Subcellular Localization of a High Affinity Binding Site for D-*myo*-Inositol 1,4,5-Trisphosphate from *Chenopodium rubrum*[1]

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It is now generally accepted that a phosphoinositide cycle is involved in the transduction of a variety of signals in plant cells. In animal cells, the binding of D-*myo*-inositol 1,4,5-trisphosphate ($InsP_3$) to a receptor located on the endoplasmic reticulum (ER) triggers an efflux of calcium release from the ER. Sites that bind $InsP_3$ with high affinity and specificity have also been described in plant cells, but their precise intracellular locations have not been conclusively identified. In contrast to animal cells, it has been suggested that in plants the vacuole is the major intracellular store of calcium involved in signal induced calcium release. The aim of this work was to determine the intracellular localization of $InsP_3$-binding sites obtained from 3-week-old *Chenopodium rubrum* leaves. Microsomal membranes were fractionated by sucrose density gradient centrifugation in the presence and absence of Mg^{2+} and alternatively by free-flow electrophoresis. An ER-enriched fraction was also prepared. The following enzymes were employed as specific membrane markers: antimycin A-insensitive NADH-cytochrome c reductase for ER, cytochrome c oxidase for mitochondrial membrane, pyrophosphatase for tonoplast, and 1,3-β-D-glucansynthase for plasma membrane. In all membrane separations, $InsP_3$-binding sites were concentrated in the fractions that were enriched with ER membranes. These data clearly demonstrate that the previously characterized $InsP_3$-binding site from *C. rubrum* is localized on the ER. This finding supports previous suggestions of an alternative non-vacuolar $InsP_3$-sensitive calcium store in plant cells.

In common with all other living organisms, plants must receive and respond to environmental signals. The phosphoinositide cycle is one of the many signaling systems known to operate in plant cells. In conjunction with signal receptors, the phosphoinositide cycle can transduce, amplify, and integrate environmental signals and thereby trigger a cellular response. Elevation of intracellular concentrations of D-*myo*-inositol 1,4,5-trisphosphate ($InsP_3$) has been reported in response to fungal elicitors (Walton et al., 1993), light (Cote and Crain, 1994), and abscisic acid (MacRobbie, 1992). $InsP_3$ has also been reported to play a role in the propagation of calcium waves in pollen tubes (Franklin-Tong et al., 1996). Physiological responses such as stomatal closure (Blatt et al., 1990, Gilroy et al., 1990), protoplast swelling (Shacklock et al., 1992), and growth inhibition of pollen tubes (Franklin-Tong et al., 1996) are all induced by injection of $InsP_3$ into plant cells. This accumulated evidence has given rise to the now generally accepted view that the phosphoinositide cycle is functionally active in plant cells. Nevertheless, our understanding of the operation of this signaling pathway in plant cells is not yet complete. For example, the intracellular localization of $InsP_3$ receptors (binding sites; $InsP_3$-R) and, hence, the site(s) of $InsP_3$-sensitive calcium stores has still to be conclusively demonstrated.

$InsP_3$-R from animals form a family of multisubunit, transmembrane proteins that function as $InsP_3$-gated calcium channels. The receptors are located predominantly on the endoplasmic reticulum (ER), which serves as a calcium store (Lytton and Nigam, 1992). In plant cells, previous studies have characterized specific high affinity $InsP_3$-binding sites, possible $InsP_3$ receptors, and demonstrated features common to the animal $InsP_3$-R (Brosnan and Sanders, 1993; Biswas et al., 1995; Scanlon et al., 1996). The majority of patch clamp experiments (Alexandre et al., 1990; Johannes et al., 1991; Alexandre and Lassalles, 1992) and calcium release studies (Canut et al., 1993; Lommel and Felle, 1996) performed using plant cells point to the vacuole as the main intracellular calcium store. However, Muir and Sanders (1997) have recently demonstrated $InsP_3$-sensitive calcium release across non-vacuolar membranes.

