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Inositol trisphosphate-stimulated calcium release from *Acer* microsomal fractions involves the uptake of potassium

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Microsome fractions from *Acer* cell-suspension cultures imported calcium from the incubation medium in an ATP-dependent manner. Intravesicular calcium was released on addition of inositol trisphosphate. Outward calcium transport was associated with inward potassium transport that was inhibited by a potassium channel blocker but not by calcium channel blockers. Therefore, there is a link between inositol trisphosphate-controlled events and the potassium channel in plants.

Ca^{2+} flux; Inositol trisphosphate; K^+ channel; Microsomal membrane; Second messenger

1. INTRODUCTION

The generation of a calcium message in response to a stimulus may involve either the massive entry of calcium from an external source or its mobilization from internal stores [1]. Such a general framework was first established in animal systems and recently extended to plant cells where calcium appears to play a pivotal role in signal transduction [2]. In this context, several lines of evidence have demonstrated the occurrence of calcium channels at the plasma membrane [3-5] and the release of calcium by IP_3 from cells [6], microsomes [7], vacuoles [8] and tonoplast fractions (the vacuolar membrane) [9]. The latter data clearly established that plants contain internal calcium stores that may be mobilized by IP_3. However, the mechanism of calcium release is still unknown and, in particular, it is unclear as to whether the process involves cation exchange. Here, we present evidence indicating that the addition of IP_3 to microsomes that have been loaded with calcium induced outward calcium transport associated with inward potassium transport that is inhibited by potassium channel blockers.

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Abbreviations: BTP, 1,3-bis[tris(hydroxymethyl)methylamino]-propane; IP_3, inositol 1,4,5-trisphosphate; TEA, tetraethylammonium chloride

2. MATERIALS AND METHODS

2.1. *Chemicals and biochemicals*

All chemicals and biochemicals were of analytical grade. IP_3, TEA, calcimycin (A23187), EGTA and potassium gluconate were purchased from Sigma (St. Louis, MO); $^{45}CaCl_2$ (54-56 mCi/mg) from Du Pont de Nemours (France); and $^{86}RbCl$ (1-12 mCi/mg Rb) from Amersham (France).

2.2. *Plant material and preparation of membrane vesicles*

Cell suspension cultures of *Acer pseudoplatanus* were performed as in [10], 8-day-old cells (exponential phase of growth) being used in all experiments.

Cells (10 g) were homogenized in 10 ml of medium containing 50 mM Hepes titrated to pH 7.3 as specified, 250 mM sucrose using a Moulinex blender (type 534). The homogenate was centrifuged at $1000 \times g$ for 5 min and the resulting supernatant fraction kept on ice while the pellet was homogenized again in 10 ml grinding medium. After a second centrifugation run, the supernatant fractions (S_1) were combined and then centrifuged for 10 min at $6000 \times g$. The resulting supernatant (S_2) was saved and the pellet (cell walls and unbroken cells) discarded. S_2 was then centrifuged for 30 min at $40\,000 \times g$ and the supernatant discarded. The $40\,000 \times g$ pellets were resuspended in grinding buffer and are referred to here as microsomes.

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2.3. *Calcium uptake and release*

Ca^{2+} uptake and release were determined by measuring Ca^{2+} trapped inside vesicles. The incubation medium contained 4 mM $MgSO_4$, 4 mM ATP, 10 μM $CaCl_2$ and 3 μCi $^{45}Ca^{2+}$/ml (0.2 mg protein/ml) in grinding buffer. $^{45}Ca^{2+}$ uptake was initiated by addition of microsomes. After 40 min incubation at 25°C, EGTA, IP_3 or specified compounds were added as indicated in the figure legends. Where indicated, 200-μl aliquots (40 μg protein) were removed and collected on Millipore HAWP filters (0.45 μm) by vacuum filtration (Millipore 1225 sample collector). Filters were washed three times with 2 ml of the same buffer and counted for radioactivity in a liquid-scintillation counter (Packard, TriCarb model 460c).

2.4. *Potassium uptake*

K^+ influx was determined by measuring $^{86}Rb^+$ trapped inside vesicles. Microsomes (0.2 mg protein/ml) were incubated as described above in grinding medium containing 50 mM Hepes, pH 7.3 (BTP), 250 mM sucrose, 4 mM $MgSO_4$, 4 mM ATP, 10 μM $CaCl_2$, 1 mM KCl and 0.8 μCi $^{86}Rb^+$/ml. After 40 min incubation at 25°C the reaction mixture was cooled down for another 10 min at 10°C, and then the specified compounds were added and 200-μl aliquots (40 μg protein) were removed periodically and filtered immediately. Filters were washed 10 times with 2 ml of the same buffer and counted for radioactivity.

