3種のCAM植物(パインアップル,セイロンベンケイ,コダカラベンケイ)葉から単離したトノプラストにおけるATPaseとPPase活性のイオン反応特性

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Effects of Various Ions on Adenosinetriphosphatase and Inorganic Pyrophosphatase in Tonoplasts Isolated from Three CAM Species, *Ananas comosus, Kalanchoë pinnata and K. daigremontiana*

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Summary

The effects of various ions on adenosinetriphosphatase (ATPase) and inorganic pyrophosphatase (PPase) in tonoplasts isolated from the leaf homogenates of the malic enzyme (ME) starch formers, *Kalanchoë pinnata* and *K. daigremontiana*, and the phosphoenolpyruvate carboxykinase (PEPCK) extrachloroplastic carbohydrate former, *Ananas comosus* (pineapple) were investigated. The ATPase activities were NO_3^- and bafilomycin A_1 sensitive. ΔNO_3^--ATPase activities were largely dependent on the presence of Mg^{2+} and Mn^{2+} could partly substitute for Mg^{2+} as divalent cation, whereas the PPase activities strictly depended on the presence of Mg^{2+} and were stimulated 7 to 10 times by K^+ at 50 mM and inhibited compeletly by KF at 5 mM and to 22 to 23% at 0.5 mM $CaCl_2$. In contrast to the ΔNO_3^--ATPase activities which were insensitive to monovalent, the PPase activities highly dependent on monovalent cations. The sequence of effectiveness was, $KCl = NH_4Cl = RbCl > CsCl > NaCl > LiCl$. Furthermore, while the ΔNO_3^--ATPase activities were found to be sensitive to anions, the PPase activities were insensitive to anions such as Cl^-, NO_3^-, ClO_3^-, SO_4^{2-}, CH_3COO^- and 2-(N-morpholino)-ethanesulfonic acid (Mes) ions. However, there were exceptions: F^- and hydrogen phthalate ions.

Key words: Adenosinetriphosphatase, CAM, Inorganic pyrophosphatase, Ion, Tonoplast.

Introduction

Nocturnal accumulation of large amounts and high concentrations of malic acid in the vacuoles of the photosynthetic cells is one of the most prominent characteristics of CAM. Malic acid is synthesized from PEP produced in glycolysis and CO_2 by dark CO_2-fixation via PEPcase. This gives oxaloacetic acid, which is reduced to malic acid, and then is transported into the vacuoles of chloroplast-containing cells. During the daytime, malic acid is removed from the vacuoles and decarboxylated. The 3-carbon product of the decarboxylation reaction, either PEP or pyruvate, is converted gluconeogenically to carbohydrate, thereby replenishing the reserve carbohydrate.

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Studies indicated that the source of the carbon skeleton of PEP, the pathway of glycolysis and gluconeogenesis, and many important enzymes varied depending on the CAM species[1-4]. For example, in starch-degrading CAM species, chloroplastic and/or cytosolic glycolysis produces 3-phosphoglycerate, which is later converted to PEP by enzymes of the cytosolic glycolytic pathway[5-6], whereas in CAM species which utilizes soluble sugars as its carbohydrate reservoir in photosynthetic cells, the degradation of soluble carbohydrates to PEP is an entirely cytosolic process that involves the complete set of cytosolic glycolytic enzymes[2,4]. Carnal and Black[7] found that species of Crassulaceae exhibit PPi-PFK activities that are either similar to or lower than ATP-PFK activities, while PPi-PFK activities in pineapple are about 15 to 20 times higher than activities of ATP-PFK.

The transport of malic acid across the tonoplast is one of the key processes in CAM. This transport is driven by the maintenance of an inside-positive electrochemical membrane potential gradient across the tonoplast energized by tonoplast ATPase and PPase[8]. Why the tonoplast should contain two enzymes apparently functioning in parallel is not very clear. Former studies suggested that the two enzymes presented in differing proportions in various tissues, species, developmental stages and environmental conditions[8-12]. Thus, the activities and characteristics of the two pumps may be different among various CAM species, especially among various CAM groups.

