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Inositol trisphosphate stimulates the release of calcium from intact vacuoles isolated from *Acer* cells

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On addition of inositol trisphosphate, intact vacuoles isolated from *Acer pseudoplatanus* cell suspension cultures release part of their calcium content. The process was specific, dose-dependent ($IC_{50} = 0.2\ \mu M$) and was inhibited by an intracellular calcium antagonist. The calcium efflux elicited by inositol trisphosphate increased with the age of the cell suspension cultures, the maximum effect being obtained when the cultures reached the stationary phase. It is suggested that vacuoles play a role as an endocellular calcium store that is responsive to inositol trisphosphate in plants.

Phosphoinositol; Second messenger; Ca^{2+}; Vacuole

1. INTRODUCTION

Calcium is now accepted to play a central role in the cascade of events that allows plant cells to convert an external stimulus into the adapted biological response [1,2]. Therefore, its mobilization, namely the control of the entry of external calcium through the plasma membrane and the release of Ca^{2+} from potential intracellular stores is crucial to modulate cytosolic concentrations. In higher plants, various Ca^{2+} transport mechanisms have been shown to occur including calcium channels and Ca^{2+}-ATPase on plasmalemma-enriched fractions and protoplasts [3,5], or in organelles such as mitochondria, plastids and vacuoles [6]. However, the role of the vacuole, the largest plant cell organelle that may accumulate up to 10 mM Ca^{2+} [7,8], has to be considered more specifically in terms of Ca^{2+} mobilization. Recent works have established that Ca^{2+}-loaded vesicles derived from the tonoplast (the vacuolar membrane) release Ca^{2+} on addition of IP_3 [9]. The present paper reports results that extend the above-mentioned data to intact organelles, points out some characteristics of the calcium efflux and demonstrates the role of the vacuole as a reservoir for second messenger.

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Abbreviations: IP_3, inositol trisphosphate; Quin 2, 2-[[2-[bis-(carboxymethyl)amino]-5-methylphenoxy]methyl]-6-methoxy-8-bis(carboxylmethyl)amino]quinoline; TMB8, 8-(*N,N*-dimethylamino)octyl 3,4,5-trimethoxybenzoate

2. MATERIALS AND METHODS

2.1. *Chemicals and biochemicals*

All the chemicals and biochemicals were analytical grade. IP_3, Quin 2, TMB8 and diethylenetriamine pentaacetic acid were purchased from Sigma (St. Louis, MO). Other chemicals were from Merck (Darmstadt, FRG).

2.2. *Vacuoles preparation*

Vacuoles were isolated from protoplasts derived from cell suspension cultures of *Acer pseudoplatanus* as described [10]. Unless stated otherwise, the isolated organelles were suspended in 25 mM Tris-Mes buffer, pH 6.5, supplemented with 0.7 M mannitol and 8% ficol.

2.3. *Measurements of calcium efflux*

The standard assays contained: $100\ \mu M$ Quin 2 and 10^6 vacuoles in 2 ml final volume. The fluorescence was measured

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(Kontron SFM 25 model) at λ_{ex} = 339 nm and λ_{em} = 492 nm (5 nm band width) [11]. Changes in fluorescence in response to the addition of the specified compounds were recorded at 25°C.

Where indicated, the concentrations of calcium released were calculated as:

$$[Ca] = K_d \times \frac{F_{max} - F}{F - F_{min}}$$

with K_d (dissociation constant), 115 nM; F, measured fluorescence of the sample; F_{max}, maximum fluorescence measured after lysis of the vacuoles by 1% Triton in the presence of 1 mM $CaCl_2$ and 100 μM diethylenetriamine pentaacetic acid to trap heavy metals [12]. F_{min}, minimum fluorescence measured after the subsequent addition of 5 mM EGTA at pH 9.

3. RESULTS AND DISCUSSION

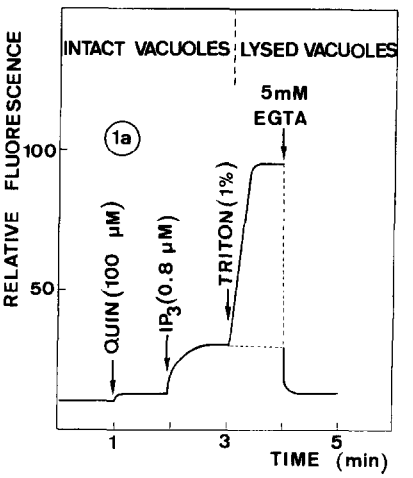
Fig.1a depicts the changes of the relative fluorescence on sequential addition of various compounds to a suspension of vacuoles from *Acer*. Quin 2 itself led to a signal which was stable for at least 4 min and may be nullified manually. The addition of 0.8 μM IP_3 elicited an increase in fluorescence that reached a steady state within 1 min. On lysis of vacuoles by 1% Triton, a dramatic increase of the fluorescence was observed, that may be quenched by an excess of alkaline solution of EGTA.