2.5. *Protein assays*

Protein was determined by the method of Smith et al. [11] with bovine serum albumin as the standard.

3. RESULTS AND DISCUSSION

As shown in fig.1, microsomes from *Acer* cells were able to import $^{45}CaCl_2$ from the incubation medium when ATP was present. Uptake was ineffective when ATP was omitted or replaced by a non-hydrolyzable analog. The process was time-dependent and reached a steady state after 30 min incubation, leading to a level of accumulation that can be maintained for at least 10 min (fig.1a,b). Consequently, subsequent experiments were performed during steady-state accumulation of calcium.

Addition of the calcium ionophore calcimycin promoted release of up to 70% of intravesicular calcium within 2 min (fig.1a), establishing that most of the calcium was in a mobilizable form. Addition of EGTA alone to the equilibrated system led to apparent efflux of calcium (3% of vesicle-associated fraction) that probably reflected the trapping of adsorbed or intervesicular ion rather than actual release (fig.1b). However, when the incubation medium was supplemented with both EGTA and IP_3, approx. 13% of the calcium was irreversibly released within 2 min (fig.1b) whereas

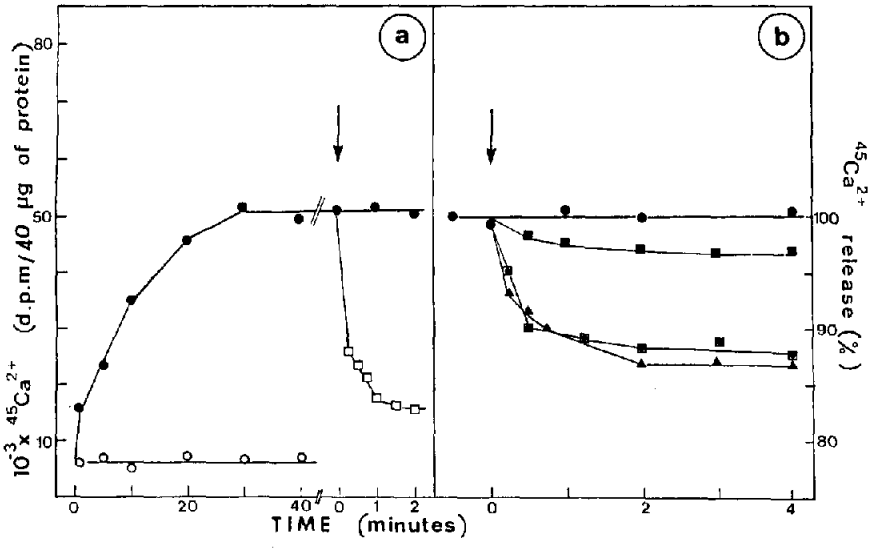

Fig.1. IP_3-induced Ca^{2+} release from microsomes. Hepes ions were titrated with KOH to pH 7.3. (a) Ca^{2+} uptake at 25°C in the presence (●) or absence (○) of ATP. At steady-state uptake (40 min incubation), aliquots were removed from the reaction mixture and incubated for another 10 min at 10°C. Where indicated (arrows), one of the following was added (final concentrations): (a) 10 μM calcimycin (□); (b) 0.75 mM EGTA (■); 20 μM IP_3 and 0.75 mM EGTA with (▲) or without (▣) 50 μM bepridil; or the same volume of buffer (●).

omission of EGTA resulted in reuptake of calcium (not shown).

In contrast to ATP-dependent uptake, calcium release from microsomes was basically temperature-independent and proceeded at the same rate at either 25 or 10°C. This result is consistent with channel-mediated efflux but calcium-channel blockers (verapamil, bepridil) that have been shown to control calcium uptake by protoplasts and bind specific receptors [4] did not inhibit IP_3-controlled calcium release (fig.1b).