Recently, we found that in tonoplasts isolated from ME starch formers, *K. daigremontiana* and *K. pinnata*, the PPase activities were greater than their ATPase and ΔNO_3^--ATPase activities, whereas in tonoplasts isolated from PEPCK extrachloroplastic carbohydrate former, *Ananas comosus* (pineapple), the ATPase and ΔNO_3^--ATPase activities were greater than its PPase activities, and compared the substrate, pH and temperature dependence of tonoplast ΔNO_3^--ATPase and PPase among the three CAM species[13].

In this paper, we investigates the effects of various ions on ATPase and PPase in tonoplasts isolated from the three CAM species.

Materials and Methods

1. Plant materials

Pineapple, *K. pinnata* and *K. daigremontiana* were propagated vegetatively and grown in pots in a greenhouse with heating under natural photoperiod. The planting date and sampling are the same as described previously[13]. The experiment was performed from 1 May to 30 September 1998.

2. Tonoplast isolations

Tonoplast preparations from the leaf homogenates of pineapple, *K. pinnata* and *K. daigremontiana* were according to the method described previously[13]. All fraction steps were performed at 4°C.

3. Analysis of tonoplast ATPase, ΔNO_3^--ATPase and PPase activities and protein content

ATPase activities were assayed according to the method of Jochem and Lüttge[14] with some modifications. The enzyme activities were assayed at 37°C for 30 min in a 0.5 ml reaction mixture containing 50 mM BTP-Mes, pH8.0, 3 mM Na_2-ATP, 3 mM $MgSO_4$, 0.02% (w/v) Triton X-100, 1 mM sodium molybdate, 50 mM KCl. The reaction was started by the addition of 0.05 ml of sample, and the reaction was stopped by the addition of 0.25 ml of 6% (w/v) sodium dodecyl sulfate (SDS), and the Pi released from the substrate was determined according to the method of Lin and Morales[15]. ΔNO_3^--ATPase was determined as the activities inhibited by 100 mM KNO_3.

PPase activities was determined according to the method of Maeshima and Yoshida[16] with some modifications. The enzyme activities were measured at 46°C for 30 min in a 0.5 ml reaction mixture containing 30 mM Tris-Mes, pH7.0, 0.16 mM Na_4PP_i, 2 mM $MgSO_4$, 0.02% (w/v) Triton X-100, 1 mM sodium molybdate, 50 mM KCl. The reaction was started by the addition of 0.05 ml of sample, and stopped by the addition of 0.25 ml of 6% (w/v) SDS, and the Pi released from the substrate was determined as described as above. To calculate PPase activities, the total Pi released was halved because hydrolysis of PPi gives 2 Pi.

Protein content was determined by the method of Bradford[17] using bovine serum albumin as the standard. The tonoplast protein content was about 200 μg ml^{-1} tonoplast suspension and diluted to 20 to 200 μg ml^{-1} tonoplast suspension according to the tonoplast ATPase and PPase activities.

Results

1. Effects of various inhibitors on tonoplast ATPase and PPase activities

The most of ATPase was sensitive to nitrate and bafilomycin A_1—inhibitors of vacuolar ATPase but insensitive to azide and vanadate, inhibitors of mitochondrial and plasmalemma ATPase, respectively (Table 1). Therefore, the membranes isolated from plants described here can be identified as tonoplasts[18-20]. As seen in Table 1, the tonoplast ATPase activities of pineapple, *K. pinnata* and *K. daigremontiana* were slightly inhibited by $KClO_3$ up to 10 mM and inhibited to 46 to 58% by adding 50 mM $KClO_3$. However, Wang and Sze[20] found that tonoplast ATPase of oat roots was inhibited by $KClO_3$ with an apparent I_{50} (concentraction of inhibitor required to give 50% inhibition) of 5 mM. It is unclear whether the tonoplast ATPase prepared from different plant species shows different responses to $KClO_3$ and further investigation is required.

