

# Inositol trisphosphate-mediated $\text{Ca}^{2+}$ release in beet microsomes is inhibited by heparin

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Microsomes from the storage root of red beet exhibit ATP-dependent, protonophore-sensitive  $\text{Ca}^{2+}$  accumulation, characteristic of the vacuolar membrane (tonoplast). A portion (20%) of this intravesicular  $\text{Ca}^{2+}$  store is specifically released by inositol 1,4,5-trisphosphate ( $\text{InsP}_3$ ) with a  $K_{0.5} = 0.54 \mu\text{M}$ .  $\text{InsP}_3$ -mediated  $\text{Ca}^{2+}$  release is eliminated by low concentrations of heparin ( $K_{0.5} = 86 \text{ nM}$ ). This result highlights the conservation between the animal and plant inositol 1,4,5-trisphosphate-mediated  $\text{Ca}^{2+}$  release mechanisms, despite the fact that they are located at different intracellular membranes.

Tonoplast; Calcium efflux; Inositol 1,4,5-trisphosphate; Heparin; (*Beta vulgaris*)

## 1. INTRODUCTION

Inositol 1,4,5-trisphosphate ( $\text{InsP}_3$ ) plays a central role in stimulus-response coupling in animal cells [1]. Agonist-induced  $\text{InsP}_3$  release from phosphatidylinositol 4,5-bisphosphate elevates cytosolic free  $\text{Ca}^{2+}$  levels. The  $\text{InsP}_3$ -sensitive  $\text{Ca}^{2+}$  pool is normally thought to reside in the endoplasmic reticulum [2].

The details of  $\text{InsP}_3$  involvement in plant signal transduction have yet to be fully established [3]. Nevertheless it is already clear, from studies on membrane vesicles [4] and on intact organelles [5], that the major  $\text{InsP}_3$ -sensitive  $\text{Ca}^{2+}$  pool in higher plants is located in the large central vacuole rather than in the endoplasmic reticulum.

The disparate sites of action of  $\text{InsP}_3$  might be indicative of a fundamentally different mechanism for  $\text{InsP}_3$ -mediated  $\text{Ca}^{2+}$  release in animals and plants. Thus, as a first step towards molecular characterization of the  $\text{InsP}_3$  receptor in the vacuolar membrane (tonoplast) of higher plants, we have studied the inhibitor-sensitivity of  $\text{InsP}_3$ -mediated  $\text{Ca}^{2+}$  release in microsomes of red beet storage root, which is a rich source of tonoplast.  $\text{Ca}^{2+}$  can be initially loaded into tonoplast vesicles via coupling to the  $\text{H}^+$  gradient set up

by the primary vacuolar  $\text{H}^+$ -ATPase. We report here that heparin is a potent inhibitor of  $\text{InsP}_3$ -induced  $\text{Ca}^{2+}$  efflux across plant membranes. Since heparin has a similar effect on animal membranes [6], our results raise the possibility that the  $\text{InsP}_3$  receptors of animals and plants share a common  $\text{Ca}^{2+}$  release mechanism despite their differing cellular locations.

## 2. MATERIALS AND METHODS

### 2.1. Preparation of red beet microsomes

Microsomes were isolated from red beet (*Beta vulgaris*), based on the protocol given in [7]. Storage root (330 g) of fresh, greenhouse-grown red beet was homogenized at pH 8.0 using a Kenwood blender (model 519). One ml of homogenization medium [7] was used per gram tissue. The homogenate was filtered through muslin and centrifuged at  $10000 \times g$  for 15 min. The supernatant was re-centrifuged at  $80000 \times g$  for 30 min to give a microsomal pellet which was resuspended in a medium containing EDTA (1 mM) at pH 8.0, as detailed in [7]. A further centrifugation step followed at  $80000 \times g$  for 30 min. The resulting pellet was then resuspended in 400 mM glycerol and 5 mM BTP/MES, pH 7.4 (EDTA-free) and allowed to equilibrate for 1 h on ice. After centrifugation ( $80000 \times g$  for 30 min), the pellet was resuspended in a small volume of the equilibration medium for use in  $\text{Ca}^{2+}$  transport studies.

