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Role of protein kinase in the effect of sound stimulation on the PM H^+-ATPase activity of *Chrysanthemum callus*

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Abstract

The relationship of PM H^+-ATPase activity and its phosphorylation is studied, using sound-treated *Chrysanthemum callus* as the material. The result shows that, after 1 h sound treatment under the strength of 100 dB and frequency of 1000 Hz, the PM H^+-ATPase activity and its phosphorylation was profoundly increased. However, it was found protein kinase inhibitory can effectively inhibit this effect. After dephosphorylation, the PM H^+-ATPase activity of the sound-treated group was similar to the control. Thus, it is concluded that the calcium-dependent protein kinase takes part in the effect of sound stimulation on the PM H^+-ATPase activity of *C. callus*.

Keywords: Calcium-dependent protein kinase; PM H^+-ATPase; Sound stimulation; Signal transduction

1. Introduction

The plant plasma-membrane H^+-ATPase plays a pivotal role in the physiology and biochemistry of plant cell. This 'master enzyme' controls the movement of solutes across the cell membrane. The PM H^+-ATPase is an electrogenic pump that directly couples ATP hydrolysis with the vectorial transport of H^+ into the apoplast. The electrochemical proton gradient so generates consequently utilized as the driving force for the secondary transport of ions and nutrients into and out of cell. Some other physiological–biochemical processes, like growth, develop, tougor pressure, and maintenance of cytoplasm value, are also under the control of PM H^+-ATPase [1–3]. Light, phytohormone, mycotoxin and many other environmental factors have effect on its activity [4–6]. Therefore, PM H^+-ATPase plays an important role in sensing the environment stimulation. Whereas, how the mechanical force affects the PM H^+-ATPase activity is seldom studied. Until recently, it was found that its activity can be controlled by phosphorylation/dephosphorylation [4,7,8]. Therefore, we studied relationship of PM H^+-ATPase activity and its phosphorylation under sound stimulation.

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2. Material and method

2.1. Material

In this experiment, the callus of flourishingly growing chrysanthemum was used as explant, cultivated in 50 ml triangular flasks, with 20 ml MS solid medium each, with 1.0 mg/l NAA and 1.5 mg/l BA. All the samples were cultured in light culture box at 26 °C.

2.2. Sound stimulation

The sound stimulation was generated by a sound generating equipment made by lab. Using this device, the materials which was cut from growing *Chrysanthemum callus* that were inoculated in culture medium, and cultivated for 3 days, were treated by the sine sound wave of certain strength and frequency for 1 h. At the same time the control group were also taken out of the light culture box and put into the same environment. Both of them were then deepfreezed in liquid nitrogen.

2.3. Separating and purification of membrane

The callus was cut into many pieces of 1 mm^2 large, and quickly homogenized in a buffer containing 0.25 mol/l Suerose, 10% (v/v) Glycerol, 2 mmol/l EGTA, 1 mmol/l DTT, 0.5% BSA. After filtrating through four layers of gauze. The homogenate was centrifuged at $12\,000 \times g$ for 10 min, and the supernatant was re-centrifuged at $50\,000 \times g$ for 30 min. Then put the deposit into suspending liquid which contains 0.25 mol/l DTT, 1 mmol/l sugar and 5 mmol/l Tris-mes (pH 6.5), and purified with 6.2% DextranT-500 and PEG-3350 mixture.

2.4. Purification of membrane H^+-ATPase

According to Qiu's method [9], the purified membrane was washed with 0.2% TritonX-100 and 0.5 mol/l KCl solution, in which, the membrane protein concentration was 0.1 mg/ml. It was then centrifuged at $11\,500 \times g$ for 45 min. The deposit was then suspended, with membrane protein of 0.5 g/l, and treated with 0.6% (W/V)Zw at room temperature for 5 min, adding sodium cholate whose final concentration was 0.9% (W/V). Being centrifuged at $1500 \times g$ for 30 min, the clear solution was put into saturated ammonia sulfate for 30 min and then centrifuged at $50\,000 \times g$ for 10 min. The clear solution was then put into saturated ammonia sulfate again for a night at 4 °C. The solution was $50\,000 \times g$ centrifuged for 10 min, and the deposit was suspended. After $111\,500 \times g$ centrifuged for 2 h, the deposit was taken and kept in liquid nitrogen.

2.5. Measurement of ATPase activity

Ten micrograms membrane protein was put into reaction medium (3 mmol/l ATP, 3 mmol/l $MgSO_4$, 100 mmol/l KCl, 25 mmol/l BTP-Mes, pH 6.5), at 38 °C for 20 min. Then put 0.5 ml, 10% SDS to end the reaction and then put 2 ml, 1% ammonium molybdate (1.6 mol/l HCl) and 0.1 ml, 0.2% naphthylamime sulfonic acid (confected with 12% $NaHSO_4$ and 1.25% Na_2SO_3). It was then put at room temperature for 30 min, and monitored in the absorbance at 700 nm. The enzyme activity was measured in μmol Pi/mg pro per h.

