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Calcium uptake by tonoplast and plasma membrane vesicles from spinach leaves

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Tonoplast- and plasma membrane-enriched fractions were simultaneously prepared from the same crude membrane suspension from spinach leaves by free-flow electrophoresis. They were used for in vitro ATP-dependent Ca^{2+}-uptake studies. The plasma membrane-enriched fraction accumulated Ca^{2+} at a rather slow rate by a mechanism which was inhibited by orthovanadate, insensitive to nitrate and activated by calmodulin. The tonoplast-enriched fraction accumulated Ca^{2+} at a high rate for 5 min, then the accumulated Ca^{2+} progressively leaked out of the vesicles. The tonoplast transport system was partly inhibited by nitrate, but also by vanadate, completely abolished by the protonophore CCCP and activated by calmodulin and the Ca^{2+} antagonist verapamil.

Free-flow electrophoresis; Membrane separation; Calmodulin; Verapamil

1. INTRODUCTION

Calcium controls many biochemical and physiological processes in plant cells [1]. Its cytosolic concentration must therefore be very precisely adjusted. It is generally believed that this concentration is kept at a low level by the operation of Ca^{2+}-transport systems located on the plasma membrane [2], tonoplast [3] and endoplasmic reticulum [4]. According to the estimations of Blumwald and Poole [5], tonoplast Ca^{2+}/H^+ antiport would be the most effective system for this regulation.

In order to assess the respective part taken by the various systems in the removal of Ca^{2+} out of cytoplasm, a parallel in vitro study using purified tonoplast and plasma membrane vesicles isolated from the same tissue would be of interest. Conventional methods for membrane purification are unfortunately unable to yield several different membranes from one homogenate. However, such a result can be obtained with preparative free-flow electrophoresis, which can be used for the simultaneous separation of tonoplast and plasma membrane vesicles [6], even with green leaves as starting material [7].

Here, tonoplast and plasma membranes were prepared from spinach leaves and their ability to transport Ca^{2+} was characterized.

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; EGTA, ethylene glycol bis(aminoethylether) *N,N'*-tetraacetic acid

2. MATERIALS AND METHODS

2.1. *Plant material*

Spinach (*Spinacia oleracea* cv. Nobel) plants were grown for 5 weeks in growth chambers under short day light periods (8 h fluorescent light, 20 W/m^2, 20°C, relative humidity 80%). They received a 24 h light period just before harvesting.

2.2. *Membrane isolation*

Leaves (25 g) were homogenized for 30 s in 100 ml of a medium containing 50 mM Hepes, 400 mM sucrose, 100 mM KCl, 1 mM $MgCl_2$, 5 mM EGTA, and 10 mM Na ascorbate (adjusted to pH 7.5 with NaOH) using a Braun blender (type MX32, Frankfurt). The homogenate was filtered through a nylon cloth (100 μm) and centrifuged at 7000 rpm for 10 min in a Sorvall SS34 rotor. The resultant supernatant was centrifuged

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at 16000 rpm for 30 min in an SS34 rotor. The pellets were resuspended in 20 ml electrophoresis chamber buffer and centrifuged again at 16000 rpm for 30 min. The final pellet was resuspended in 2 ml chamber buffer.

2.3. *Free-flow electrophoresis*

The membrane suspension was injected into the separation chamber of a VaP 21 free-flow electrophoresis unit (Bender and Hobein, Munich) at a rate of 0.9 ml/h. Electrophoresis conditions were as follows: chamber buffer contained 10 mM ethanolamine, 250 mM sucrose, 2 mM KCl, 10 mM acetic acid, 10 μM $CaCl_2$, pH 7.5 (NaOH); electrode buffer comprised 100 mM triethanolamine, 100 mM acetic acid, pH 7.5 (NaOH); the buffer flow through the chamber was fixed at 3 ml/h per fraction, and the temperature in the separation chamber at 6°C. The separation was carried out at a constant current of 120 mA and a potential of about 1800 V. The membranes separated by the electrical field during their migration through the chamber wer collected at the bottom of the chamber. The separation profile was read at 280 nm and the fractions, pooled as indicated in each case, were centrifuged at 16000 rpm for 30 min to collect the membranes which were then resuspended in the appropriate buffer.

2.4. *Ca^{2+} uptake*

Membranes were resuspended in 25 mM Mops [3-(*N*-morpholino)propanesulfonic acid] equilibrated at pH 7.5 with Tris containing 10% (w/v) sucrose. ATP-dependent Ca^{2+} uptake by membrane vesicles was measured by a filtration method essentially as in [8], in the presence of 5 mM $MgCl_2$, 3 mM NaN_3, 10 μM $CaCl_2$ (with 250 Bq $^{45}CaCl_2$), 1 mM NaATP and 50 μg membrane protein at 20°C. At the end of the incubation, membrane vesicles were retained on Whatman glass microfiber filters (2–4 cm GF/B), previously dipped for 2 h in 0.3% polyethylenimine and washed with 5 ml resuspension buffer. The radioactivity present on the filters was determined by liquid scintillation counting. Active transport was defined as the difference between uptake in the presence or absence of ATP. Appropriate controls without ATP were prepared in each case. Each experiment was repeated at least 3 times.