Fig.1b shows that the addition of buffer, after IP_3 led either to no change (10 μl buffer) or a slight decrease in fluorescence due to the dilution afforded by the buffer (50 μl). In contrast to IP_3, the compounds listed in table 1 were without effects on the Ca^{2+} release.

Fig.2 shows that the effects of IP_3 may be inhibited by the intracellular Ca^{2+} antagonist, TMB8 [13]. The inhibition was dose-dependent and led maximally to 80% decrease with respect to the control value.

From these various experiments it is concluded that the vacuoles are rather stable in the experimental conditions used and exhibit minor alteration (if any). More importantly, IP_3 appears to elicit calcium release from vacuole suspensions and to act in a specific manner when compared to non-substituted *myo*-inositol or to other phosphorylated compounds including inositol 1-phosphate.

Different characteristics of the process have been further examined. Thus, the IP_3-released Ca^{2+} was dependent upon IP_3 concentrations



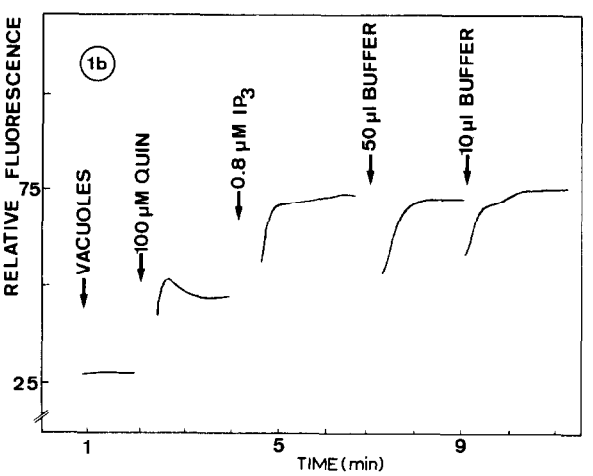


Fig.1. Effects of IP_3 on the calcium release from vacuoles isolated from *Acer pseudoplatanus*. (a) Kinetics of the process. 9-day-old cell suspension cultures of *Acer* were used as vacuole source. (b) Effects of sequential additions of IP_3 and buffer.

Table 1

Effect of different compounds on the efflux of calcium

Compounds	Maximal concentration used (μM)	Relative effect
Inositol trisphosphate	1	100
Ribulose 1,5-bisphosphate	10	0
Glucose 6-phosphate	100	0
Inositol	100	0
Inositol 1-phosphate	10	0
Fructose 1,6-bisphosphate	20	0

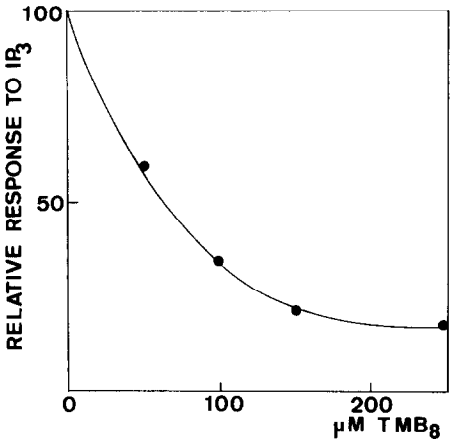


Fig.2. Effect of TMB8 on the IP_3-dependent Ca^{2+} release. Quin 2 (100 μM) was added to a suspension of vacuoles as for fig.1. Then, the preparation was supplemented with increasing concentrations of TMB8 and allowed to equilibrate for 2 min. Finally, IP_3 (1 μM) was added and the resulting increase in fluorescence recorded. The data are expressed as a percentage of Ca^{2+} efflux in the absence of TMB8.