As shown in Table 1, the tonoplast PPase activities strictly depended on the presence of Mg^{2+} and were stimulated 7 to 10 times by K^+ at 50 mM and inhibited completely by KF at 5 mM and to 20 to 23% by $CaCl_2$ at 0.5 mM, which appeared to be characteristic features of the tonoplast PPase[8,16,21-25]. The effects of several ATPase inhibitors on tonoplast PPase activities were examined. These inhibitors had no inhibitory effects on tonoplast PPase activities observed. These properties of PPase in tonoplasts isolated from the three CAM species were the same as those of the tonoplast PPase from pumpkin cotyledons[26],

Table 1. Effects of different inhibitors on ATPase and PPase activities of pineapple, *K. daigremontiana* and *K. pinnata* tonoplasts. ATPase and PPase activities were assayed as described in "Materials and Methods". Values are means of 3 to 6 experiments±SD. The numbers in parentheses indicate the relative ATPase and PPase activities % of control.

Inhibitor	ATPase activities (μmol Pi mg^{-1} protein h^{-1})			PPase activities (μmol Pi mg^{-1} protein h^{-1})		
	Pineapple	*K. daigremontiana*	*K. pinnata*	Pineapple	*K. daigremontiana*	*K. pinnata*
Control	56.7±7.1(100)	48.0±5.3(100)	32.2±4.3(100)	43.9±3.3(100)	96.7±10.1(100)	60.2±7.2(100)
-KCl *	43.7±3.7(77)	36.4±4.1(76)	22.9±2.9(71)	6.1±0.7(14)	9.7±1.0(10)	7.8±0.6(13)
-$MgSO_4$ *	1.1±0.2(2)	0.5±0.1(1)	0.6±0.1(2)	0	0	0
KNO_3 50mM	14.2±2.1(25)	6.7±0.9(14)	4.2±0.5(13)	44.8±3.7(102)	108.3±9.4(112)	61.4±4.4(102)
100mM	4.0±0.7(7)	3.8±0.5(8)	1.3±0.1(4)			
Vanadate 1mM	51.6±4.7(91)	47.0±5.2(98)	29.6±3.3(92)	41.7±5.7(95)	98.6±6.7(102)	62.6±8.0(104)
Azide 1mM	53.3±6.3(94)	44.2±3.8(92)	29.0±3.1(90)	42.1±4.4(96)	99.6±10.1(103)	60.2±6.8(100)
Bafilomycin A_1 100nM	2.3±0.3(4)	0	0.3±0.1(1)	44.3±3.9(101)	94.8±7.6(98)	62.6±5.1(104)
$KClO_3$ 5mM	52.2±6.1(92)	42.7±6.3(89)	29.3±2.5(91)			
10mM	48.2±2.9(85)	39.4±3.7(82)	27.7±2.3(86)			
50mM	32.9±3.0(58)	25.9±2.3(54)	14.8±1.1(46)			
KF 5mM				0	0	0
$CaCl_2$ 0.5mM				10.1±0.9(23)	21.3±2.4(22)	13.8±1.1(23)
NaCl 50mM				43.9±4.8(100)	93.8±9.9(97)	57.2±5.9(95)

* ATPase and PPase activities were assayed as given "Materials and Methods" except that KCl or $MgSO_4$ were omoitted.

radish seedlings[23], storage roots of red beet[27] and purified from mung bean hypocotyls[16,21]. NaCl was found to be an inhibitor of tonoplast PPase from oat roots[28] and storage roots of red beet[29]. However, tonoplast PPase activities from plants described here were not inhibited by NaCl (Table 1 and 3), and was similar to tonoplast PPase from *K. daigremontiana* leaves[22] and purified from mung bean hypocotyls[16].

2. Effects of various ions on tonoplast ΔNO_3^--ATPase and PPase activities

The ΔNO_3^--ATPase activities were insensitive to monovalent cations such as K^+, NH^{4+}, Rb^+, Li^+, Na^+, and Cs^+, which supported similar ΔNO_3^--ATPase activities (Table 2).