2.6. Measurement of free Ca^{2+} concentration

It was measured with S3606-Detector, using F_{2112} Ca^{2+} as the pole. $CaCl_2$ and EGTA were used to adjust required Ca^{2+} concentration.

2.7. In-vitro dephosphorylation of PM H^+-ATPase

Three hundred micrograms PM H^+-ATPase was put into 5 ml suspending medium (30 mmol/l Tris–HCl, pH 8.5; 1.5 mmol/l EGTA, 0.1 mol/l KCl), and centrifuged at $200\,000 \times g$ for 15 min. The deposit was then suspended in 40 μl reaction medium (30 mmol/l Tris–HCl, pH 8.5; 0.1 mmol/l KCl, 1 mmol/l $MgSO_4$), adding alkaline phosphatase, at room temperature for 20 min. Then put 20 μl buffer solution (30 mmol/l Hepes–Tris, pH 7.0; EGTA 1.5 mmol/l, 0.1 mol/l KCl) to end the reaction. After being supercentrifuged at

200 000 × *g* for 15 min, the deposit was collected for the activity measuring.

2.8. Treating the plasma membrane vesicle with sound stimulation

The plasma membrane vesicle was put into reaction medium (3 mmol/l ATP, 3 mmol/l $MgSO_4$, 100 mmol/l KCl, 25 mmol/l BTP-Mes, pH 6.5, with final volume of 2 ml, with membrane protein concentration of 5 μg/ml. After being treated in 1000 Hz, 100 dB sound wave for 20 min, 0.5 ml, 10% SDS was put to end the reaction. Then the membrane activity was measured.

2.9. Measurement of PM H^+-ATPase phosphorylation

The callus was put into culture medium, which contains P^{32}, treated with sound stimulation, and then measured according to Zhang's method [10].

3. Result

3.1. Effect of sound stimulation on the PM H^+-ATPase activity

Fig. 1 shows that, when the callus was treated with sound stimulation, its PM H^+-ATPase in plasma membrane activity was profoundly increased. But Fig. 2 shows that it was inhibited when sound treatment was used to the plasma membrane vesicle. It shows that the promotion of

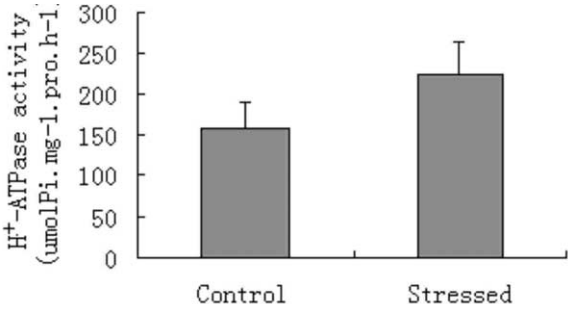

Fig. 1. Effect of sound stimulation on the PM H^+-ATPase activity from *C. callus*.

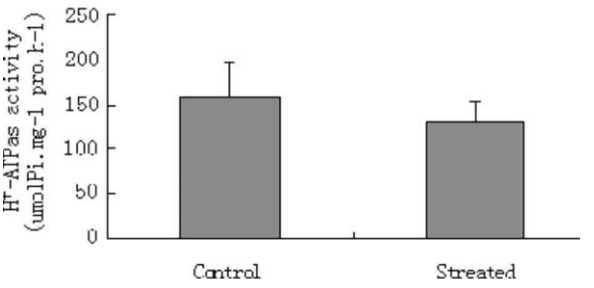

Fig. 2. Effect of sound stimulation on H^+-ATPase activity of plasma membrane vesicle isolated from *C. callus*.

sound stimulation to the callus PM H^+-ATPase activity is not a direct effect of stimulating the conformation but may affect via a series of physiological–biochemical reaction.

Plenty of research shows that, many enzyme activities were controlled by some other factors, such as second messenger. Ca^{2+} acting as an important messenger molecule, together with CaM, takes in part controlling many physiological processes. Therefore, we set some different Ca^{2+} ion concentration in culture medium via EGTA chelation, in order to study its effect to the PM H^+-ATPase activity.

The callus was cultivated in Ca^{2+}-chelated-by-EGTA culture medium. Treated with sound for 3 days, its PM H^+-ATPase activity was measured. Fig. 3 shows that, when the Ca^{2+} in the cell wall was chelated, the effect of sound on the PM H^+-ATPase activity was partly deliquesced, indicating that the change of PM H^+-ATPase activity is tied to Ca^{2+}.