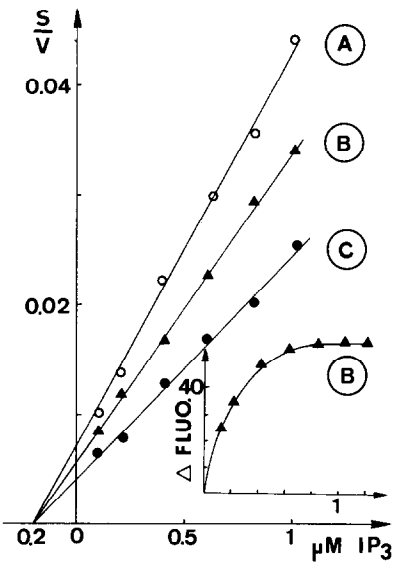


Fig.3. Effects of increasing concentrations of IP_3 in various osmotic conditions on the efflux of vacuolar Ca^{2+}. The osmolarity was obtained by manipulating the concentrations of mannitol in the assay media (10^6 vacuoles). The rate of release was expressed as changes in fluorescence (Δ fluo) per min. (A) 0.75 M mannitol; (B) 0.7 M mannitol; (C) 0.65 M mannitol.

(fig.3). The process was saturable with apparent $IC_{50} = 0.2$ μM. Moreover, the osmotic pressure influenced the efflux of Ca^{2+} without changing the affinity constant. In this way, on decreasing the concentration of osmoticum (mannitol), the rate of Ca^{2+} release was stimulated in parallel. The lower limit (0.6 M mannitol) has been chosen to avoid any lysis of the vacuoles due to the osmotic shock.

The rate of IP_3-dependent calcium efflux changed with the age of the cell suspension cultures (fig.4). It clearly appears that IP_3 was essentially ineffective on vacuoles isolated from young cells (up to 5 days) which contain more than one vacuole per cell (not shown). The release of calcium dramatically increased with the age of the cultures, reached a maximum value (day 10) and decreased slightly (up to day 13), whereas the IC_{50} remains essentially constant throughout the culture period (not shown). The maximum effect was observed when the cell cultures reached the stationary phase. No efforts have been made to study vacuoles from cells older than 13 days due to the fragility of the organelles at these later stages. Moreover, IP_3 was without effect on the transtonoplastic pH gradient which may be involved in the Ca^{2+}/H^+ exchange as judged by measurements with the quinacrine probe (not shown).

Data obtained with different plant systems suggest that phosphoinositides play a role in the transduction of stimuli. Such a conclusion has been drawn from the following observations: (i) phospholipase C and different inositol phosphates derived from phospholipids exist in plants [14–16]; (ii) the amounts of IP_3 may vary in response to different stimuli, including light [17] and auxin [18]; (iii) IP_3 evokes Ca^{2+} release from Ca^{2+}-loaded vesicles [9,19,20].

This experimental evidence is in line with the observed situation in animal cells where IP_3 is an important link between the receptor-activated phosphoinositide breakdown and Ca^{2+} mobilization from internal stores. In animal cells, as far as Ca^{2+} mobilization is concerned, experiments have been essentially performed through the addition of IP_3 to permeabilized cells and microsomal fractions. These treatments triggered the rapid release of Ca^{2+} [21]. In plant cells, the large central vacuole is one of the major intracellular Ca^{2+} stores [7] namely up to 85% of the protoplasmic

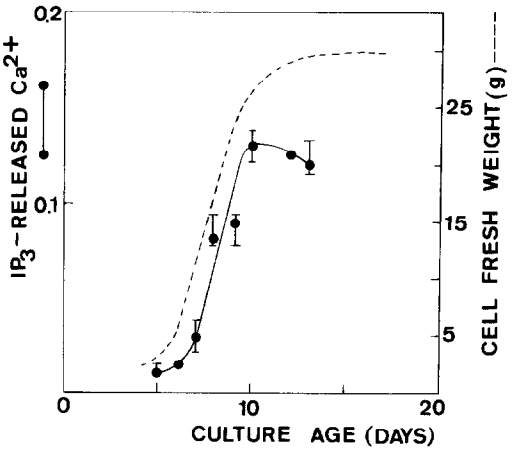


Fig.4. Effects of IP_3 at saturating concentrations on the Ca^{2+} efflux with the age of the cell cultures. The amount of calcium released was expressed as 10^{-2} nmol calcium per 10^6 vacuoles.

Ca^{2+} is trapped in these organelles in *Acer* cells [8]. The results described in this paper along with data already obtained with tonoplast vesicles [9] show that some of the vacuolar Ca^{2+} may be mobilized in response to IP_3.

Despite their fragility, isolated intact vacuoles display major advantages: (i) the membrane is right-side out; (ii) they have not to be artificially loaded with radioactive solutes. However, the most important advantage in the present study lies in the fact that they keep their endogenous content. Therefore any registered efflux of solutes reveals that the corresponding compound is present in the vacuole in a releasable form and then can be potentially mobilized in the cytoplasm.