In contrast to the tonoplast ΔNO_3^--ATPase activities of the three CAM species, the tonoplast PPase activities were widely affected by monovalent cations. The sequence of effectiveness was, KCl= NH_4Cl=RbCl>CsCl>NaCl>LiCl. This indicates that the stimulation of PPase activities by salts of monovalent ions is due to the cations rather than the anions. Rb^+, the hydrated cation of which has the same ionic radius as the hydrated K^+, and which behaves like K^+ in membrane transport[22], also had the similar effectiveness as K^+. NH^{4+} stimulated PPi-hydrolysis as K^+, which was believed that NH^{4+} increased membrane permeability and stimulated hydrolysis by dissipating proton electrochemical gradient[22]. Cs^+ was much less effective, and there were very low activities with Na^+ and Li^+, which in other cases had been believed to be inhibitors[28-29]. This agrees with the results of Marquardt and Lüttge[22] and Pugliarello et al.[23].

Divalent cations were examined in the presence of 50 mM KCl using 3 mM chloride and/or sulfate salts. As shown in Table 2, the tonoplast ΔNO_3^--ATPase activities was largely dependent on the presence of Mg^{2+}, only Mn^{2+} could partly substitute for Mg^{2+} as divalent cation. The order of effectiveness was $Mg^{2+} \gg Mn^{2+} \gg Ca^{2+}$, Co^{2+}, Zn^{2+}; no effect were observed with Cu^{2+}, Ba^{2+} and Hg^{2+}. Very similar results had been obtained with ATPase in tonoplasts isolated from *K. daigremontiana*[30-31], corn[32] and red beet[33]. As shown in Table 1 and 3, divalent cations were examined in the presence of 50 mM KCl using 2 mM chloride and/or sulfate salts. Contrast to the tonoplast ΔNO_3^--ATPase, the tonoplast PPase activities were specifically dependent on Mg^{2+}. Co^{2+} was very poor substitute for Mg^{2+} and allowed only 8 to 12% of the tonoplast PPase activities observed

Table 2. Effects of ions on NO_3^--ATPase activities of pineapple, *K. daigremontiana* and *K. pinnata* tonoplasts. NO_3^--ATPase activities were assayed as given in "Materials and Methods". Values are means of 3 to 6 experiments±SD. The numbers in parentheses indicate the relative NO_3^--ATPase activities % of control.

Effectors	NO_3^--ATPase activities (μmol Pi mg^{-1} protein h^{-1})		
	Pineapple	*K. daigremontiana*	*K. pinnata*
Effects of univalent cations			
50mM KCl	61.1±7.2(100)	41.8±3.9(100)	32.7±4.1(100)
50mM NH_4Cl	65.4±5.3(107)	42.2±4.4(101)	31.4±3.9(96)
50mM RbCl	62.3±4.2(102)	39.7±2.7(95)	34.3±3.5(105)
50mM LiCl	59.3±6.7(97)	39.3±2.9(94)	32.7±2.3(100)
50mM NaCl	65.4±7.7(107)	43.1±3.5(103)	36.6±4.2(112)
50mM CsCl	55.6±3.8(91)	37.2±3.8(89)	30.4±2.4(93)
Effects of divalent cations			
3mM $MgSO_4$	56.1±4.3(100)	43.3±4.1(100)	30.1±3.2(100)
-$MgSO_4$	0	0.4±0.1(1)	0.6±0.1(2)
3mM $MgCl_2$	57.2±5.2(102)	43.7±4.7(101)	30.7±2.7(102)
3mM $ZnSO_4$	5.0±0.6(9)	4.3±0.3(10)	3.3±0.5(11)
3mM $CuSO_4$	0	0	0
3mM $MnSO_4$	49.4±5.8(88)	35.5±5.1(82)	24.1±1.6(80)
3mM $CoCl_2$	10.7±0.9(19)	6.9±0.8(16)	5.4±0.6(18)
3mM $MnCl_2$	33.7±3.6(60)	23.7±2.5(55)	17.8±1.2(59)
3mM $CaCl_2$	11.2±1.4(20)	7.8±0.6(18)	5.1±0.7(17)
3mM $BaCl_2$	0.6±0.1(1)	0	0.3±0.0(1)
3mM $HgCl_2$	0	0	0
Effects of anions			
50mM KCl	53.8±5.1(100)	38.9±3.5(100)	33.3±2.9(100)
-KCl	40.4±3.5(75)	30.0±3.1(77)	24.3±1.3(73)
50mM KF	46.3±2.8(86)	30.7±3.1(79)	28.3±3.0(85)
50mM KBr	49.5±4.6(92)	35.8±4.1(92)	31.6±2.5(95)
50mM KI*	47.3±3.4(88)	35.4±2.9(91)	29.6±2.7(89)
50mM $KHCO_3$	50.6±4.7(94)	37.7±3.3(97)	31.6±3.6(95)
50mM K_2SO_4	23.1±2.1(43)	18.7±2.0(48)	15.7±1.5(47)
25mM K_2SO_4	38.7±3.6(72)	30.3±3.4(78)	23.3±1.5(70)
50mM CH_3COOK	50.0±3.4(93)	32.7±2.6(84)	29.0±2.1(87)
50mM K-Mes	43.0±3.8(80)	32.7±3.1(84)	27.3±2.8(82)
50mM $KClO_3$	33.4±4.3(62)	22.2±1.7(57)	18.3±1.9(55)
50mM Potassium hydrogen phthalate	19.9±1.7(37)	15.9±0.9(41)	10.3±1.2(31)

