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Effect of Ca^{2+} on H^+-ATPase activity of plasma membrane in wheat root

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Abstract

Phosphorylation/dephosphorylation of plasma membrane H^+-ATPase acts as a regulator to control the H^+-ATPase activity. In this work a plasmalemma-enriched fraction from wheat root and a partially purified H^+-ATPase were used to investigate the effects of Ca^{2+} and calmodulin on the H^+-ATPase activity and its phosphorylation status. The hydrolytic activities increased approximately 40% by μmol l^{-1} Ca^{2+}, while calmodulin did not have any effect neither alone nor in the presence of Ca^{2+}, indicating that calmodulin is not involved in the process. However, when adding stauosporine, a kinase inhibitor, the stimulatng effect of Ca^{2+} was eliminated. Moreover, partially purified H^+-ATPase has the same characteristics. These results show that the H^+-ATPase activity is influenced by its phosphorylation.

Keywords: PM H^+-ATPase; Ca^{2+}; Protein phosphorylation

1. Introduction

PM H^+-ATPase, acting as a kind of transmembrane glucoprotein, plays an important role in the development of plant cell. PM H^+-ATPase is an electrogenic pump that directly couples ATP hydrolysis with the vectorial transport of H^+ into the appolast. The electrochemical proton gradient acts as a driving force for the secondary transport of ions and nutrients into and out of cell. It also controls some major physiological-biochemical process, e.g. growth, develop, maintenance of cell turgor pressure and intracellular pH [1–3]. In order to have more understanding of these important physiological functions, PM H^+-ATPase has been a hot topic in the past two decades and profound improvements have been achieved. It is found that phosphorylation/dephosphorylation, reversible covalent modification, can also control its activity [4–6]. However, there is still some divarication in the study of this field, e.g. research of Takemoto [7] and Suzuki [8] indicates that phosphorylation promotes the PM H^+-ATPase activity, while some other research shows an inhibition [5,9,10]. Zocchi conjectured that Ca^{2+} and CaM may have taken part in this process, but unfortunately was not able to purify membrane in then [11,12]. However, using advanced technology to isolate membrane, this work has proved this conjecture.

2. Material and methods

2.1. Material

Wheat '8866' (*Triticum aestivum* L) was used in the experiment. The seeds were placed in 1% sodium hypochlorite solution for 20 min, washed with distilled water for three times after biocidal treatment. They were then put in darkness at temperature of 25 °C for 24 h. After bourgeon, they were put on filter paper, and then fostered in 1/2 Houglandn culture solution for 7 days, with light intensity of 36 μmol m^{-2} s^{-1}, for 13 h per day. The root was collected for further experiment.

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2.2. Isolation and purification of plasma membrane

According to method of [13], plasma membrane vesicle purified by sucrose gradient centrifugation.

2.3. Purification of plasma membrane H^+-ATPase

According to method of Qiu [12], using the Zwittergent3-14 help solution.

2.4. Measurement of H^+-ATPase activity

According to the paper [6], activity of hydrolytic ATPase was determined by measuring the release of Pi from ATP.

Proton-activity was monitored as the decrease in the absorbance of acridine orange at 492 nm. The assay medium consisted of 140 mM KCl, 10 mM Mops-BTP (pH 7.0), 2 mM ATP, 1 mM EDTA, 1 mM DTT, 1 mg ml^{-1} BSA (fatty-acid free), 5 μM acridine orange and 50–100 μg PM protein in a final volume of 1 ml. After 5 min pre-incubation at 25 °C, the reaction was started by addition of $MgCl_2$ to final concentration of 4 mM. Absorbance was monitored using a Jasco V550 spectrophotometer. Calcium chloride and EGTA to obtain 8 μM free Ca^{2+}, calmodulin (CaM 5 μg ml^{-1}) and stauosporine (1 μM) were added to the standard assay medium.

2.5. Measurement of free-Ca^{2+} concentration

According to the paper [6], using S3606 radiometer F2171Ca Selectrode to measure the concentration.

2.6. Dephosphorylation

PM H^+-ATPase (300 μg) was added into 5 ml suspend medium (30 mmol l^{-1} Tris–HCl pH 8.5, 1.5 mmol l^{-1} EGTA, 0.1mol l^{-1} KCl), and centrifugated at 200 000 × *g* for 15 min. The deposit was then suspended in 40 μl reaction medium (30 mmol l^{-1} Tris–HCl pH 8.5, 0.1 mmol l^{-1} KCl, 1 mmol l^{-1} $MgSO_4$). A certain quantity (μg) alkaline phosphatase was added, reacted at room temperature for 20 min. Then 20 μl buffer solution (30 mmol l^{-1} Hepes–Tris pH 7.0, EGTA 1.5 mmol l^{-1}, 0.1 mol l^{-1} KCl) was added to stop the reaction. After ultracentrifugated at 200 000 × *g* for 15 min, the deposit was collected to measure.