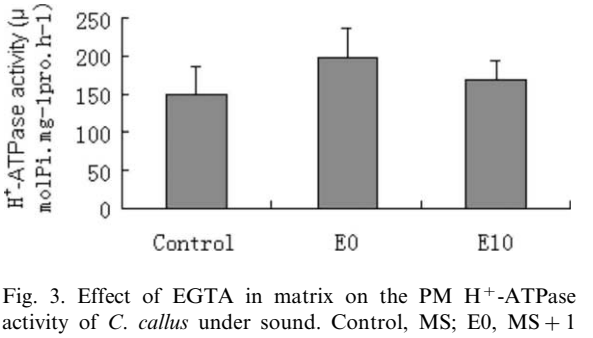

Fig. 3. Effect of EGTA in matrix on the PM H^+-ATPase activity of *C. callus* under sound. Control, MS; E0, MS + 1 mmol/l EGTA + sound; E10, MS + 10 mmol/l EGTA + sound.

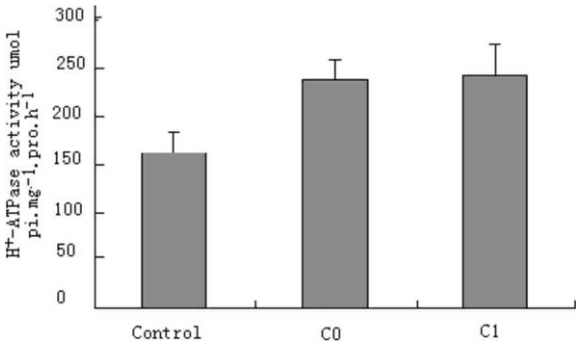

Fig. 4. Effect of CPZ in matrix on the PM H^+-ATPase activity of *C. callus* under sound. Control, MS; C0, MS + sound; C1, MS + 1 μmol/l CPZ + sound.

Fig. 4 shows that, no matter whether chloropromazine (CPZ) inhibits CaM, sound stimulation can profoundly promote the PM H^+-ATPase activity, indicating that CaM does not influence the effect of sound stimulation on the PM H^+-ATPase activity. Effect of protein kinase inhibitor on the *C. callus* plasma membrane H^+-ATPase activity under sound stimulation.

Fig. 5 shows that if *C. callus* was treated with protein kinase inhibitor (staurosporine), the effect of sound on the PM H^+-ATPase activity was completely deliquesced showing that protein kinase has influence to the effect.

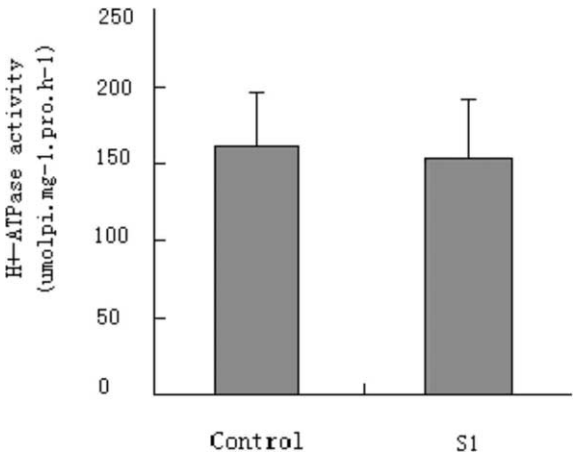

Fig. 5. Effect of Staurosporine on the *C. callus* plasma membrane H^+-ATPase activity under sound. Control, MS; S1, MS + 1 μmol Staurporine + sound.

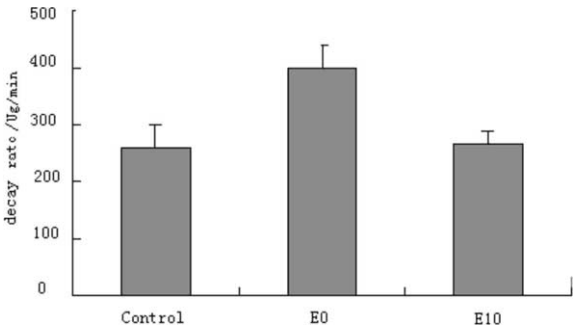

Fig. 6. Effect of phosphorylation on the *C. callus* plasma membrane H^+-ATPase activity under sound. Fig. 3 effect of calcium in matrix on the PM H^+-ATPase activity of *C. callus* under sound.

3.2. Phosphorylation of PM H^+-ATPase in sound stimulation

In order to get more understanding how sound stimulation affects the PM H^+-ATPase activity; we studied the change in PM H^+-ATPase phosphorylation level after sound treatment. As Fig. 6 shows that, it increased significantly, while deliquesced when Ca^{2+} was chelated by EGTA, indicating that sound stimulation promotes the phosphorylation of PM H^+-ATPase, which is tied to Ca^{2+}.

