -ATP concentrations above 100 μM [42]. Moreover, for sarcoplasmic reticulum Ca^{2+} -ATPase X-ray crystallography shows binding of an ATP analogue to the transition state of dephosphorylation (E2-P^*). The authors suggest that ATP binds in exchange for ADP already in the E2P state [43]. Khalid *et al.* [42] showed that in the absence of K^+ , Mg -ATP bound to the E2P state of PM Na^+/K^+ -ATPase plays a modulatory role by decreasing the rate of dephosphorylation. For PM Na^+/K^+ -ATPase it is now clear “that the enzyme only has a single ATP binding site which fulfills both allosteric and catalytic functions in different parts of the reaction cycle” [42].

Competitive inhibition of PM Na^+/K^+ -ATPase by Mg^{2+} -free ATP has been first described by Hexum *et al.* in 1970 [44]. Robinson [45] confirmed this finding. His experiments were performed at pH 7.8, at which the concentration of $(\text{H-ATP})^{3-}$ is negligible. He did not discriminate between $(\text{K-ATP})^{3-}$ and ATP^{4-} and determined an apparent K_i of 4.8 mM. For PM H^+ -ATPase in maize we obtained considerably lower values between 0.01 mM and 1.1 mM depending on the ionic conditions in the assay. Competitive inhibition requires that Mg^{2+} -free ATP binds at or in the ATP-binding pocket of the nucleotide-binding domain (N-domain). For PM Na^+/K^+ -ATPase site-directed mutagenesis was exploited to identify specific amino acid residues in the N-domain which contribute to high-affinity binding of Mg^{2+} -free ATP [46]. Indeed, an Arg residue at the mouth of the ATP-binding pocket was proven to be important for binding of Mg^{2+} -free ATP as well as Mg -ATP.

Inhibitory ATP species

At inhibitory ATP concentrations changes of K^+ concentration did not influence PM ATPase activity (Figure 2B), although the concentration of all major Mg^{2+} -free ATP species changed (Table 2). Does this observation rule out a model of inhibition by a single ATP species? The answer has to account for the important observation that the K_m for Mg-ATP decreased at low $[K^+]$ (Table 1: Exp. 5 compared to Exp. 4). This increase of substrate affinity should increase hydrolytic activity in the presence of the inhibitory ATP species. Because this did not occur, obviously affinities increased for Mg-ATP as well as for the inhibitor. Indeed, in any mechanism of competitive inhibition a change of K_m is likely to affect K_i as well. When regression analysis is not forced to use the same K_m and K_i for both K^+ concentrations, the K^+ -dependent concentration change of a given ATP species is completely compensated by a change in K_i . As a consequence good fits are obtained for any ATP species and Akaike weights are similar for all of them (Table 2). Therefore, inhibition by a single ATP species cannot be ruled out.

Physiological relevance of inhibition by Mg^{2+} -free ATP

Apparent K_i values for competitive inhibition by Mg^{2+} -free ATP are in the range of the K_m values for Mg-ATP. This means that at a given Mg-ATP concentration activity declines by 50% upon withdrawal of half the Mg^{2+} from Mg-ATP (in addition to the decrease due to smaller substrate concentration). It is evident that this mechanism is particularly useful for down-regulating PM H^+ -ATPase activity when Mg-ATP concentrations are in the saturating range in which changes in substrate concentration have little influence on activity. Moreover, this mechanism does not require disposal or removal of Mg^{2+} -free ATP, which remains available as a regulator for other processes or for fast reactivation by Mg^{2+} .

Our investigation revealed differences between leaves and roots with respect to K_i and K_m (Table 1: Exp. 2 and 3). The two parameters changed in the same direction, but K_i/K_m was smaller for leaves. As explained in the Results section and utilizing a K_d for Mg-ATP of 71 μM a decrease of Mg^{2+} activity to a level of 63 μM ($[Mg^{2+}]_{50}$) would cause 50% inhibition of PM H^+ -ATPase in leaves, while in roots a lower Mg^{2+} activity of 37 μM would be required to achieve 50% inhibition. For the leaves the control potential of ATP inhibition is substantially higher at high K^+ concentration with a $[Mg^{2+}]_{50}$ of 127 μM (Exp. 4). It is particularly high at pH 7.5 with a $[Mg^{2+}]_{50}$ of 222 μM . This is well within the range of estimated Mg^{2+} activities in leaf mesophyll cells [8]. Control by cellular Mg^{2+} could play a physiological role in leaf cells once cellular turgor has been established. Inhibition by Mg^{2+} -free ATP of E1 phosphorylation could act in concert with K^+ -stimulated dephosphorylation of the E1P state [3], both preventing E1P accumulation under conditions in which the membrane potential is close to the reversal potential of the pump. Since the E1P state can phosphorylate ADP (is ADP-sensitive), E1P accumulation could cause the pump to run backward [3]. We did not observe the stimulatory effect of K^+ on ATP hydrolysis of root vesicles. An explanation could be that hydrolytic stimulation of root isoforms is already saturated at 55 mM K^+ .