Clear localization of possible $InsP_3$ target(s) would contribute to a better understanding of the complicated mosaic of plant cellular signaling. In this paper we describe the subcellular localization of a previously characterized (Scanlon et al., 1996) $InsP_3$ high affinity binding site from *Chenopodium rubrum.*

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RESULTS

Distribution of $InsP_3$-Binding and Marker Enzyme Activities in Suc Density Gradients in the Absence of Mg^{2+}

Microsomal membranes prepared from *C. rubrum* leaves were separated on an 18% to 38% (w/w) Suc gradient. Membrane vesicles were recovered from each fraction. The resulting distributions of total protein (Fig. 1A), marker enzymes (Fig. 1, B and C), and [^{3}H]$InsP_3$-binding sites (Fig. 1D) were determined as described in "Materials and Methods." Glucan synthase II and cytochrome c oxidase, the markers for plasma membrane and mitochondria, respectively, were concentrated in the higher density fractions, 8 to 11. Pyrophosphatase, the tonoplast marker, was found at slightly lower density with a distinct peak in fraction 7. Antimycin A-insensitive cytochrome c reductase, the ER marker, was found in the lower density fractions, 1 to 3, and was well resolved from the other markers. The distribution of [^{3}H]$InsP_3$-binding sites was very similar to that of cytochrome c reductase with a clear peak in fraction 1 that tailed off over fractions 2 to 5. Chloroplast membranes were situated in fractions 8 to 9 (identified by green coloration). The data depicted in Figure 1 are typical results from an experiment repeated on three further occasions with independent preparations of M.

Distribution of $InsP_3$-Binding and Marker Enzyme Activities in Suc Density Gradients in the Presence of Mg^{2+}

A similar Suc density gradient separation was carried out but with the incorporation of $MgCl_2$ at a final concentration of 4 mM. Under these conditions a membrane pellet was routinely observed (fraction P in Fig. 2), whereas the amount of proteins in fraction 1 (smooth ER) was so low (Fig. 2A) that we were not able to measure either the activities of marker enzymes nor [^{3}H]$InsP_3$ binding (Fig. 2D). The distributions of total protein (Fig. 2A) and the markers for plasma membrane (Fig. 2A), tonoplast (Fig. 2B), and mitochondria (Fig. 2C) were similar to that observed in the absence of Mg^{2+}. However, the presence of Mg^{2+} resulted in a marked difference in the distributions of antimycin A-insensitive cytochrome c reductase and [^{3}H]$InsP_3$-binding sites. Three peaks of cytochrome c reductase activity were observed, in fractions 1, 7, and 10. The maximum activity was found in fraction 7. Significant ER marker activity was also measured in the pellet. The profile of [^{3}H]$InsP_3$-binding sites was similar to that of the cytochrome c reductase except that no peak in fraction 10 was observed. The experiment was performed five times with independent MF preparations and yielded similar results.

