ACTIVE CALCIUM TRANSPORT IN PLASMA MEMBRANE VESICLES FROM DEVELOPING COTYLEDONS OF COMMON BEAN*

Huang Jianzhong (黄建中)** and Chen Ziyuan(陈子元)

(*Institute of Nuclear Agricultural Sciences, Zhejiang Agricultural University, Hangzhou 310029*)

ABSTRACT

Plasma membrane vesicles were prepared from the developing cotyledons of common bean (*Phaseolus vulgaris* L cv Diyundou) by aqueous two-phase partitioning and characterized as to their purity by assaying marker enzymes for other membranes. The putative plasma membrane fraction was minimally contaminated by membranes other than plasma membrane and hence was of high purity. It exhibited a Ca^{2+}-dependent ATPase activity, which was inhibited by 1 μmol/L EB and promoted by calcium ionophore A23187. Such an activity was responsible for the observed ATP-dependent $^{45}Ca^{2+}$ uptake into inside-out plasma membrane vesicles. This process was stimulated by 0.6 μmol/L CaM and 20 μmol/L IAA but inhibited by 2 μmol/L ABA and abolished by A23187. Possible role of cytoplasmic Ca^{2+} in mediating phytohormones activity is discussed.

Keywords $^{45}Ca^{2+}$ uptake, Plasma membrane vesicles, Phytohormones, Developing cotyledons of common bean, Tracer techniques

1 INTRODUCTION

It is well recognized in animal cells that the free cytoplasmic Ca^{2+} acts as a second messenger in transducing extracellular signals to cellular responses[1]. In plant cells, a similar role for Ca^{2+} has recently been established[2]. Since the cytoplasmic Ca^{2+} concentration of plant cells is at a level approaching 10^{-7} mol/L whereas those of organelles and cell exteriors are at millimolar range[2], continuous operation of Ca^{2+} transport is evidently required to maintain the submicromolar cytoplasmic Ca^{2+} concentration. Both passive and active transport systems are involved in either sequestering Ca^{2+} into organelles or mediating Ca^{2+} efflux from the cell. One of them is the Ca^{2+}-ATPase localized at the plasma membrane (PM), which pumps Ca^{2+} out of the cell by making use of the free energy released during ATP hydrolysis, and has only recently been convincingly verified in several plant tissues[3]. We present here the ATP-dependent Ca^{2+} transport in PM vesicles purified from developing cotyledons of common bean. As the sink-controlled photosynthates partitioning in a plant has been suggested to be mediated by endogenous phytohormones[4], the possible mechanism of hormonal activity by cellular Ca^{2+} signalling is also discussed.

*This work was supported in part by a grant from the Foundation of Natural Sciences of Zhejiang Province

**Current add: Section of Plant Physiology, Zhejiang Agricultural University

Manuscript received date: 1994–08–02

2 MATERIALS AND METHODS

2.1 Radioisotope tracer

[^{45}Ca]$CaCl_2$ (1.4 GBq/g) was from China Institute of Atomic Energy, Beijing.

2.2 Plant material

Bush bean (*Phaseolus vulgaris* L. cv. Diyundou) plants were grown in gravel culture as previously described[5]. Pods were harvested 25 d after flowering.

2.3 Membrane preparation

Cotyledons were separated from the other parts of pods and prechilled at 10°C. All the subsequent steps were carried out at 0–5°C. Cotyledons were homogenized in a blender for 1–2 min in a homogenization medium with a ratio of 1:3(w/v). The homogenization medium contained 20 mmol/L Hepes/10 mmol/L Btp/15 mmol/L Tris (pH 7.5), 5 mmol/L EDTA, 0.2 mmol/L PMSF, 2 mmol/L DTT, 0.2%(w/v) defatted BSA, 1%(w/v) PVP and 250 mmol/L sorbitol. The brei was passed through 4 layers of gauze and the filtrate centrifuged for 15 min at 15 000xg. The supernatant was centrifuged again for 60 min at 95 000xg. The resulting microsomal pellets were suspended to 10–15 mg of protein per mL in a suspension buffer containing 2.5 mmol/L Hepes/Btp (pH 7.8), 10%(w/v) glycerol and 250 mmol/L sorbitol.

Aliquots of this microsomal fraction were added to phase systems with a final polymer composition of 6.5%(w/w) Dextran T500, 6.5%(w/w) PEG3350, 250 mmol/L sorbitol, 3 mmol/L KCl, 1 mmol/L DTT, 0.1 mmol/L EGTA in 5 mmol/L KPi (pH 7.8). The following steps of two-phase partitioning were essentially the same as described[6]. The final upper and lower phases were diluted 10-fold with dilution buffer containing 2 mmol/L Hepes/Btp (pH 7.8), 2 mmol/L K_2SO_4, 1 mmol/L DTT and 250 mmol/L sorbitol, and pelleted by centrifugation for 60 min at 115 000 xg. The pellets were resuspended in the same buffer. Membranes were kept on ice for immediate assays or stored frozen in liquid nitrogen. The final upper phase was enriched in plasma membrane vesicles which were mostly right-side-out. These vesicles were turned into sealed and inside-out by the procedure of freeze-thaw[7].