Mechanisms which decrease cytosolic Mg^{2+} activity

ATP inhibition, *in vivo*, of PM ATPases by Mg^{2+} -free ATP depends on the existence of mechanisms, which force the cytosolic Mg^{2+} activity down to the range of 30 μM , the dissociation constant of $(Mg-ATP)^2$. Two types of mechanisms have to be considered [7]: (a) Mg^{2+} transport into organelles or to the extracellular space through membrane transporters or channels and (b) Mg^{2+} binding by other strong chelators or macromolecules.

For the small thin cytosolic compartment of a leaf mesophyll cell the voluminous chloroplasts with their ability to take up Mg^{2+} appear to represent a competitive Mg^{2+} sink. Ion translocation within the chloroplast is known to have a profound influence on ionic conditions in the cytosol and on PM H^+ -ATPase activity. After switching off the light or when the substomatal CO_2 concentration suddenly increases massive proton release from the thylakoid lumen to the chloroplast stroma occurs. Also within the cytosol proton activity transiently increases [47]. At the plasma membrane both stimuli immediately invoke a transient hyperpolarisation [47, 48]. At the same time apoplastic pH decreases [49] consistent with a transient stimulation of PM H^+ -ATPase. While protons pass the thylakoid membrane, Mg^{2+} ions move in the opposite direction for charge compensation. When the light is turned on, Mg^{2+} is transported to the stroma and is bound to the *N*-carboxylated Rubisco

thereby activating it. It is clear that upon light-off Mg^{2+} must be released to be bound again upon the next exposure to light. Release probably causes a transient increase in stromal Mg^{2+} activity [8]. Evidence indicates that after switching off the light the stromal Mg^{2+} concentration eventually decreases by several mM. Taking into consideration that Mg^{2+} can freely move through the outer chloroplast membrane, not only protons, but also Mg^{2+} may leave the chloroplast upon light-off and may contribute to the transient stimulation of PM H^+ -ATPase by complexing Mg^{2+} -free ATP.

For plant cells information on Mg^{2+} transport, its affinity for Mg^{2+} and its regulation is very limited [50]. There is one report on a candidate Mg^{2+} transporter of *Arabidopsis thaliana* which restores intramitochondrial Mg^{2+} concentration in a yeast mutant. Membrane transporters for Mg^{2+} are better investigated in mammalian cells. The channel kinase TRPM7 mediates Mg^{2+} uptake from the extracellular space into the cytosol and is essential for cellular viability [51]. This channel is strongly down-regulated by intracellular Mg^{2+} activity, MgATP and Mg-nucleotides thereby restricting cytosolic access of Mg^{2+} . Massive, cAMP-mediated Mg^{2+} release from mitochondria into the cytosol occurs upon catecholamine administration [7]. Mg^{2+} release from isolated mitochondria upon administration of cAMP is accompanied by an increase in respiration rate. The underlying activity changes of mitochondrial enzymes could be caused by a decrease of matrix Mg^{2+} activity. In the intact cell, relief of ATP inhibition of the Na^+/K^+ -ATPase, which may consume 20-30% of the cellular ATP [46], by additional Mg^{2+} could boost ATP hydrolysis and thus contribute to enhanced respiration. Besides ATP a number of Mg^{2+} chelators are related to the regulation of metabolic activity. In chloroplasts Mg-chelatase activity and Mg-protoporphyrin concentration increase upon abscisic acid application [13] suggesting a mechanism for hormone-stimulated Mg^{2+} withdrawal from the cytosol. In non-photosynthetic tissue the cytosolic Mg^{2+} activity may be decreased by synthesis of phytic acid. Phytic acid probably exists mostly as a *penta*- Mg^{2+} salt *in vivo* [52]. Abscisic acid induces the enzyme inositol-3-phosphate synthase leading to the accumulation of phytic acid in duckweed [53]. For roots of maize seedlings it has been proven that exogenous phosphate is readily incorporated into phytic acid [54]. The level of the phytic acid precursor *myo*-inositol increased to 75% of soluble carbohydrates in roots of salt-adapted tomato plants [55]. Another physiological process involving Mg^{2+} binding to phosphate groups is polyamine catabolism. Upon degradation of these abundant polycations their binding sites at DNA, RNA and proteins will adsorb free Mg^{2+} [56].