2.5. *Enzyme assays*

The H^+-translocating inorganic pyrophosphatase was used as tonoplast marker [9]. Its activity was measured in a medium containing 0.33 mM EGTA, 200 mM sorbitol, 5 mM $MgSO_4$, 0.1 mM sodium pyrophosphate, 50 mM KCl, 2 μM quinacrine and 50 μg membrane protein. H^+ accumulation in the vesicles was quantified by the quenching of quinacrine fluorescence, using a Bausch & Lomb spectrofluorimeter (excitation at 420 nm, emission at 495 nm). Plasma membrane vesicles were detected in the electrophoresis fractions using Ca^{2+}-dependent 1,3-β-glucan synthase activity [10]. Briefly, the incubation medium contained 50 mM Tris-HCl buffer (pH 7.0), 20 mM cellobiose, 16% (w/v) glycerol, 0.04% (w/v) digitonin, 0.1 mM $CaCl_2$, 0.8 mM UDP-glucose with 925 Bq UDP-[^{14}C]glucose and 50 μg membrane protein. After 20 min at 25°C, the reaction was stopped in boiling water and the contents of the tubes were absorbed with pieces of 3 MM Whatman paper. The papers were washed in a mixture of 350 mM ammonium acetate and 30% ethanol. The labelled product remaining on the papers was counted. Antimycin-resistant NADPH-cytochrome *c* reductase was used to detect endoplasmic reticulum [11]. The amount of membrane proteins was estimated by using the dye-binding method [12], with bovine serum albumin as a standard.

3. RESULTS

3.1. *Membrane separation by free-flow electrophoresis*

The distribution of the various membranes extracted from spinach leaves after separation by free-flow electrophoresis has been described in detail [7]. Fig.1 shows a typical separation profile. The amount of material in each fraction was quantified by the absorbance at 280 nm. Pyrophosphate-dependent H^+ pumping and 1,3-β-glucan synthase activities were taken as biochemical markers of tonoplast [9] and plasma membranes [10], respectively. The least electronegative fractions on the right of the profile appeared to be enriched in plasma membrane vesicles and fractions near the anode on the left of the profile were rich in tonoplasts. This observation was consistent with previous analysis [6,7]. However, the tonoplast marker was not found in fractions at the very right of the separation, indicating a possible biochemical differentiation among tonoplast vesicles. The highest specific activity of NADPH-cytochrome *c* reductase, the usual marker of endoplasmic reticulum [11], was found in the main central peak, as expected [6]. Some activity was also measured in tonoplast- and plasma membrane-enriched fractions.

Membrane vesicles present in the electrophoresis fractions were collected by centrifugation and assayed for ATP-dependent Ca^{2+} uptake after a long (fig.2) or short (fig.3) incubation period. Fig.2 shows that after 45 min incubation, membranes from all parts of the elution profile were able to accumulate Ca^{2+} in the presence of ATP. On a membrane protein basis, fractions on both sides of the profile were hardly more active than the central ones. The situation was quite different after 5 min incubation (fig.3). In this case, the most anodic fractions, enriched in tonoplasts, were very active, while those corresponding to plasma membranes exhibited weak Ca^{2+} accumulation. These data indicated a net difference in the rate of Ca^{2+} accumulation between fractions enriched in tonoplasts and plasma membranes.

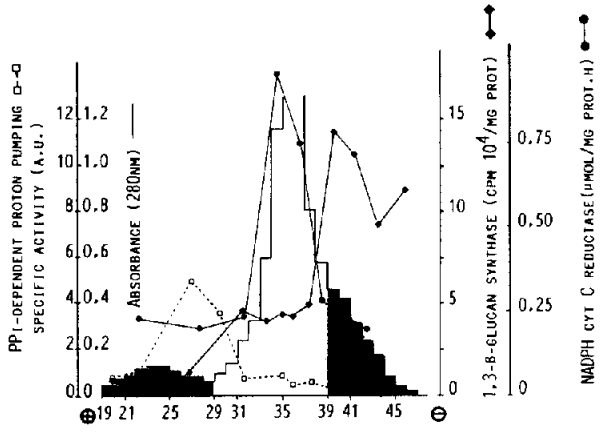

Fig.1. Free-flow electrophoresis separation of a crude membrane suspension from spinach leaves showing the distribution of three biochemical markers. Stippled areas show how fractions were pooled for the preparation of tonoplasts (fractions 19–29) and plasma membranes (39–45).