* Pi released from substrate was determined according to the method of Jochem and Lüttge[14].

with Mg^{2+}. Other divalent cations (Zn^{2+}, Cu^{2+}, Mn^{2+}, Ca^{2+}, Ba^{2+} and Hg^{2+}) were almost ineffective in replacing Mg^{2+}.

To make comparisons, various anions were used as 50 mM K-salts except that K_2SO_4 was 25 and 50 mM (Table 2). As shown in Table 2, the tonoplast ΔNO_3^--ATPase was anion sensitive and maximally stimulated by Cl^-, Br^- and HCO_3^-; CH_3COO^-, I^- and F^- were slight less effective. ΔNO_3^--ATPase activities were unaffected by Mes ion and 25 mM K_2SO_4, and inhibited by 50 mM K_2SO_4, ClO_3^- and hydrogen phthalate ion . These results were basically consistent with the characteristics described for ATPase in tonoplasts isolated from red beet[33], oat roots[20] and *K. daigremontiana*[31]. However, rather inconsistent data have been reported for ClO_3^-, which tonoplast ATPase activity of peanut

Table 3. Effects of various ions on PPase activities of pineapple, *K. daigremontiana* and *K. pinnata* tonoplasts. PPase activities were assayed as given in "Materials and Methods". Values are means 3 to 6 experiments±SD. The numbers in parentheses indicate the relative PPase activities % of control.