Fig. 7 shows the PM H^+-ATPase of the sound-treated group, when dephosphorylated by alkaline phosphatase, its activity was similar to the con-

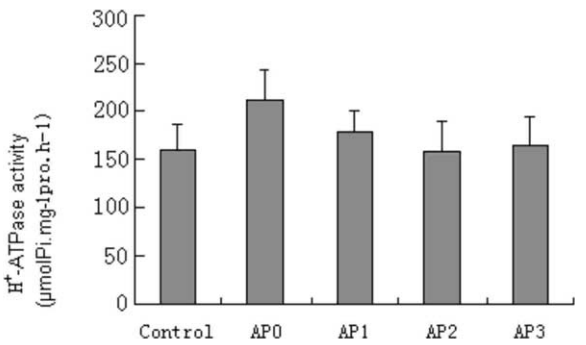

Fig. 7. Effect of alkaline phosphatase on the *C. callus* plasma membrane H^+-ATPase activity under sound. Control, vesicles; AP0, after sound vesicles; AP1, after sound vesicles + 1 unit; AP2, after sound vesicles + 2 unit AP2; AP3, after sound vesicles + 3 unit.

trol. Therefore, it is concluded that, the PM H^+-ATPase activity is directly related to its phosphorylation.

4. Discussion

The study of PM H^+-ATPase physiological–biochemical functions shows that, like light, phytohormone, mycotoxin, many environmental factors have effect on its activity [5,6]. In this experiment, it is found that, sound stimulation can also change the PM H^+-ATPase activity. It indicates that PM H^+-ATPase also plays an important role in the response of *C. callus* to the mechanical stimulation. In this experiment it is found that when sound treatment is used for the plasma membrane vesicle (Fig. 2), the change of H^+-ATPase activity is opposite to that of *C. callus* (Fig. 1), showing that the promotion of sound stimulation to the callus PM H^+-ATPase activity is not a direct effect of stimulating the enzyme molecule conformation, but may affect via a series of physiological–biochemical reaction. Ca^{2+}, acting as an important second messenger molecule in cells, via combining with target protein, controls the functions and configurations of other proteins and effect plant's physiological–biochemical functions in the 'stimulation-response' coupling system [11]. Former research has shown that, mechanical stimulation would increase Ca^{2+} concentration in cell, notwithstanding, it is unknown if the increase of PM H^+-ATP as activity is tied to the change of Ca^{2+} and CaM concentration. In our experiment, it is shown that while the Ca^{2+} in cell wall was chelated by Ca^{2+}-chelater, the effect of sound stimulation on the callus PM H^+-ATPase acivity was partly eliminated (Fig. 3). Therefore, it shows that Ca^{2+} takes part in the effect of sound stimulation on the PM H^+-ATPase activity, and the important role of Ca^{2+} in cell wall, acting as extraculler Ca^{2+} store, in the sound stimulation. It is also found that, in our experiment, CaM inhibitor in the culture medium does not influence the promotion of sound stimulation to the PM H^+-ATPase activity, which clearly shows that the promotion is tied to Ca^{2+}, but not CaM. We also found that sound stimulation can increase the phosphorylation of PM H^+-ATPase. The activity of PM H^+-ATPase significantly decreased after the dephosphorylation reaction with alkaline phosphatase. On the other hand, when the *C. callus* was treated with protein kinase inhibitor (staurosporine), the effect of sound to the PM H^+-ATPase activity was completely deliquesced showing that protein kinase has influence on the effect. Similar effect was found when the Ca^{2+} in the cell wall was chelated. Which points out that the sound stimulation may promote the phosphorylation of PM H^+-ATPase, and thus increase its activity. The Ca^{2+}/CaM-Depedent Protein Kinase, Ca^{2+}/Phospholipid-Dependent Protein Kinase and cAMP-Dependent Protein Kinase widely exist in animal cell signal transduction, but are still not found in the plant cell [12]. It has been found, in plant cell, a kind of Ca^{2+}-Dependent Protein Kinase, which, depend on Ca^{2+}, but not on CaM nor phospholipid [13]. And the Protein Kinase catalyzing PM H^+-ATPase found in this experiment is believed to be of this kind.

Recent research has specified the significant function of the protein reversible phosphorylation process in the cell signal transduction of animal and prokaryote cells, that, it is not only the passageway of message in cell, but also the central tache of interaction of systems of messages in cell [14,15]. Nonetheless, the understanding of this process in plant cell is much less than this. This experiment clearly shows that the reversible phosphorylation process may also play an important role in the plant cell signal transduction. The protein kinase includes many families and species, with their own particular substrates, and takes part in corresponding signal transducting processes. Our study is the first to reveal the Ca^{2+}-dependent protein kinase, one of the most important receptor of Calcium ion in plant cell, can transduct the sound signal to the H^+-ATPase in plasma membrane, change its activity, and thus realize the response of cell to the sound stimulation.

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