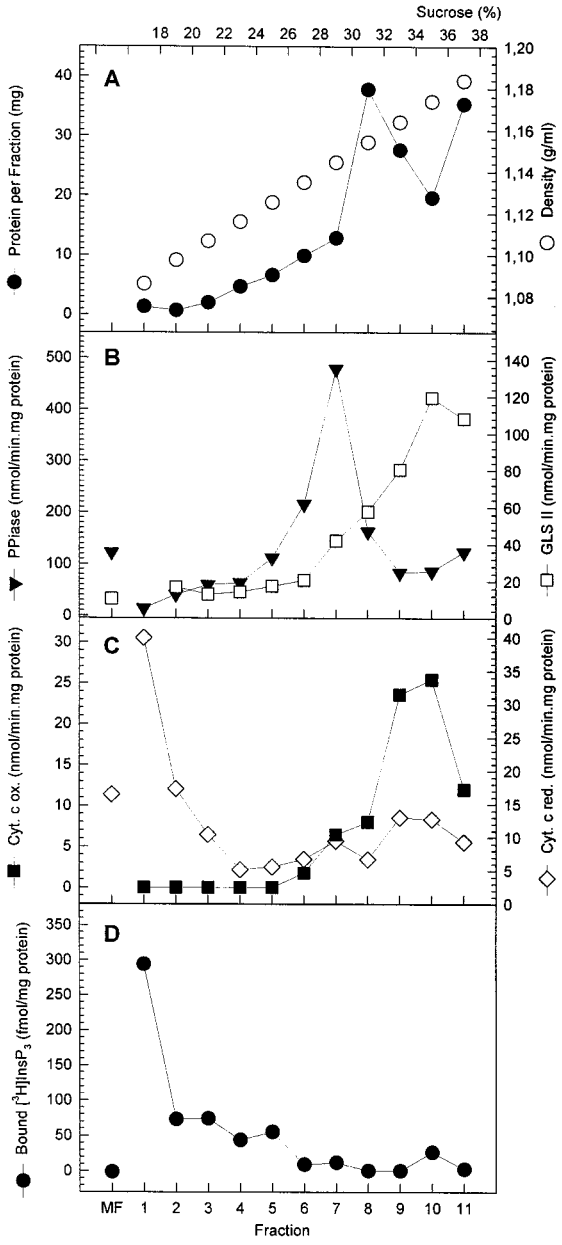

Figure 1. Microsomal fractions from *C. rubrum* were separated on an 18% to 38% (w/w) linear Suc density gradient in the absence of Mg^{2+} (3 mM EDTA). Marker enzyme activities and [^{3}H]$InsP_3$ binding were estimated in each fraction as described in "Materials and Methods." MF, Microsomal fraction. Fraction 1 represents the top of the gradient. Data are from a typical experiment. Similar results were obtained from three other independent experiments. A, ●, Protein profile of separated membranes. B, □, 1,3-β-D-glucan synthase II (plasma membrane marker); ▼, pyrophosphatase (tonoplast marker). C, ■, Cytochrome c oxidase (mitochondria marker); ◇, antimycin A-insensitive cytochrome c reductase (ER marker). D, ●, [^{3}H]$InsP_3$ binding.

Distribution of Marker Enzyme Activities and $InsP_3$ Binding following Free-Flow Electrophoresis

Free-flow electrophoresis is a powerful method for the separation of intracellular membranes (Zeiller et