2.4 Enzyme assays

ATPase activity was assayed as described[8] with some modifications. The standard reaction mixture consisted of 20 mmol/L Tris/Mes (pH 6.5), 4 mmol/L $MgSO_4$, 50 mmol/L KCl, 3 mmol/L ATP, 1 mmol/L NaN_3, 1 mmol/L molybdate, 0.02%(w/v) Triton X-100 and 5–10 μg of membrane protein in a final volume of 125 μL. The reaction was started by adding ATP and proceeded for 10–30 min at 30°C, then terminated with 125 μL of ice-cold 10%(w/v) TCA. Aliquots of the reaction mixture were removed for Pi assay, which was based on the binding of malachite green[9].

The vanadate- or nitrate- sensitive ATPase activity was referred respectively to the decrease in activity due to the inclusion of 100 μmol/L vanadate or 50 mmol/L $NaNO_3$ in the assay medium. Other marker enzymes were assayed as described: cytochrome c oxidase (CCO)[10], antimycin A- insensitive NADH-cytochrome c-reductase (CCR)[11]

and latent IDPase[12].

2.5 Ca^{2+} transport

Uptake of Ca^{2+} into membrane vesicles was assayed by the filtration method[13] at 20°C with $^{45}Ca^{2+}$ used as a tracer for Ca^{2+} accumulation. Transport solution included 250 mmol/L sorbitol, 25 mmol/L Hepes/Btp (pH 7.5), 4 mmol/L $MgSO_4$, 0.4 mmol/L NaN_3, 20 μmol/L $CaCl_2$ (10 kBq/mL $^{45}Ca^{2+}$) and $\pm$3 mmol/L ATP. Uptake was initiated by adding membrane vesicles (200 μg protein/mL) to the reaction mixture. At 20 min following the initiation, the reaction mixture was rapidly filtered through 0.45 μm nitrocellulose filters previously dipped for 2 h in a rinse solution containing 2.5 mmol/L Hepes/Btp (pH 7.5), 250 mmol/L sorbitol, and 0.1 mmol/L $CaCl_2$. Filters were quickly washed thrice with 1 mL each of ice-cold rinse solution. The solution remaining upon the filter was aspirated away by a mild vacuum to the tap.

The filters were thoroughly dried and the radioactivity determined in 5 mL of scintillation cocktail (4.2 g of PPO+80 mg of POPOP in one litre of toluene) using a Packard 1900 TR[14]. The accumulation of Ca^{2+} in the membrane vesicles was then calculated. Active uptake was defined as the difference between uptake in the presence and absence of 3 mmol/L ATP.

2.6 Protein

Protein was determined by the dye-binding method[15] with BSA as a standard.

2.7 Pigment extraction

Pigment extraction and absorption spectrum scan were performed as described in Ref.[16].

2.8 Chemicals

ATP, IDP, calcium ionophore A23187, CaM, Hepes, Mes, Btp, antimycin A, cytochrome c, PEG 3350 were purchased from Sigma. Dextran T500 was obtained from Pharmacia. PVP (K30) was a product of Fluka. All other chemicals were of analytical or the highest grade commercially available.

2.9 Data presentation

Assays were run in triplicate. Each experiment was carried out at least two times, but values shown here are from one representative experiment.

3 RESULTS AND DISCUSSION

3.1 Preparation of plasma membrane vesicles

The final upper phase from aqueous phase partitioning of membranes from developing bean cotyledons was characterized as to PM purity by assaying marker enzymes for other membranes (Table 1). The data indicate that contamination by membranes other than PM is minimal and correspond well with other studies where aqueous two-phase partitioning was used to obtain PM vesicles of high purity[6,8,17]. For example, the nitrate-sensitive ATPase, which is localized on the tonoplast[18], was only about 1.5% of

the vanadate-sensitive ATPase, a marker for PM[19]. The specific activity of the latter in the final upper phase was about 4.5 times greater than that found in the microsomal

