Volume 260, number 1, 70–72 FEB 08010 January 1990

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

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Received 16 October 1989; revised version received 20 November 1989

Microsomes from the storage root of red beet exhibit ATP-dependent, protonophore-sensitive Ca^{2+} accumulation, characteristic of the vacuolar membrane (tonoplast). A portion (20%) of this intravesicular Ca^{2+} store is specifically released by inositol 1,4,5-trisphosphate ($InsP_3$) with a $K_{0.5}$ = 0.54 μM. $InsP_3$-mediated Ca^{2+} release is eliminated by low concentrations of heparin ($K_{0.5}$ = 86 nM). This result highlights the conservation between the animal and plant inositol 1,4,5-trisphosphate-mediated 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 ($InsP_3$) plays a central role in stimulus-response coupling in animal cells [1]. Agonist-induced $InsP_3$ release from phosphatidylinositol 4,5-bisphosphate elevates cytosolic free Ca^{2+} levels. The $InsP_3$-sensitive Ca^{2+} pool is normally thought to reside in the endoplasmic reticulum [2].

The details of $InsP_3$ 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 $InsP_3$-sensitive 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 $InsP_3$ might be indicative of a fundamentally different mechanism for $InsP_3$-mediated Ca^{2+} release in animals and plants. Thus, as a first step towards molecular characterization of the $InsP_3$ receptor in the vacuolar membrane (tonoplast) of higher plants, we have studied the inhibitor-sensitivity of $InsP_3$-mediated Ca^{2+} release in microsomes of red beet storage root, which is a rich source of tonoplast. Ca^{2+} can be initially loaded into tonoplast vesicles via coupling to the H^+ gradient set up by the primary vacuolar H^+-ATPase. We report here that heparin is a potent inhibitor of $InsP_3$-induced Ca^{2+} efflux across plant membranes. Since heparin has a similar effect on animal membranes [6], our results raise the possibility that the $InsP_3$ receptors of animals and plants share a common Ca^{2+} release mechanism despite their differing cellular locations.

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Abbreviations: BTP, 1,3-bis[tris(hydroxymethyl)methylamino]-propane; $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_2$, inositol 1,4-bisphosphate; $InsP_4$, inositol 1,3,4,5-tetrakisphosphate; TMB-8, 8-(*N,N*-diethylamino)-octyl 3,4,5-trimethoxybenzoate-HCl; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone

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 × *g* for 15 min. The supernatant was re-centrifuged at 80000 × *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 × *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 × *g* for 30 min), the pellet was resuspended in a small volume of the equilibration medium for use in Ca^{2+} transport studies.

2.2. *Ca^{2+} transport assay*

Microsomes were loaded with Ca^{2+} at 22°C in 1 ml incubation medium (IM) comprising 400 mM glycerol, 50 mM KCl, 3 mM $MgSO_4$, 3 mM BTP/ATP, 5% polyethylene glycol (M_r = 6000), 0.3 mM NaN_3 and 5 mM BTP/MES, pH 7.4. Protein concentration was 50 μg/ml.

Microsomes were equilibrated in IM for 5 min to allow a H^+ gradient to be formed via ATPase activity before Ca^{2+} uptake was initiated by the addition of 10 μM $CaCl_2$ plus 3 μCi $^{45}Ca^{2+}$/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 μl were placed on a nitrocellulose filter (0.22 μm pore size: Sartorius, Göttingen, FRG) which had been pre-wetted with wash medium (400 mM

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00145793/90/$3.50

glycerol, 0.2 mM $CaCl_2$, 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). $^{45}Ca^{2+}$ (10–40 mCi/mg on arrival) was from Amersham International (Amersham, England).

3. RESULTS AND DISCUSSION

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

Fig.2A shows that $InsP_3$ elicits Ca^{2+} release amounting to 20% of A23187-sensitive Ca^{2+} uptake. The basic features of $InsP_3$-induced 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 Ca^{2+}, the $InsP_3$-induced release is transient; the $K_{0.5}$ for $InsP_3$ is 0.54 ± 0.11 μM (fig.2B); the effect is specific since neither $InsP_2$ nor $InsP_4$ at 20 μM release Ca^{2+} (data not shown); Ca^{2+} efflux is completely inhibited by the endomembrane Ca^{2+} channel blocker, TMB-8, applied at 200 μM (data not shown).

In a search for a high affinity inhibitor which could be used in subsequent purification of the $InsP_3$ receptor, we tested a number of Ca^{2+} channel antagonists characterized on animal systems. Nifedipine (which blocks plasma membrane Ca^{2+} channels [11]) and ryanodine (which blocks sarcoplasmic reticulum Ca^{2+} channels [12]) are both ineffective as inhibitors of $InsP_3$-mediated Ca^{2+} release in red beet. However, the glycosaminoglycan, heparin, proved to be a potent inhibitor of $InsP_3$-induced Ca^{2+} efflux. When applied to red beet microsomes at 5 μg/ml (≡ 1 μM assuming M_r = 5000), heparin eliminated the $InsP_3$ effect (fig.3A). The $K_{0.5}$ for heparin inhibition is 86 ± 20 nM (fig.3B). Similar observations have been reported for heparin action on rat hepatocyte vesicles ($K_{0.5}$ = 40 nM [6]) and permeabilized rabbit tracheal cells ($K_{0.5}$ = 160 nM [13]). The $K_{0.5}$ value for red beet is thus comparable with these two animal tissues.