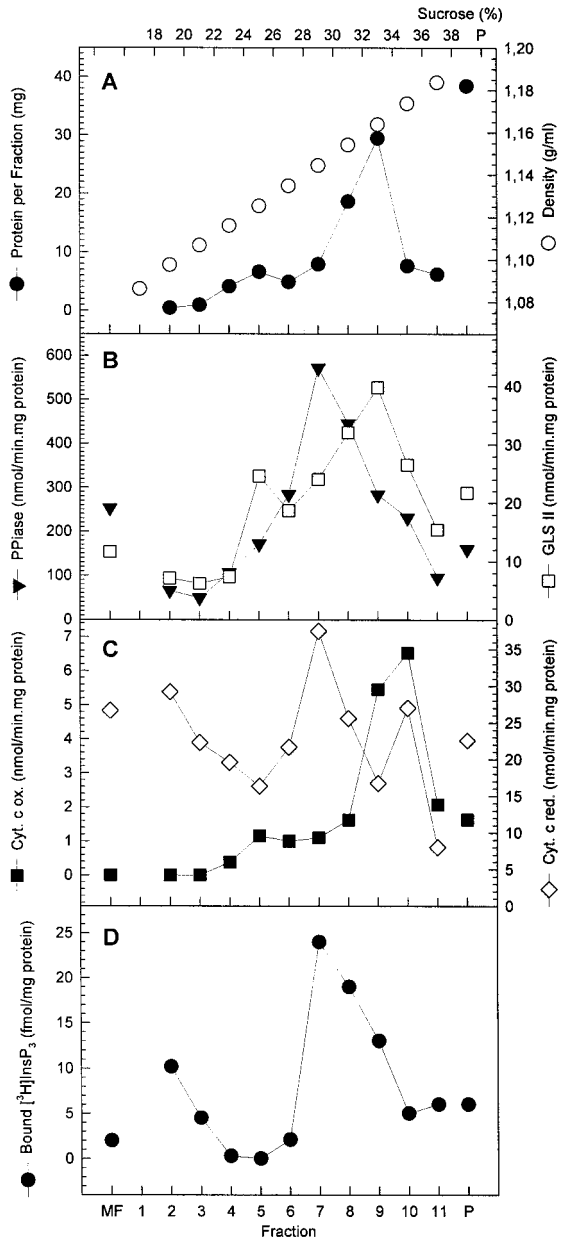

Figure 2. Microsomal fractions from *C. rubrum* were separated on an 18% to 38% (w/w) linear Suc density gradient in the presence of 4 mM $MgCl_2$. Marker enzyme activities and [^{3}H]$InsP_3$ binding were estimated in each fraction as described in "Materials and Methods." Fraction 1 represents the top of the gradient. Data are from a typical experiment. Similar results were obtained from three other independent experiments. A, ●, Protein profile of separated membranes. B, □, 1,3-β-D-glucan synthase II (plasma membrane marker); ▼, pyrophosphatase (tonoplast marker). C, ■, Cytochrome c oxidase (mitochondria marker); △, antimycin A-insensitive cytochrome c reductase (ER marker). D, ●, [^{3}H]$InsP_3$ binding.

al., 1975; Morré et al., 1987). In particular it is possible to obtain very good resolution of tonoplast and plasma membrane fractions, with the center of the separation field usually containing a mixture of other intracellular membranes. The separation of microsomal membranes from *C. rubrum* conformed to this expectation (Fig. 3). The maximum activities of pyrophosphatase (Fig. 3B) and glucan synthase II (Fig. 3B) were observed in fractions 1 and 9, respectively.