### 2.2. $\text{Ca}^{2+}$ transport assay

Microsomes were loaded with  $\text{Ca}^{2+}$  at  $22^\circ\text{C}$  in 1 ml incubation medium (IM) comprising 400 mM glycerol, 50 mM KCl, 3 mM  $\text{MgSO}_4$ , 3 mM BTP/ATP, 5% polyethylene glycol ( $M_r = 6000$ ), 0.3 mM  $\text{NaN}_3$  and 5 mM BTP/MES, pH 7.4. Protein concentration was  $50 \mu\text{g/ml}$ .

Microsomes were equilibrated in IM for 5 min to allow a  $\text{H}^+$  gradient to be formed via ATPase activity before  $\text{Ca}^{2+}$  uptake was initiated by the addition of  $10 \mu\text{M}$   $\text{CaCl}_2$  plus  $3 \mu\text{Ci}$   $^{45}\text{Ca}^{2+}/\text{ml}$ . Uptake was allowed to reach steady-state levels (15–20 min; see fig.1) before additions were made with rapid mixing. Aliquots of  $50 \mu\text{l}$  were placed on a nitrocellulose filter (0.22  $\mu\text{m}$  pore size: Sartorius, Göttingen, FRG) which had been pre-wetted with wash medium (400 mM

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**Abbreviations:** BTP, 1,3-bis[tris(hydroxymethyl)methylamino]propane;  $\text{InsP}_3$ , inositol 1,4,5-trisphosphate;  $\text{InsP}_2$ , inositol 1,4-bisphosphate;  $\text{InsP}_4$ , inositol 1,3,4,5-tetrakisphosphate; TMB-8, 8-(*N,N*-diethylamino)-octyl 3,4,5-trimethoxybenzoate-HCl; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazine

glycerol, 0.2 mM  $\text{CaCl}_2$ , 5 mM BTP/MES, pH 7.4), and filtered using a Millipore microfiltration unit. The filters were washed once with 5 ml ice cold wash medium, placed in scintillation vials and radioactivity determined by liquid scintillation spectrometry.

### 2.3. Protein assay

Protein concentration was determined using the Bradford method [8], with bovine serum albumin as a standard.

Reagents were obtained from Sigma (Dorset, England).  $^{45}\text{Ca}^{2+}$  (10–40 mCi/mg on arrival) was from Amersham International (Amersham, England).

## 3. RESULTS AND DISCUSSION

ATP-dependent, azide-insensitive  $\text{Ca}^{2+}$  accumulation by red beet microsomes is abolished by the  $\text{Ca}^{2+}$  ionophore, A23187, but is insensitive to orthovanadate (fig.1). These results show that ATP-dependent  $\text{Ca}^{2+}$  transport is not into plasma membrane or endoplasmic reticulum vesicles (vanadate-sensitive processes [9]) or via azide-sensitive  $\text{Ca}^{2+}$  uptake by mitochondrial contaminants. However, the protonophore, FCCP, abolishes ATP-dependent  $\text{Ca}^{2+}$  uptake (fig.1). The  $\text{Ca}^{2+}$  accumulation mechanism therefore has the characteristics of  $\text{Ca}^{2+}/\text{H}^+$  antiport activity which relies on an ATP-generated  $\text{H}^+$  electrochemical gradient for energization.  $\text{Ca}^{2+}/\text{H}^+$  antiport is localized dominantly in the tonoplast of plant cells [10]. The pathways of  $\text{Ca}^{2+}$  release from this microsomal fraction were studied using vesicles which had been loaded with  $\text{Ca}^{2+}$  to steady-state levels (15–20 min) in the presence of ATP.

Fig.2A shows that  $\text{InsP}_3$  elicits  $\text{Ca}^{2+}$  release amounting to 20% of A23187-sensitive  $\text{Ca}^{2+}$  uptake. The basic features of  $\text{InsP}_3$ -induced  $\text{Ca}^{2+}$  efflux are similar to those previously reported in other plant tissues [4,5]: in the absence of FCCP, which prevents re-uptake of  $\text{Ca}^{2+}$ , the  $\text{InsP}_3$ -induced release is transient; the  $K_{0.5}$  for  $\text{InsP}_3$  is  $0.54 \pm 0.11 \mu\text{M}$  (fig.2B); the effect is specific since neither  $\text{InsP}_2$  nor  $\text{InsP}_4$  at  $20 \mu\text{M}$  release  $\text{Ca}^{2+}$  (data not shown);  $\text{Ca}^{2+}$  efflux is completely inhibited by the endomembrane  $\text{Ca}^{2+}$  channel blocker, TMB-8, applied at  $200 \mu\text{M}$  (data not shown).