3. Results

3.1. Separating and purifying of plasma membrane

Plasma membrane vesicle was purified via sucrose gradient centrifugation. Purity of plasma membrane we obtained was identified by various ATPase-inhibitors. The results (Table 1) shows that plasma membrane ATPase inhibitor Na_3VO_4 inhibits at 63%, vacuolar membrane ATPase inhibitor $NaNO_3$ inhibits at 2.9%, mitochondria ATPase inhibitor NaN_3 inhibits at 3.2%, and acid phosphatase inhibitor $NaMO_4$ inhibits at 4.3%. These result shows that the plasma membrane obtained, has high purity and is rarely polluted by vacuolar membrane, mitochondria nor acid phosphatase.

Table 1
Effect of inhibitor on plasma membrane H^+-ATPase from wheat roots

	ATPase activity (μmol Pi mg^{-1} protein h^{-1}) plasma membranes
Control	126 ± 8.9
Na_3VO_4 (0.1 mmol l^{-1})	49.1 ± 3.4 (63%)
$NaNO_3$ (50 mmol l^{-1})	122.2 ± 4.7 (2.9%)
NaN_3 (1 mmol l^{-1})	121.9 ± 5.3 (3.2%)
$NaMoO_4$ (0.1 mmol l^{-1})	120.6 ± 3.6 (4.3%)

3.2. Regulatory mechanism to control PM H^+-ATPase activity

Isolated plasma membrane vesicle was used to evaluate the effect of Ca^{2+} to PM H^+-ATPase activity. As shown in Fig. 1 μmol l^{-1} Ca^{2+} is enough to promote PM H^+-ATPase activity, while a concentration of 8 μmol l^{-1} shows the greatest effect. This result was same to Patrizia's experiment [6], and a concentration of 8 μmol l^{-1} was subsequently used throughout the study.

In the 'Stimulation–Response' coupling system, Ca^{2+}, acting as a important second messenger in cell, via its reaction with target protein, controls the function protein, and so that influences the physiological–biochemical behavior in plant cells. The following experiment is to evaluate if CaM take part in Ca^{2+}'s influence to PM H^+-ATPase activity.

Table 2 shows that PM H^+-ATPase hydrolytic activity increased about 40% when Ca^{2+} has a concentration of 8 μmol l^{-1}, while CaM shows no effect. In

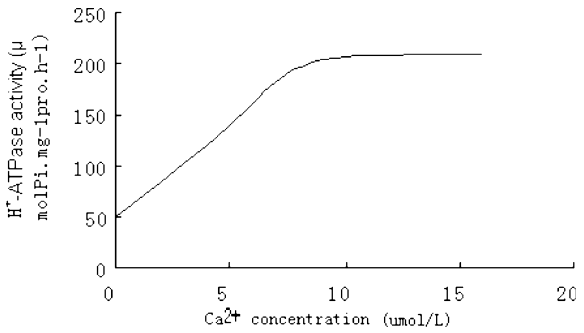

Fig. 1. Effect of μmol concentration of Ca^{2+} on the H^+-ATPase activity of PM vesicles from wheat roots.

Table 2
Effect of CaM on the H^+-ATPase activity of PM vesicles from wheat roots (Ca^{2+}, 8 μmol l^{-1})

	H^+-ATPase activity (μmol Pi mg^{-1} protein h^{-1})
Control	172±26
Ca^{2+}	240±19[a]
CaM (3 μg l^{-1})	174±23
CaM (5 μg l^{-1})	168±31
CaM (7 μg l^{-1})	170±25
Ca^{2+},CaM (3 μg l^{-1})	246±22[a]
Ca^{2+},CaM (5 μg l^{-1})	243±24[a]
Ca^{2+},CaM (7 μg l^{-1})	249±18[a]

[a] $P < 0.05$.

addition, when Ca^{2+} and CaM was added at the same time, the effect to the activity of PM H^+-ATPase is similar to that using Ca^{2+} alone. Cytoplasm was used in order to verify if something take part in the effect of CaM was lost in the separation of membrane. However, as shown in Table 3, CaM does not influence either, indicating that CaM might not take part in the effect of Ca^{2+} on PM H^+-ATPase activity. Of course, it is not sure if there is endogenous CaM combined in membrane, which has influence to the effect of CaM added. In order to get further verification, calmodulin antagonists W_5, W_7 was added. Table 3 shows that they have not influence in the effect of Ca^{2+}, indicating CaM does not take part in this process.