There is evidence that ABA responsiveness of guard cells requires attenuation of H^+ pumping in addition to activation of anion channels [13]. For activation of the guard cell anion channel GCAC1 ATP is required, but for this PM transporter the Mg^{2+} -free nucleotide is sufficient for activation [57]. Because a large number of ABA responsive genes are triggered by Mg^{2+} deficiency [58], it is clear that ABA responses are not hampered by Mg^{2+} deficiency. It is also conceivable that ABA responses may impose a physiological Mg^{2+} deficiency on the cytosolic compartment.

For PM Na^+/K^+ -ATPase not only inhibition by Mg^{2+} -free ATP was found, but also inhibition by Mg-ADP in a competitive manner [45]. In our assays ADP was also inhibitory (Fatima, unpublished). This is evident from assays which were performed with root vesicles at pH 6.5 in the presence of 5 mM ATP, 5 mM Mg^{2+} and 155 mM K^+ , but without ATP-regenerating system. Derived from the increase in phosphate concentration, ADP accumulated to a concentration of 0.15 mM. Omission of the ATP-regenerating system decreased PM H^+ -ATPase activity by $15\% \pm 2\%$ ($n = 4$). Possibly, the inhibition mechanisms of Mg^{2+} -free ATP and of Mg-ADP are closely related. Certainly, these mechanisms contribute to the complex metabolic and mineral requirements of PM H^+ -ATPase for maximum hydrolytic activity. With respect to mineral requirements, kinetic properties of PM H^+ -ATPase and emerging patterns of regulation of several intracellular Mg^{2+} chelators indicate that ATP inhibition of PM H^+ -ATPase could be involved in regulatory mechanisms for reducing the activity of this “powerhouse” enzyme during stages of growth reduction, growth arrest or during the transition from active growth to dormancy.

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FIGURE LEGENDS

Figure 1 Effect of Mg^{2+} -free ATP on plasma membrane ATPase activity from roots

Assays were performed with vesicles (cytosolic side outside) at pH optimum (6.5) using $\text{Na}_2\text{-ATP}$ adjusted to pH 6.5. Released ADP was converted to ATP with pyruvate kinase and PEP. A) Experiment at ATP concentrations up to 5 mM at 5 mM Mg^{2+} and 55 mM K^+ . Upper error bar denotes SE of original data, lower error bar SE of normalised data ($n = 3$). Regression curves by DynaFit assuming Michaelis-Menten kinetics (dashed line) or inhibition by Mg^{2+} -free ATP (solid line, script in Supplementary Annex 1). Curves were identical for competitive, uncompetitive and non-competitive inhibition. B) Experiment at ATP concentrations up to 13 mM at 5 mM Mg^{2+} and 155 mM K^+ . Gramicidin was used to prevent a pH gradient at the vesicle membrane. Data are means + SE of normalised data ($n = 3$). Regression curves calculated by DynaFit were identical for all tested inhibition types. C) Mg^{2+} -free ATP is a competitive inhibitor. Thick line: activity in the presence of 2 mM Mg^{2+} -free ATP. Thin line: activity with 3 mM Mg^{2+} in excess of total ATP at all ATP concentrations (no inhibition). K^+ concentration was 155 mM. Data are means $\pm$ SE of original data ($n = 3$). Regression curves by DynaFit assuming competitive inhibition.

Figure 2 Effect of Mg^{2+} -free ATP on plasma membrane ATPase activity from leaves

Assays were performed with ATP regeneration at pH 6.5 and at 5 mM Mg^{2+} . A) Inhibition with two different ATP reagents: $\text{Na}_2\text{-ATP}$ (circles) and diTRIS-ATP (squares). K^+ concentration was 155 mM. Data are means + SE of normalised data ($n = 3$). For diTRIS-ATP SE was omitted for clarity and is shown in Figure 2B. Regression curves calculated by DynaFit were identical for all tested inhibition types. B) Inhibition at different K^+ concentrations: 155 mM (squares) and 55 mM (triangles). ATP was provided as diTRIS-ATP. Data from experiment at 155 mM K^+ were already shown in Fig. 2A. Data are means $\pm$ SE ($n = 3$).

Figure 3 Influence of $[\text{Mg}^{2+}]$

A) Recovery of ATPase activity after addition of Mg^{2+} . The assay was performed with vesicles from leaves at Mg^{2+} concentrations of 5 mM (squares) and 15 mM (circles) with 150 mM K^+ at pH 6.5. ATP was provided as diTRIS-ATP. The two Mg^{2+} concentrations were tested in the same assay. No ATP-regenerating system was used. B) Inhibition of plasma membrane ATPase activity at low $[\text{Mg}^{2+}]$. Assays were performed with vesicles from leaves at 2 mM Mg^{2+} and 155 mM K^+ at pH 6.5. ATP was provided as diTRIS-ATP. Data are means $\pm$ SE ($n = 4$).