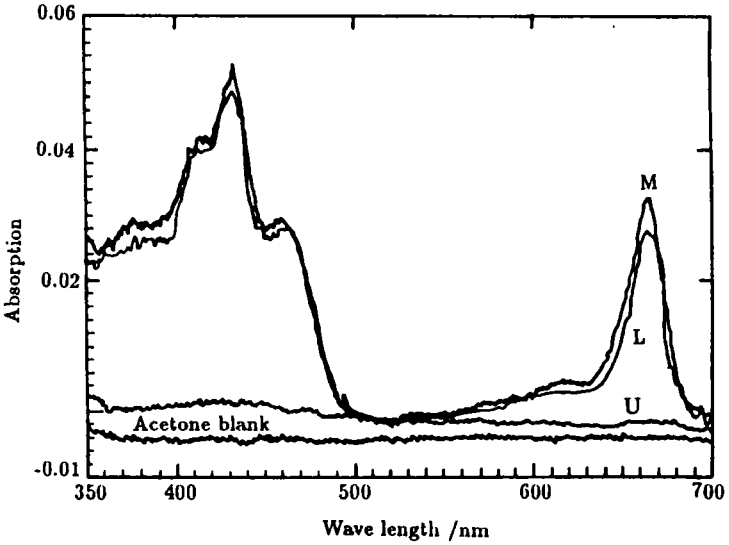

Fig.1 Absorption spectra of acetone extracts from a bean microsomal fraction (M) and from the upper (U) and lower (L) phases after aqueous two-phase partitioning

Table 1
Characterization of plasma membrane enriched vesicles purified by aqueous two-phase partitioning. Membranes were isolated from developing bean cotyledons (n=3)

Parameter	Total/nmol·min^{-1}		Recovery	Specific activity/nmol·mg^{-1}·min^{-1}		
	M	U	(U/M,%)	M	U	U/M ratio
Prot /mg	23.52±0.25	0.85±0.02	3.6	–	–	–
CCO /Mit	931.4±58.1	7.0±0.3	0.8	39.6±2.5	8.2±0.4	0.21
CCR(ER)	891.4±52.1	9.6±1.0	1.1	37.9±2.2	11.3±1.2	0.30
Latent IDPase(Golgi)	1926.3±81.6	23.9±1.4	1.2	81.9±3.5	28.1±1.6	0.34
ATPase, vanadate sensitive(PM)	668.0±42.8	108.1±8.0	16.2	28.4±1.8	127.2±9.4	4.48
ATPase, nitrate sensitive(Tp)	89.8±5.3	1.6±0.1	1.8	3.8±0.2	1.9±0.1	0.5

Table 2
Effect of Ca^{2+}, EB and calcium ionophore A23187 on PM ATPase activity assayed at pH7.5

Additions	ATPase activity		
	EGTA(0.5 mol/L)	Ca^{2+}/nmol Pi·mg^{-1}·min^{-1}	Ca^{2+}-dependent
None	98±2.1	105±2.5	7
EB(1 μmol/L)	85±2.8	80±2.9	5
A23187(2μmol/L)	99±2.7	114±3.3	15

PM vesicles were from developing bean cotyledons
Ca^{2+} was added as 30 μmol/L $CaCl_2$ in the absence of EGTA

fraction. It was also found that pigments were almost absent from the final upper phase (Fig.1). Absorption in the 600–700 nm and 400–500 nm regions is mainly caused respectively by chlorophylls and carotenoids. Both of them are situated in the thylakoid

membranes. Little absorption in these regions demonstrates the minimal contamination by thylakoids in the membrane preparation from the final upper phase. It is clear that PM vesicles with high purity was obtained by the aqueous two-phase partitioning method. These PM vesicles, however, were mostly right-side-out[7] whereas inside-out PM vesicles were required in the Ca^{2+} transport experiments. Therefore, where necessary, the freeze-thaw protocol was used to turn the right-side-out vesicles into the sealed, inside-out ones[7].

3.2 Ca^{2+}-ATPase and Ca^{2+} uptake of PM vesicles

High concentration of Ca^{2+} strongly inhibited the ATPase activity (not shown). Nevertheless, low level of Ca^{2+} promoted the total ATPase activity when assayed at pH 7.5 (Table 2). Since 1 μmol/L erythrosin B (EB) completely blocks ATP-dependent Ca^{2+} uptake but has only minor effect upon the activity of PM H^+-ATPase[20,21], the greater inhibition of ATPase activity by EB measured in the presence of Ca^{2+} than in its absence might reflect a Ca^{2+}-dependent ATPase activity. This activity was very low compared with that of PM H^+-ATPase and would be masked by the inhibitory effect of Ca^{2+} on the PM H^+-ATPase. In accordance, the calcium ionophore A23187 significantly stimulated the Ca^{2+}-dependent ATPase activity (Table 2) because of the collapse of the Ca^{2+} gradient induced by the ionophore. The notion that the PM vesicles could not maintain the Ca^{2+} gradient across PM, i.e., was leaky to Ca^{2+} at the presence of calcium ionophore A23187, was confirmed in the $^{45}Ca^{2+}$ uptake experiment (Fig.2). It was also found that the activity of Ca^{2+} uptake into inside-out PM vesicles was promoted by 0.6 μmol/L CaM and 20 μmol/L IAA but inhibited by 2 μmol/L ABA (Fig.2).