Influence of phosphorylation on the effect of Ca^{2+} to H^+-ATPase activity is also studied. Stauosporine, a kind of kinase inhibitor, was added in the reaction medium, and promotion of Ca^{2+} to H^+-ATPase activity was eliminated (Table 4). This indicates the stimulation effect is probably related to the phosphorylation. Because when stauosporine was added, kinase was inhibited, which restrained the phosphorylation of PM H^+-ATPase, and so that eliminated the effect of Ca^{2+}. It is clear that there is a kind of kinase, which is sensitive to Ca^{2+}, and independent of CaM, with PM H^+-ATPase acting as its substrate.

Table 4
Effect of kinase inhibitor (staurosporine) on PM H^+-ATPase activity from wheat roots in the presence of Ca^{2+} (8 μmol l^{-1})

	H^+-ATPase activity (umol Pi mg^{-1} protein h^{-1}) (Staurosporine 1 μmol l^{-1})
Control	159±29
Ca^{2+}	146±13
CaM	151±32
Ca^{2+}, CaM	139±26

Table 5
Effects of Ca^{2+} (8 μmol l^{-1}), calmodulin (5 μmol l^{-1}) and staurosporine (1 μmol l^{-1}) on the purified H^+-ATPase activity

	H^+-ATPase activity (μmol mg^{-1} protein h^{-1})
Control	254±16
Ca^{2+}	408±24[a]
CaM	250±12
Ca^{2+}, CaM	419±16[a]
Ca^{2+}, CaM, staurosporine	244±19

[a] $P < 0.01$.

In order to know more on the effect of Ca^{2+} and CaM on H^+-ATPase activity, partially purified H^+-ATPase via Zw-helped solution and ammonium-sulfate-deposit method was used to repeat the experiment above. Table 5 shows that purified PM H^+-ATPase activity increases significantly. Ca^{2+} (8 μmol l^{-1}) promotes PM H^+-ATPase activity at about 60%, while CaM has no effect. However, stauosporine can eliminated this effect, by the comparing of Tables 2 and 4, adding stauosporine in membrane plasma obtained can inhibit PM H^+-ATPase activity. This is however due to the remained Ca^{2+} in membrane plasma obtained, and can be eliminated when PM H^+-ATPase is fully purified (Table 5). Moreover, stauosporine cannot directly inhibit PM H^+-ATPase activity.

The measurement of proton pumping activity shows that (Table 6), Ca^{2+} can promote PM H^+-ATPase

Table 3
Effect of calmodulin antagonists on PM H^+-ATPase activity from wheat roots in the presence of Ca^{2+} (8 μmol l^{-1}) and calmodulin (5 μg ml^{-1})

	H^+-ATPase activity (umol Pi mg^{-1} protein h^{-1})					
	W_5 (50 μM)	W_5 (100 μM)	W_5 (150 μM)	W_7 (50 μM)	W_7 (100 μM)	W_7 (150 μM)
Control	173±32	168±19	177±18	186±32	174±25	181±31
Ca^{2+}	242±19[a]	247±31[a]	239±26[a]	231±17[a]	236±18[a]	236±24[a]
CaM	182±25	190±17	184±19	170±24	173±22	179±11
Ca^{2+}, CaM	239±24[a]	235±26[a]	241±21[a]	246±14[a]	238±27[a]	243±19[a]
Ca^{2+}, CaM, cytoplasm (1 μmol l^{-1})	244±29[a]	248±13[a]	238±16[a]	239±36[a]	241±31[a]	247±18[a]

[a] $P < 0.05$.

Table 6
Effect of Ca^{2+} (8 μmol l^{-1}) CaM (5 μmol l^{-1}) staurosporine (1 μmol l^{-1}) on the proton-pumping activity in PM vesicles

	Proton-pumping activity
Control	15.2±2.1
Ca^{2+}	22.3±3.2**
CaM	14.7±1.9
Ca^{2+},CaM	21.6±1.6**
Ca^{2+}, CaM, staurosporine	15.8±2.4

Proton-pumping activity represented by relative fluorescence quenching of protein mg^{-1} min^{-1}.

proton transport activity, while CaM has no influence to it. Kinase inhibitor can reduce the promotion of Ca^{2+}. Therefore, the effect of Ca^{2+} on PM H^+-ATPase hydrolytic activity is similar to its proton pumping activity.