In a search for a high affinity inhibitor which could be used in subsequent purification of the  $\text{InsP}_3$  receptor, we tested a number of  $\text{Ca}^{2+}$  channel antagonists characterized on animal systems. Nifedipine (which blocks plasma membrane  $\text{Ca}^{2+}$  channels [11]) and ryanodine (which blocks sarcoplasmic reticulum  $\text{Ca}^{2+}$  channels [12]) are both ineffective as inhibitors of  $\text{InsP}_3$ -mediated  $\text{Ca}^{2+}$  release in red beet. However, the glycosaminoglycan, heparin, proved to be a potent inhibitor of  $\text{InsP}_3$ -induced  $\text{Ca}^{2+}$  efflux. When applied to red beet microsomes at  $5 \mu\text{g}/\text{ml}$  ( $\approx 1 \mu\text{M}$  assuming  $M_r = 5000$ ), heparin eliminated the  $\text{InsP}_3$  effect (fig.3A). The  $K_{0.5}$  for heparin inhibition is  $86 \pm 20 \text{ nM}$  (fig.3B). Similar observations have been reported for heparin action on rat hepatocyte vesicles ( $K_{0.5} = 40 \text{ nM}$  [6]) and permeabilized rabbit tracheal cells ( $K_{0.5} =$

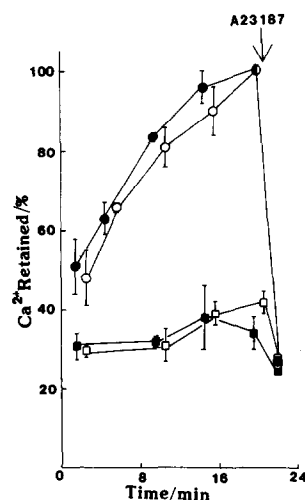


Fig.1. Characteristics of  $\text{Ca}^{2+}$  uptake in red beet microsomes. Microsomes were incubated for the times indicated in IM (●), IM minus ATP (■), IM plus  $100 \mu\text{M}$  vanadate (○) or IM plus  $10 \mu\text{M}$  FCCP (□). A23187 was added at the times shown to a final concentration of  $5 \mu\text{M}$ . 100%  $\text{Ca}^{2+}$  retained on filter =  $13.0 \pm 2.3 \text{ nmol}/\text{mg}$ . Results are means  $\pm$  SEM from 3 experiments.

160 nM [13]). The  $K_{0.5}$  value for red beet is thus comparable with these two animal tissues.

Inhibition by heparin of  $\text{InsP}_3$  binding proteins is not axiomatic, despite superficial structural similarities between  $\text{InsP}_3$  and heparin. Thus, although  $\text{InsP}_3$  3-kinase