The following experiments were performed in order to address the question of possible cooperation between Ca^{2+} release and another cation pathway within the same membranes. In this way, calcium uptake was performed in Hepes-BTP buffer that contains no other inorganic salt. Such a buffer change altered neither the ability of microsomal fractions to import labelled calcium from the incubation mixture nor their sensitivity to calcimycin that elicited release of vesicular calcium (fig.2a). Addition of IP_3 to calcium-loaded vesicles resulted in no efflux (fig.2b). Such negative data were also obtained when the medium was supplemented with Na^+ by using either NaOH (as Hepes-NaOH buffer) or NaCl (up to 100 mM). In all such cases, calcimycin remained active in inducing release of calcium, suggesting that intravesicular ion was mobilizable but that the microsomes somehow became insensitive to IP_3.

For recovery of the initial sensitivity, it was necessary to supplement the incubation medium with potassium. Indeed, when KOH (instead of BTP or NaOH) was used to titrate Hepes, then sensitivity to IP_3 was observed (fig.1b). This remained valid when Hepes-BTP was supplemented with KCl but not potassium gluconate (which dissociates very slowly), suggesting that only 'mobile' or 'exchangeable' K^+ is suitable for IP_3 to be active (fig.3a,b).

To test such a hypothesis, a set of complementary experiments was performed. Thus, the potassium channel blocker tetraethylammonium (as the chloride salt) had no effect on steady-state accumulation of calcium but almost completely abolished its IP_3-controlled efflux even when the medium was supplemented with KCl (fig.3c). A more direct correlation has been obtained by determining the uptake of a radioactive analog of potassium ($^{86}Rb^+$) under various experimental conditions.

When microsomes were incubated in medium

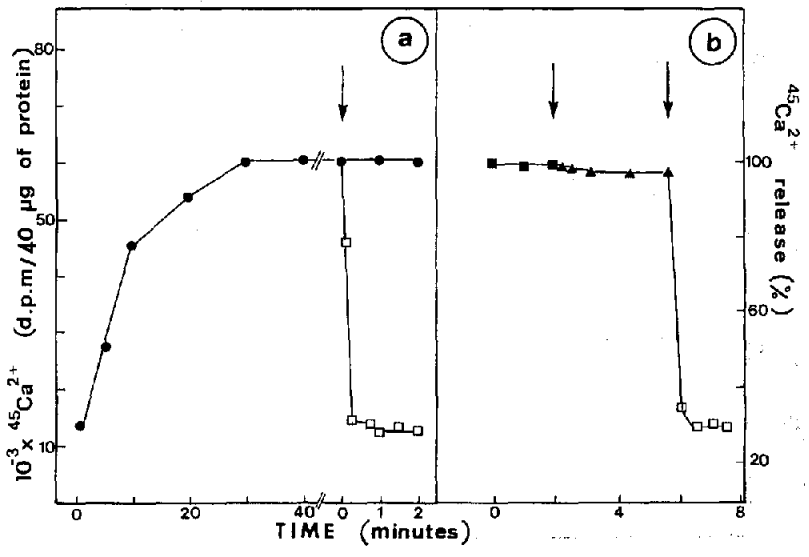

Fig.2. Effects of potassium removal on sensitivity of microsomes to IP_3. Hepes ions were titrated with BTP to pH 7.3. (a) Ca^{2+} uptake at 25°C in the presence of ATP (●). At steady state (40 min uptake), two aliquots were removed from the reaction mixture and incubated for another 10 min at 10°C. Where indicated (arrow), 10 µM calcimycin (final concentration) (□) was added. (b) The second aliquot was made 0.75 mM EGTA (■) and the following were added (arrows) successively (final concentrations): 20 µM IP_3 (▲) or 10 µM calcimycin (□).