In order to verify the influence of phosphorylation to PM H^+-ATPase, alkaline phosphatase was added, reacting with PM H^+-ATPase to study its activity. Alkaline phosphatase can catalyze dephosphorylation. Alkaline phosphatase of different concentration was added, and the activity of PM H^+-ATPase activity was measured after 20 min. The result shows that (Fig. 2), the dephosphorylation was promoted when alkaline phosphatase concentration increased, while the activity of PM H^+-ATPase was decreased. It obviously shows that the promotion of PM H^+-ATPase activity is indeed directly related to phosphorylation.

4. Discussion

In the study, it is found that μmol l^{-1} Ca^{2+} is enough to promote PM H^+-ATPase activity. Ca^{2+} with a concentration of 8 μmol l^{-1} has the greatest effect, and this effect does not change even if Ca^{2+} concentration increases (Fig. 1), indicating that the promotion of Ca^{2+} to PM H^+-ATPase is depending on the concentration. This is however opposite to the result of Barbara, using beet as material [5], which is probably due to the different regulation mechanisms of PM H^+-ATPase activity in different species. Analysis of the primary structure of PM H^+-ATPase shows that there is serine and threomine residue on its multipeptide [14], which has already been proved to be specific substrate of kinase by recent studies [15]. PM H^+-ATPase is, therefore, possibly the substrate of phosphorylase. Certainly, it does not mean that phosphorylation has a control effect to its activity. But the existence of these two specific amino acids is an important precondition of the phosphorylation PM H^+-ATPase. The research of Biduay [7] shows that the effect of syringomycin to beet PM H^+-ATPase activity is related to its effect on kinase phosphorylate PM H^+-ATPase process. This effect of syringomycin disappeared when deoxycholate eluted kinase, indicating that kinase can regulate PM H^+-ATPase activity. In addition, it was found by Vander Hoeven [16] that PM H^+-ATPase of oat coleoptile can be inhibited by PCK (kinase C) inhibitor, indicating the relation between PM H^+-ATPase and its phosphorylation. In this study, it is found that, adding kinase inhibitor will completely eliminate the promotion of Ca^{2+} (Table 4). Therefore, the promotion of PM H^+-ATPase activity is related to phosphorylation. In addition, in order to verify the effect Ca^{2+} to PM H^+-ATPase activity, alkaline phosphatase, which has dephosphorylation function, was added to the reaction medium. Result shows that the activity of PM H^+-ATPase decreased when dephosphorylation is enhanced (Fig. 2). It is clearly shown that Ca^{2+} affect PM H^+-ATPase activity via phosphorylation. Acting as a major message molecular, Ca^{2+} usually take part many physiological processes together with CaM. Wheat root was used as material in this experiment. It is found in this experiment that CaM does not take part in the phosphorylation of PM H^+-ATPase, for it does not influence the promotion of Ca^{2+} to PM H^+-ATPase activity (Table 3). Whereas, Ca^{2+}/CaM-depended kinase, Ca^{2+}/phospholipid-depended kinase and cAMP-depended kinase, which widely exist in animal cell signal transdution are not found in plant cells [15]. Nevertheless, it is found that there is another kind of Ca^{2+}-depended kinase, which depends on Ca^{2+}, but not on CaM and phosphatides [17]. The kinase, which catalyzes PM H+-ATPase found in this experiment, may also be of this kind. However, different conclusions of the effect dephosphorylation on the activity of H^+-ATPase may be found in relevant researches using different materials. According to Tokemoto's research [8], mycotoxin promoted the activity of PM H^+-ATPase via phosphorylation, which accords to our result. However, opposite results were also found in other experiments using corn, beet, or potato suspend cells [5]. This is possibly because of the different positions where kinase-catalyzed phosphorylation of amino acid residue at.

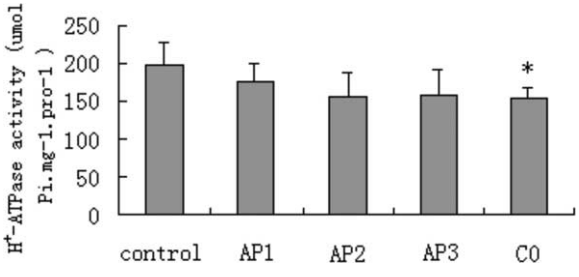

Fig. 2. Effect of alkaline phosphatase on the H^+-ATPase activity of PM vesicles from wheat roots. Control, vesicles+8 μMCa^{2+}; AP1, vesicles+8 μMCa^{2+}+1 unit AP; AP2, vesicles+8 μMCa^{2+}+2 unit AP; AP3, vesicles+8 μMCa^{2+}+3 unit AP; C0, vesicles.

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