Recently, ATP-dependent $^{45}Ca^{2+}$ uptake experiments have been successfully carried out in PM vesicles isolated from radish seedlings, red beet storage tissue, spinach leaves and *Commelina communis* leaves[3]. These results indicated the presence of a primary Ca^{2+}-translocating ATPase which transports Ca^{2+} from the cytoplasm to the cell exterior. In our study, such an activity was shown to exist in the PM vesicles from developing cotyledons of common bean and was responsible for the uptake of Ca^{2+} into the inside-out PM vesicles. Our results also suggested that the PM Ca^{2+}-ATPase and hence the active Ca^{2+} transport across PM might be subject to the regulation by CaM and phytohormones (Fig.2). In particular relevance to this point is the recent finding that phytohormones indeed changed the cytoplasmic Ca^{2+} concentration probed with Ca^{2+} selective microelectrode[22,23]. These lines of evidences,

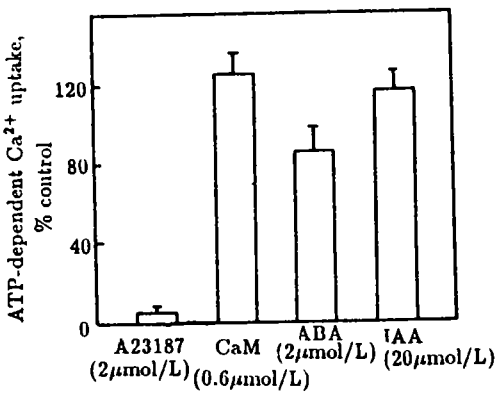

Fig.2 Effect of ABA, IAA and CaM on ATP-dependent Ca^{2+} uptake by PM vesicles from developing cotyledons
100 % uptake (control) was 1.9 nmol Ca^{2+} per mg protein in 20 min

taken together, imply that the phytohormones may regulate the cellular events by the mediation of the Ca^{2+} second messenger system.

REFERENCES

1 Rasmussen H, Barrett P Q. Physiol Rev, 1984; 64:938–984
2 Poovaiah B W, Reddy A S N. CRC Crit Rev Plant Sci, 1987; 6:47–103
3 Briskin D P. Plant Physiol, 1990; 94:397–400
4 Thomas T H. In: Bopp M, ed. Plant growth substances 1985. Berlin Heidelberg: Springer-Verlag, 1986:350–359
5 Huang Jianzhong, Chen Ziyuan. Proceedings of Sino-International Colloquium on Soilless Culture'94. Hangzhou: China Agricultural Sci Tech Press, 1994:192–193
6 Hodges T K, Mills D. Methods Enzymol, 1986; 118:41–54
7 Huang Jianzhong. H^+/sucrose symport in plasma membrane vesicles from developing cotyledons of *Phaseolus vulgaris* and its regulation by plant hormones. Ph D Thesis, Zhejiang Agric Univ, 1992
8 Blum W, Key G, Weiler E W. Physiol Plant, 1988; 72:279–287
9 Lanzetta P A, Alvarez L J, Reinach P S. Anal Biochem, 1979; 100:95–97
10 Hodges T K, Leonard R T. Methods Enzymol, 1974; 32:392–406
11 Tolbert N E. Methods Enzymol, 1974; 31(part A):734–746
12 Sandelius A S, Penel C, Auderset G *et al.* Plant Physiol, 1986; 81:177–185
13 Bush D R, Sze H. Plant Physiol, 1986; 80:549–555
14 Allen J D, Parsons S M. Anal Biochem, 1979; 92:22–30
15 Bradford M M. Anal Biochem, 1976; 72:248–254
16 Williams L E, Nelson S J, Hall J L. Planta, 1990; 182:532–539
17 Larsson C, Widell S, Kjellbom P. Methods Enzymol, 1987; 148:558–568
18 Mandala S, Taiz L. J Biol Chem, 1986; 261:12850–12855
19 Gallagher S R, Leonard R T. Plant Physiol, 1982; 70:1335–1340
20 Cocucci M C. Plant Sci, 1986; 47:21–27
21 Rasi-Caldogno F, Pugliarello M C, de Michelis M I. Plant Physiol, 1987; 83:994–1000
22 Blatt M R, Thiel G. Ann Rev Plant Physiol Plant Mol Biol, 1993; 44:543–567
23 Wang M, van Duijn B, Schram A W . FEBS Lett, 1991; 278:69