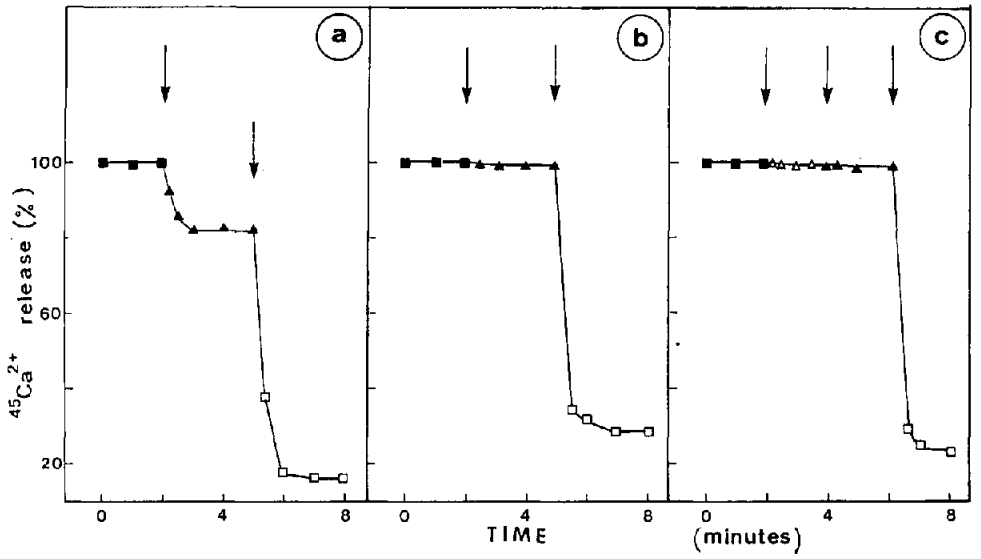

Fig.3. Effect of K^+ salts and K^+ channel blocker on IP_3-induced Ca^{2+} release. Intravesicular Ca^{2+} uptake, removal at steady state and incubation of aliquots from the reaction mixture were performed as described in fig.2b. Where indicated (arrows), the following were added successively (final concentrations); (a) 50 mM KCl (■), 20 μM IP_3 (▲), 10 μM calcimycin (□). (b) 50 mM K^+ gluconate (■), 20 μM IP_3 (▲), 10 μM calcimycin (□). (c) 50 mM KCl (■), 2 mM TEA (△), 20 μM IP_3 (▲), 10 μM calcimycin (□).

containing rubidium, equilibrium was rapidly reached which was not perturbed by addition of IP_3 (not shown). Conversely, when membranes were preloaded with unlabelled calcium (either by the ATPase system or by vesicularization in calcium-containing buffer), then IP_3 induced Rb^+ uptake that was inhibited by the potassium channel blocker (fig.4).

From all of these data, it appears that calcium-loaded microsomes respond to IP_3 with calcium efflux as well as potassium uptake. Since calcium channel blockers have no effect on IP_3-controlled efflux whereas potassium channel blocker inhibits the process, there is a link between the IP_3-controlled event and the potassium channel in plants. From a more general point of view, the

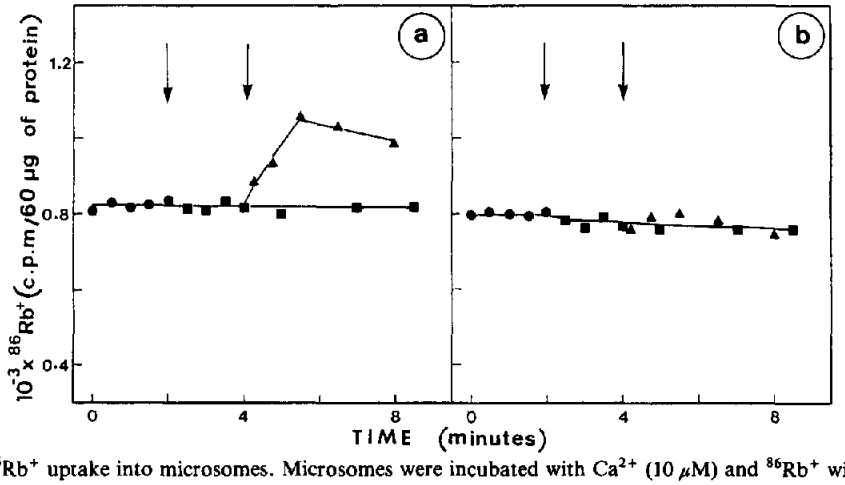

Fig.4. IP_3-induced $^{86}Rb^+$ uptake into microsomes. Microsomes were incubated with Ca^{2+} (10 μM) and $^{86}Rb^+$ with ATP at 25°C for 40 min. After a further 10 min incubation at 10°C (●) the following were added (arrows) successively (final concentrations): (a) 0.75 mM EGTA (■), 20 μM IP_3 (▲). (b) 0.75 mM EGTA plus 2 mM TEA (■), 20 μM IP_3 (▲). 60 μg protein samples were filtered at the indicated times.

present results extend data obtained on animal systems such as the rough endoplasmic reticulum from liver [12] and microsomes from brain [13]. The process of calcium mobilization is therefore conserved in plants and the next step would be the localization of all plant compartments that contain IP_3 receptors and IP_3-sensitive calcium pools. It is already clear that the tonoplast is a very good candidate [8,9] but other membranes (ER,...) may also be involved in the control of cellular Ca^{2+}.

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