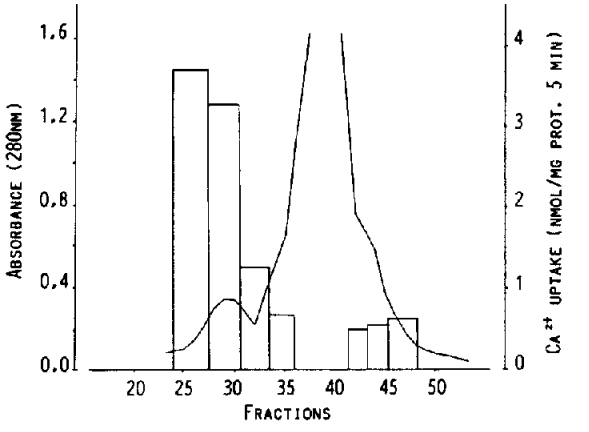

Fig.3. Free-flow electrophoresis separation showing the level of ATP-dependent Ca^{2+} uptake by pooled fractions after incubation for 5 min.

3.2. *Ca^{2+} uptake by tonoplasts or plasma membranes*

Fractions pooled as indicated in fig.1 were used as tonoplast- or plasma membrane-enriched preparations for further studies. Ca^{2+} uptake by both preparations was measured after different times of incubation (fig.4). When the Ca^{2+} content of plasma membrane vesicles continuously increased for 45 min in the presence of ATP, the maximum uptake by tonoplast vesicles was already reached after 5 min and then the content in Ca^{2+} progressively decreased. Although the absolute values observed in different experiments may be different, the time courses of Ca^{2+} uptake by tonoplast and plasma membrane fractions always exhibited similar shapes.

The effect of increasing the concentration of exogenous Ca^{2+} on the rate of Ca^{2+} accumulation was tested after incubation for 5 min for tonoplasts and 45 min for plasma membranes (fig.5). Both membrane transports were mediated by saturable systems. Saturation was reached at rather high Ca^{2+} concentrations. It is generally assumed that Ca^{2+} cytosolic concentration is lower. This explains why Ca^{2+} concentrations for all other measurements were fixed at 10 μM added $CaCl_2$.

Various substances known to affect Ca^{2+}

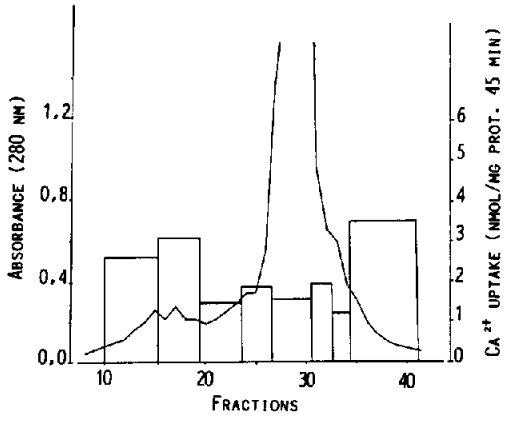

Fig.2. Free-flow electrophoresis separation showing the level of ATP-dependent Ca^{2+} uptake by pooled fractions after incubation for 45 min.

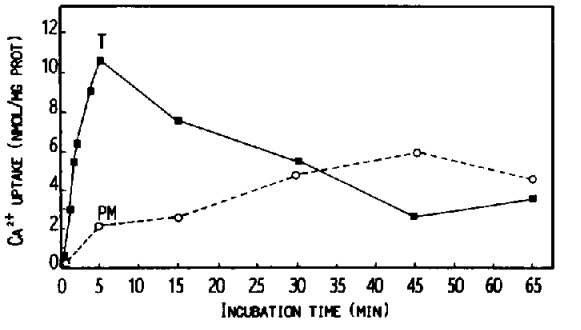

Fig.4. ATP-dependent Ca^{2+} uptake by fractions enriched in tonoplasts (T) or plasma membranes (PM) prepared as shown in fig.1.

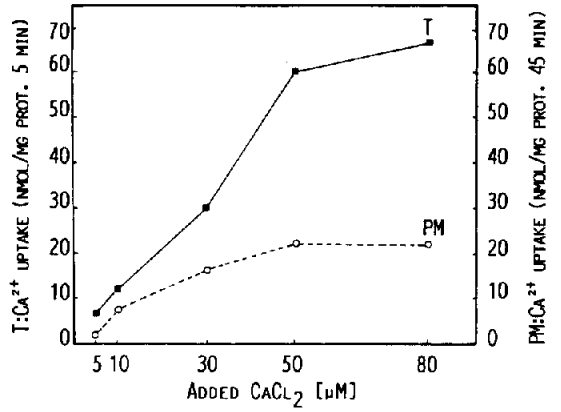

Fig.5. Effect of the concentration of added $CaCl_2$ on the ATP-dependent Ca^{2+} uptake by fractions enriched in tonoplasts (T) or plasma membranes (PM) prepared as shown in fig.1.