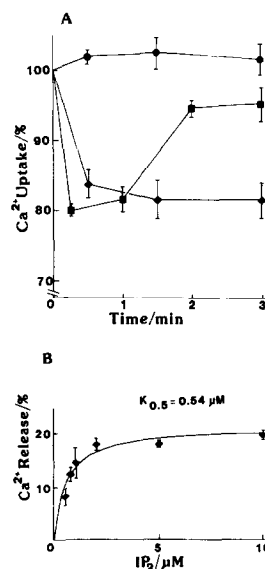


Fig.2. (A)  $\text{InsP}_3$ -induced  $\text{Ca}^{2+}$  release from red beet microsomes. After steady-state  $\text{Ca}^{2+}$  accumulation had been reached, additions were made at a time designated  $t = 0 \text{ min}$ . 100%  $\text{Ca}^{2+}$  uptake (after subtraction of A23187-insensitive binding) =  $10.1 \pm 3.0 \text{ nmol}/\text{mg}$ . The following additions were made: (■)  $5 \mu\text{M}$   $\text{InsP}_3$  ( $n = 3$ ); (●)  $10 \mu\text{M}$  FCCP ( $n = 3$ ); (◆)  $5\text{--}10 \mu\text{M}$   $\text{InsP}_3$  and  $10 \mu\text{M}$  FCCP ( $n = 10$ ). (B) Dose-response curve for  $\text{InsP}_3$ -induced  $\text{Ca}^{2+}$  release. 100%  $\text{Ca}^{2+}$  release =  $6.8 \pm 1.3 \text{ nmol}/\text{mg}$ . Data are means  $\pm$  SEM for 3 experiments. Solid line shows least-squares fit [18] to a Michaelis-Menten relationship, and yields a maximal release =  $20.8 \pm 0.8\%$  and  $K_{0.5} = 0.54 \pm 0.11 \mu\text{M}$   $\text{InsP}_3$ .

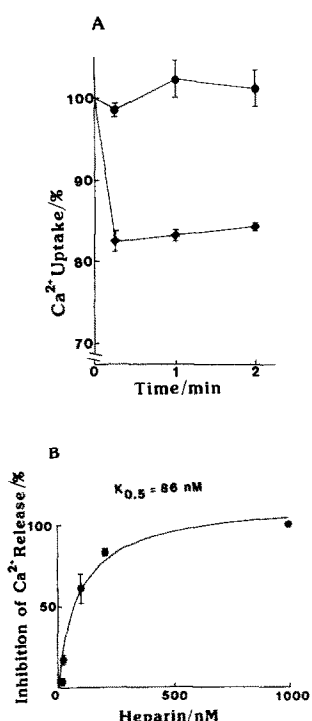


Fig.3. (A) Effect of heparin (1  $\mu\text{M}$ ) on  $\text{InsP}_3$ -mediated  $\text{Ca}^{2+}$  release from red beet microsomes. Microsomes were incubated in IM until steady-state  $\text{Ca}^{2+}$  accumulation was attained, and 10  $\mu\text{M}$   $\text{InsP}_3$  added at time designated  $t = 0$  min. (●) Absence of heparin; (●) heparin added at  $t = -1$  min. 100%  $\text{Ca}^{2+}$  uptake (after subtraction of A23187-insensitive binding)  $\approx 6.4 \pm 1.1$  nmol/mg. Data are means  $\pm$  SEM from 3 experiments. (B) Dose-response curve for heparin inhibition of  $\text{InsP}_3$ -mediated  $\text{Ca}^{2+}$  release.  $\text{InsP}_3$  (10  $\mu\text{M}$ ) was added to  $\text{Ca}^{2+}$ -loaded microsomes with varying concentrations of heparin present. 100% inhibition  $\approx 0.90 \pm 0.05$  nmol/mg ( $n = 3$ ). Solid line shows least-squares fit [19] to a rectangular hyperbola, with a  $K_{0.5} = 86 \pm 20$  nM heparin.

is sensitive to nM concentrations of heparin [14],  $\text{InsP}_3$  phosphatase from animal tissues is not [15]. Furthermore,  $\text{InsP}_3$ -mediated  $\text{Ca}^{2+}$  release in fungi is heparin-insensitive [16]. It will be interesting to see if the apparent similarity in the mechanism of  $\text{InsP}_3$ -mediated  $\text{Ca}^{2+}$  release between animals and plants is maintained at the protein level, as is suggested by the effect of heparin. The high degree of inhibition exhibited by heparin on  $\text{InsP}_3$ -induced  $\text{Ca}^{2+}$  release will enable use of affinity chromatography for purification of the red

beet  $\text{InsP}_3$  receptor, as has already been done with rat cerebellum [17].

Recent evidence from parotid and pancreatic acinar cells reveals that the animal  $\text{InsP}_3$ -sensitive  $\text{Ca}^{2+}$  store is accumulated by an ATP-dependent, protonophore-inhibited process rather than via a  $\text{Ca}^{2+}$ -ATPase [18]. The tonoplast exhibits analogous  $\text{Ca}^{2+}$  uptake properties, thereby complementing the common features of the  $\text{InsP}_3$ -induced  $\text{Ca}^{2+}$  efflux pathway described here.

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