Effectors	PPase activities (μmol Pi mg^{-1} protein h^{-1})		
	Pineapple	*K. daigremontiana*	*K. pinnata*
Effects of univalent cations			
50mM KCl	40.3±3.8(100)	99.1±11.1(100)	53.2±5.8(100)
50mM NH_4Cl	37.8±2.9(94)	89.2±9.7(90)	51.1±3.2(96)
50mM RbCl	37.8±3.3(94)	87.2±8.0(88)	47.9±3.8(90)
50mM LiCl	8.1±0.7(20)	11.9±0.9(12)	8.0±0.6(15)
50mM NaCl	14.5±1.2(36)	26.8±1.9(27)	18.1±1.5(34)
50mM CsCl	24.2±1.8(60)	56.5±4.7(57)	31.4±3.3(59)
Effects of divalent cations			
2mM $MgSO_4$	41.8±4.7(100)	89.5±8.5(100)	58.9±4.3(100)
2mM $MgCl_2$	39.7±3.7(95)	91.3±9.2(102)	58.9±6.7(100)
2mM $ZnSO_4$	1.3±0.2(3)	1.8±0.3(2)	0.6±0.1(1)
2mM $CuSO_4$	0.4±0.1(1)	0	0
2mM $MnSO_4$	1.3±0.3(3)	1.8±0.4(2)	1.8±0.2(3)
2mM $CoCl_2$	5.0±0.4(12)	7.2±1.1(8)	6.5±0.7(11)
2mM $CaCl_2$	0.4±0.1(1)	0	0
2mM $MnCl_2$	0.8±0.2(2)	0.9±0.1(1)	2.4±0.3(4)
2mM $BaCl_2$	0	0.9±0.1(1)	0
2mM $HgCl_2$	0.8±0.1(2)	0.9±0.2(1)	0.6±0.1(1)
Effects of anions			
50mM KCl	45.3±3.4(100)	96.2±10.3(100)	61.4±7.1(100)
50mM KF	0	0	0
50mM KBr	48.0±3.8(106)	97.2±6.2(101)	60.8±6.8(99)
50mM KI*	44.8±4.3(99)	93.3±8.3(97)	59.6±5.1(97)
50mM KNO_3	48.0±5.6(106)	99.1±8.6(103)	63.2±4.2(103)
50mM $KClO_3$	47.1±3.2(104)	103.9±9.6(108)	67.5±5.4(110)
50mM K_2SO_4	46.7±5.9(103)	99.1±11.1(103)	63.2±7.1(103)
25mM K_2SO_4	43.9±4.1(97)	90.4±8.4(94)	58.1±4.4(95)
50mM CH_3COOK	47.6±5.6(105)	97.2±7.1(101)	63.9±5.6(104)
50mM K-Mes	48.0±3.3(106)	99.1±5.9(103)	69.4±8.2(113)
50mM Potassium hydrogen phthalate	19.5±1.3(43)	38.5±4.8(40)	28.2±2.4(46)

* Pi content released from substrate was determined according to the method of Marquardt and Lüttge[14].

totally lacked when $KClO_3$ substituted KCl[34].

In contrast to the tonoplast ΔNO_3^--ATPase of the three CAM species that was sensitive to anions, the PPase activities in tonoplasts isolated from the three CAM species were insensitive to anions such as Cl^-, Br^-, I^-, NO_3^-, ClO_3^-, SO_4^{2-}, CH_3COO^- and Mes ion which supported similar tonoplast PPase activities[22,23,28]. The exceptions were F^-, which was ineffective in stimulating tonoplast PPase activities and in fact, inhibited tonoplast PPase activities[16,23], and hydrogen phthalate ion which allowed only 40 to 46% of the tonoplast PPase activities examined with Cl^- (Table 3). Walker and Leigh[29] reported that the tonoplast PPase activities tested with potassium hydrogen phthalate was 50.0% of that tested with KCl. This was virtually identical to our result.

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3種のCAM植物（パインアップル，セイロンベンケイ，コダカラベンケイ）葉から単離したトノプラストにおけるATPaseとPPase活性のイオン反応特性

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摘　　要

セイロンベンケイソウ（*Kalanchoe pinnata* Pers.），コダカラベンケイ（*K. daigremontiana* R. Hamet & Perrier），パインアップル（*Ananas comosus* (L) Merr.）の葉から単離したトノプラストにおけるATPaseとPPaseに対する各種イオンの影響を調査した．調査したATPaseはNO_3^-とバフィロマイシンA_1に感受性を示した．ΔNO_3-ATPase活性は明らかなMg^{2+}依存性を示し，Mn^{2+}も同様な効果を示した．しかし，PPaseのMg^{2+}依存性は特異的でその他の2価陽イオンによる置換はできなかった．またPPaseは50mMK^+により7～10倍の活性阻害を受け，0.5mM$CaCl_2$で約20%の活性阻害を受けた．ATPaseが1価の陽イオンに殆ど反応しないのとは対称的にPPaseは1価の陽イオンに対し様々な感受性を示し，その程度は$KCl = NH_4Cl = RbCl > CsCl > NaCl > LiCl$であった．またATPaseが陰イオンに反応性を示すのに対し，PPaseはCl^-，NO_3^-，SO_4^{2-}，CH_3COO^-それにMesに殆ど感受性を示さなかったが，F^-とフタル酸水素イオンにより阻害された．