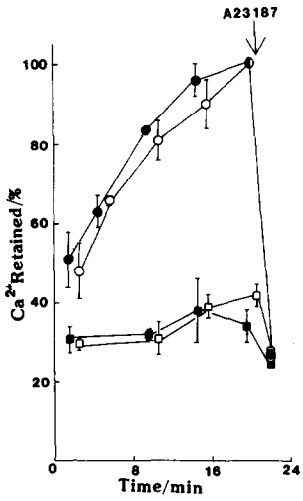

Fig.1. Characteristics of Ca^{2+} uptake in red beet microsomes. Microsomes were incubated for the times indicated in IM (●), IM minus ATP (■), IM plus 100 μM vanadate (○) or IM plus 10 μM FCCP (□). A23187 was added at the times shown to a final concentration of 5 μM. 100% Ca^{2+} retained on filter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

Inhibition by heparin of $InsP_3$ binding proteins is not axiomatic, despite superficial structural similarities between $InsP_3$ and heparin. Thus, although $InsP_3$ 3-kinase

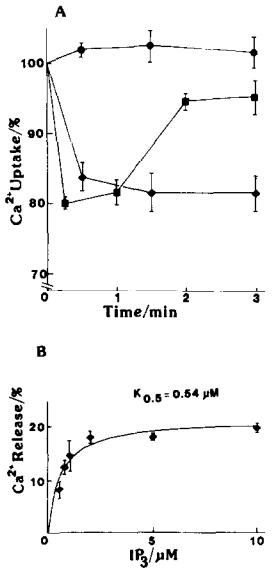

Fig.2. (A) $InsP_3$-induced Ca^{2+} release from red beet microsomes. After steady-state Ca^{2+} accumulation had been reached, additions were made at a time designated t = 0 min. 100% Ca^{2+} uptake (after subtraction of A23187-insensitive binding) ≡ 10.1 ± 3.0 nmol/mg. The following additions were made: (■) 5 μM $InsP_3$ (n = 3); (●) 10 μM FCCP (n = 3); (◆) 5–10 μM $InsP_3$ and 10 μM FCCP (n = 10). (B) Dose-response curve for $InsP_3$-induced Ca^{2+} release. 100% Ca^{2+} release ≡ 6.8 ± 1.3 nmol/mg. Data are means ± SEM for 3 experiments. Solid line shows least-squares fit [18] to a Michaelis-Menten relationship, and yields a maximal release = 20.8 ± 0.8% and $K_{0.5}$ = 0.54 ± 0.11 μM $InsP_3$.

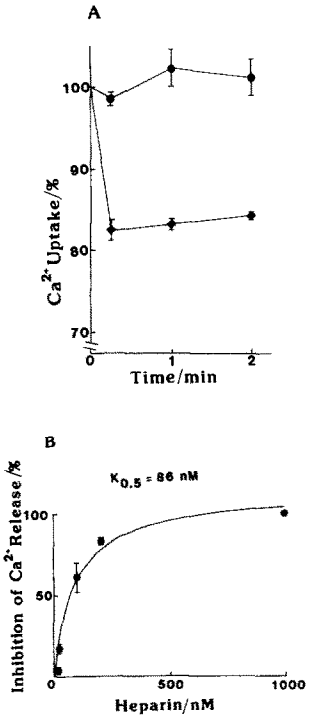

Fig.3. (A) Effect of heparin (1 μM) on $InsP_3$-mediated Ca^{2+} release from red beet microsomes. Microsomes were incubated in IM until steady-state Ca^{2+} accumulation was attained, and 10 μM $InsP_3$ added at time designated t = 0 min. (♦) Absence of heparin; (●) heparin added at t = −1 min. 100% Ca^{2+} uptake (after subtraction of A23187-insensitive binding) ≡ 6.4 ± 1.1 nmol/mg. Data are means ± SEM from 3 experiments. (B) Dose-response curve for heparin inhibition of $InsP_3$-mediated Ca^{2+} release. $InsP_3$ (10 μM) was added to Ca^{2+}-loaded microsomes with varying concentrations of heparin present. 100% inhibition ≡ 0.90 ± 0.05 nmol/mg (n = 3). Solid line shows least-squares fit [19] to a rectangular hyperbola, with a $K_{0.5}$ = 86 ± 20 nM heparin.

is sensitive to nM concentrations of heparin [14], $InsP_3$ phosphatase from animal tissues is not [15]. Furthermore, $InsP_3$-mediated Ca^{2+} release in fungi is heparin-insensitive [16]. It will be interesting to see if the apparent similarity in the mechanism of $InsP_3$-mediated 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 $InsP_3$-induced Ca^{2+} release will enable use of affinity chromatography for purification of the red beet $InsP_3$ receptor, as has already been done with rat cerebellum [17].

Recent evidence from parotid and pancreatic acinar cells reveals that the animal $InsP_3$-sensitive Ca^{2+} store is accumulated by an ATP-dependent, protonophore-inhibited process rather than via a Ca^{2+}-ATPase [18]. The tonoplast exhibits analogous Ca^{2+} uptake properties, thereby complementing the common features of the $InsP_3$-induced Ca^{2+} efflux pathway described here.

Acknowledgement: We thank the Science and Engineering Research Council for provision of an Earmarked Studentship to J.M.B.

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