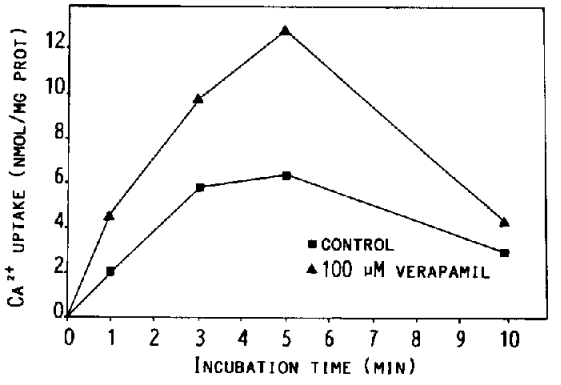

Fig.6. ATP-dependent Ca^{2+} uptake by a tonoplast-enriched fraction in the absence or presence of 100 μM verapamil.

transport by plant membranes were tested on the two membrane preparations (table 1). Calmodulin, a Ca^{2+}-dependent protein which was shown to activate the transport of Ca^{2+} by plasma membrane vesicles [2], enhanced Ca^{2+} uptake by plasma membranes but also by tonoplast vesicles. The Ca^{2+}-channel blocker verapamil, which specifically binds to plant plasma membranes and, possibly, to tonoplasts [13], did not change the Ca^{2+} uptake by plasma membranes, but increased the accumulation by tonoplast preparations. In the presence of this substance, the rate of Ca^{2+} uptake by tonoplasts was higher from the beginning of the incubation. However, verapamil did not prevent the progressive loss of Ca^{2+} occurring after 5 min (fig.6). Table 1 also shows that the accumulation of Ca^{2+} by both types of membranes was absolutely dependent on the presence of Mg^{2+} and that the accumulated Ca^{2+} was completely released when the ionophore A23187 was added for 1 min at the end of the incubation. Other substances exhibited a different effect. Na vanadate, known to inhibit plasma membrane ATPase [14], completely suppressed Ca^{2+} uptake by the plasma membrane-enriched fraction, but also inhibited that by the tonoplast-enriched fraction. The effect of CCCP on the tonoplast preparation indicated that in this case the Ca^{2+} transport was achieved by a Ca^{2+}/H^+ antiport dependent on the presence of an H^+ gradient [3,5]. This effect and the inhibition observed in the presence of nitrate [$Mg(NO_3)_2$ instead of $MgCl_2$ in the incubation medium] may be interpreted as confirmation that this preparation actually was rich in tonoplasts.

Table 1

Effect of various substances on Ca^{2+} uptake by membrane vesicles prepared by free-flow electrophoresis (in %)

	Tonoplasts (5 min incubation)	Plasma membranes (45 min incubation)
Incubation medium[a]	100	100
+ calmodulin (5 μg/ml)	200	227
+ CCCP (10 μM)	0	85
+ A23187 (10 μM)	0	0
+ verapamil (100 μM)	215	97
+ vanadate (50 μM)	37	0
− $MgCl_2$	0	0
− $MgCl_2$ + 5 mM $Mg(NO_3)_2$	35	98

[a] See section 2

4. DISCUSSION

The present results demonstrate that free-flow electrophoresis is a suitable method for obtaining sealed membrane vesicles which can be used for in vitro transport studies. It possesses the advantage of yielding in one operation and from one homogenate active fractions rich in tonoplasts and plasma membranes. It was shown that tonoplasts and plasma membranes prepared from spinach leaves by this technique actively accumulate Ca^{2+} in the presence of ATP. The characteristics of this accumulation were essentially similar to those described for other plant materials [2,3,5,14,15].

The existence of a plasma membrane Ca^{2+}-ATPase, which has been questioned [16], is confirmed by the present results which also revealed that calmodulin, in addition to this plasma membrane Ca^{2+} pump, also activated the Ca^{2+}-transport system of tonoplasts. The regulation by calmodulin, which may be important for the control of cytosolic Ca^{2+} concentration, could be explained by activation of either the H^+-ATPase or the Ca^{2+}/H^+ antiporter. Another original point is the activation of tonoplast Ca^{2+} uptake by the Ca^{2+} antagonist verapamil. This activation probably resulted from a change in permeability of vesicles to Ca^{2+}. The possible physiological role of this effect of verapamil is under investigation.

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