Non-permeant fluorescent indicators may be used to test Ca^{2+} efflux from these natural membrane vesicles in a non-invasive and non-destructive way. The Ca^{2+} chelator Quin 2 is a convenient probe in this respect as it does not cross membranes and therefore reports on extravacuolar Ca^{2+}. Consequently quantitative estimates in different conditions are possible including physiological age and physical constraints.

From the data reported in this paper, the half-maximal Ca^{2+} release is obtained at 0.2 μM IP_3 (compare with 0.6 μM for tonoplast vesicles [9] and 10 μM for crude membranes [18,19]). Moreover, at day 10, IP_3 elicits the release of 140 pmol calcium within 1 min from 10^6 vacuoles (7 μl). Assuming an average volume of 0.7 μl for the corresponding cytoplasm, it means that within 1 min the cytosolic Ca^{2+} may reach 2×10^{-4} M which is more than enough to trigger Ca^{2+}-dependent events. Thus the mobilized Ca^{2+} may potentially play a role in signal transduction.

In our experiments, the effect of osmotic pressure on the rate of IP_3-evoked calcium is of particular interest. Since the efficiency of the inositol phosphate increases with the surface of the vacuole without any change in affinity it can be suggested that the number of accessible IP_3 sites of action increases as a function of turgor.

As in animal cells the release of Ca^{2+} is rapid and occurs at less than micromolar concentrations of IP_3. It seems clear therefore, that the vacuole is one major source of IP_3-released Ca^{2+} in plant cells and can be compared in this way to the endoplasmic reticulum in animal cells. The identification of the vacuoles as a Ca^{2+} internal store sensitive to IP_3 appears however more direct than in the case of endoplasmic reticulum which has only been characterized by fractionation studies and autoradiography.

REFERENCES

[1] Hepler, P.K. and Wayne, R.O. (1985) Annu. Rev. Plant Physiol. 36, 397–439.
[2] Ranjeva, R. and Boudet, A.M. (1987) Annu. Rev. Plant Physiol. 38, 73–93.
[3] Marmé, D. (1983) in: Inorganic Plant Nutrition, Encyclopedia of Plant Physiology (Laüchli, A. and Bielskied, R.L. eds) vol.15B, pp.599–625, Springer, Berlin.
[4] Andrejauskas, E., Hertel, R. and Marmé, D.J. (1985) J. Biol. Chem. 260, 5411–5414.
[5] Graziana, A., Fosset, M., Ranjeva, R., Hetherington, A. and Lazdunski, M. (1988) Biochemistry, in press.
[6] Kauss, H. (1987) Annu. Rev. Plant Physiol. 38, 47–72.
[7] Moore, A.L. and Akerman, K.E.O. (1984) Plant Cell Environ. 7, 423–429.
[8] Alibert, G., Boudet, A.M., Canut, H. and Rataboul, P. (1985) in: The Physiological Properties of Plant Protoplasts (Pilet, P.E. ed.) pp.105–115, Springer, Berlin.
[9] Schumaker, K.S. and Sze, H. (1987) J. Biol. Chem. 262, 3844–3946.
[10] Alibert, G., Carrasco, A. and Boudet, A.M. (1982) Biochim. Biophys. Acta 721, 22–29.
[11] Grynkiewicz, G., Poenie, M. and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440–3450.

[12] Arslan, P., Di Virgilio, F., Beltrame, M., Tsien, R.Y. and Pozzan, T. (1985) J. Biol. Chem. 260, 2719–2727.
[13] Clapper, D.L. and Lee, H.C. (1985) J. Biol. Chem. 260, 13947–13954.
[14] Heim, S. and Wagner, K.G. (1986) Biochem. Biophys. Res. Commun. 134, 1175–1181.
[15] Coté, G.G., Morse, M.J., Crain, R.C. and Satter, R.L. (1986) Plant Cell Rep. 6, 352–355.
[16] Reddy, A.S.N., McFadden, J.J., Friedmann, M. and Poovaiah, B.W. (1987) Biochem. Biophys. Res. Commun. 149, 334–339.
[17] Dillenschneider, M., Hetherington, A., Graziana, A., Alibert, G., Berta, P., Haiech, J. and Ranjeva, R. (1986) FEBS Lett. 208, 413–417.
[18] Ettlinger, C. and Lehle, L. (1988) Nature 331, 176–178.
[19] Droback, B.K. and Ferguson, I.B. (1985) Biochem. Biophys. Res. Commun. 130, 1241–1246.
[20] Reddy, A.S.N. and Poovaiah, B.W. (1987) J. Biochem. 101, 569–573.
[21] Muto, Y., Tohmatsu, T., Yoshioka, S. and Nozawa, Y. (1986) Biochem. Biophys. Res. Commun. 135, 46–51.