Figure 4 Inhibitory effect of Mg^{2+} -free ATP with different buffers

A) Inhibition in the presence of divalent (BTP, circles) or monovalent (TRIS, squares) amino buffer cations. The concentration of buffer cations was 30 mM. In both cases MES was used as acidic buffer component. The assay was performed with vesicles from leaves at 5 mM Mg^{2+} , 55 mM K^+ with diTRIS-ATP at pH 6.5. The two buffer cations were tested in the same assay procedure. Regression curve refers to BTP. B) Inhibition at pH 7.5 (circles) in comparison with pH 6.5 (triangles, from Figure 2B). Assays were performed with vesicles from leaves in BTP buffer at 5 mM Mg^{2+} and 55 mM K^+ with diTRIS-ATP. Data are means + SE ($n = 2$).

TABLES

Table 1 Kinetic constants of plasma membrane ATPase from maize roots and leaves

Assays were performed with different ATP reagents under the specified ionic conditions (number denotes ion concentration in mM). Catalytic rate constant k_r (s^{-1}), Michaelis-Menten constant K_m (μM) and inhibition constant K_i (μM) were calculated assuming competitive (c), uncompetitive (u) and non-competitive (n) inhibition without discriminating between ATP species (mean $\pm$ SD). k_r and K_m refer to (c), but deviation with (u) and (n) was less than 3% (all data in Supplementary Annex 4). In lines "3sep" and "4sep" mean value and SD was calculated from kinetic constants determined separately for each replicate. n. d.: not determined because of small range of inhibitory ATP concentrations (see results).

<i>Exp.</i>	<i>ATP reagent</i>	<i>Ionic conditions</i>	<i>n</i>	<i>k_r</i>	<i>K_m</i>	<i>K_i</i>	<i>K_i/K_m</i>	
Root	1	Na ₂ -ATP	5 Mg, 55 K	3	72 ± 2,8	203 ± 28	n. d.	
	2	Na ₂ -ATP	5 Mg, 155 K	3	64 ± 2.2	410 ± 46	c: 787 ± 108 u: 9637 ± 1386 n: 10410 ± 1464	c: 1.92
Leaf	3	Na ₂ -ATP	5 Mg, 150 K	3	179 ± 5.1	977 ± 70	c: 1102 ± 80 u: 5666 ± 458 n: 6749 ± 507	c: 1.13
	3sep				181 ± 18	1041 ± 268	c: 1133 ± 180	
	4	diTRIS-ATP	5 Mg, 155 K	3	144 ± 8.8	907 ± 155	c: 511 ± 68 u: 2843 ± 398 n: 3346 ± 419	c: 0.56
	4sep				147 ± 19	972 ± 426	c: 512 ± 37	
	5	diTRIS-ATP	5 Mg, 55 K	3	108 ± 2.5	128 ± 30	c: 129 ± 26 u: 5085 ± 407 n: 5207 ± 403	c: 1.01
	6	diTRIS-ATP	2 Mg, 155 K	4	101 ± 7.7	340 ± 77	c: 419 ± 95 u: 2493 ± 670 n: 2900 ± 730	c: 1.23
	7	diTRIS-ATP	5 Mg, 55 K, pH 7.5	2	67 ± 3.1	24 ± 11	c: 8 ± 4 u: 1645 ± 457 n: 1652 ± 458	c: 0.32

Table 2 **Discrimination between different Mg^{2+} -free ATP species**

The enzyme activity data from the experiment at different $[\text{K}^+]$ (Figure 2B) were analysed based on three models of competitive inhibition by a single ATP species. For each ATP species K_i (μM) was calculated (Supplementary Annex 2). Percentage values denote ratio between K_i of the species and the sum of K_i values at the given $[\text{K}^+]$. This ratio reflects the molar composition of the Mg^{2+} -free ATP pool. The fit of each of the models to the experimental data was expressed as Akaike weight. Model (ATP species) with highest Akaike weight is most plausible, but here differences are negligible.

<i>ATP species</i>	<i>K_i at 155 mM K^+</i>		<i>Akaike weight at 155 mM K^+</i>	<i>K_i at 55 mM K^+</i>		<i>Akaike weight at 55 mM K^+</i>
(H-ATP) ³⁻	150	28%	0.34380	60	41%	0.35495
(K-ATP) ³⁻	269	50%	0.31140	38	26%	0.28737
ATP ⁴⁻	119	22%	0.34481	47	33%	0.35768

Fig. 1

Fig. 2

Fig. 3

Fig. 4