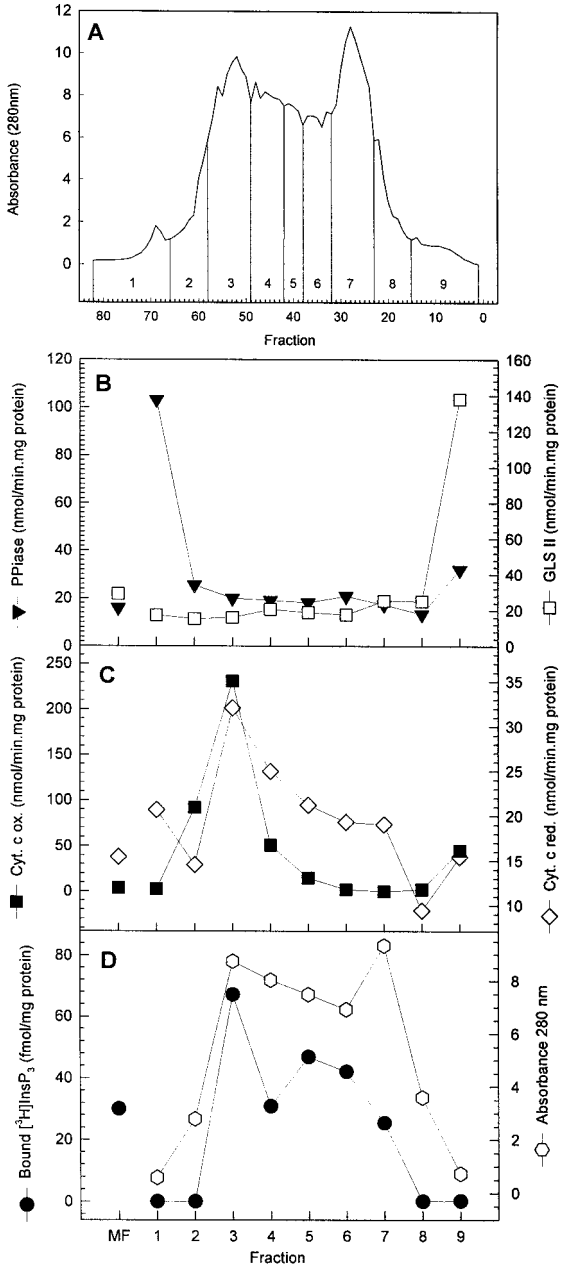

Figure 3. A, Microsomal fractions from *C. rubrum* were separated using free-flow electrophoresis in an electric field. A_{280} was recorded. Individual fractions were pooled into nine final fractions (marked regions 1–9). B, C, and D, Marker enzyme activities and [^{3}H]$InsP_3$ binding were estimated in each fraction as described in "Materials and Methods." Data are from a typical experiment. Similar results were obtained from two other independent experiments. B, □, 1,3-β-D-glucan synthase II (plasma membrane marker); ▼, pyrophosphatase (tonoplast marker). C, ■, Cytochrome c oxidase (mitochondria marker); ◇, antimycin A-insensitive cytochrome c reductase (ER marker). D, ●, [^{3}H]$InsP_3$ binding.

Cytochrome c oxidase and antimycin A-insensitive cytochrome c reductase activities (Fig. 3C) were distributed across fractions 2 to 7 with a distinct peak in fraction 3. [^{3}H]InsP_3-binding sites had a distribution profile closely resembling that of cytochrome c reductase (Fig. 3D). Data shown in Figure 3 are from one of three independent experiments, each of which gave similar results.

Preparation of ER-Enriched Fraction

The association and dissociation of ribosomes from ER in the respective presence and absence of Mg^{2+} leads to a shift in the buoyant density of ER membranes (Ray, 1977; Jones, 1980a, 1980b). This property was utilized to design a stepwise Suc density gradient separation protocol that yielded a membrane preparation enriched in ER membranes. The enrichment in ER membranes, as compared with the MF membrane fraction, was assessed by measuring a range of marker enzyme activities. Compared with marker enzymes from plasma membrane, tonoplast, and mitochondria, this preparation was enriched in ER membranes by a factor of five (Fig. 4). [^{3}H]InsP_3 binding was also enriched in this preparation and the enrichment factor varied between 15 and 30 times in separate experiments.

DISCUSSION

The data obtained in the present study demonstrate that the previously characterized high affinity

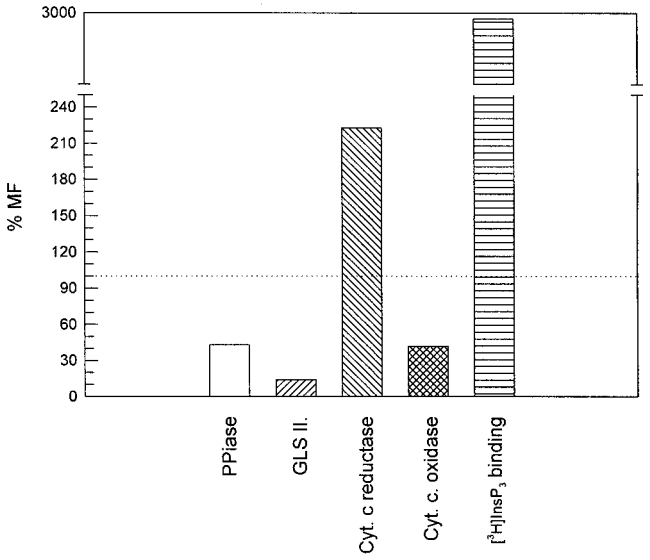

Figure 4. A membrane fraction enriched in ER was prepared as described in "Materials and Methods." Marker enzyme activities and [^{3}H]InsP_3 binding were estimated in the enriched fraction. Activities and binding are expressed as percentages of that measured in the MF, which was set to 100%. □, Pyrophosphatase; ▨, 1,3-β-glucan synthase II; ▧, antimycin A-insensitive cytochrome c reductase; ▩, cytochrome c oxidase; ▤, [^{3}H]InsP_3 binding. Data are from a representative experiment (see text for details).

[^{3}H]InsP_3-binding site found in *C. rubrum* leaf membrane preparations (Scanlon et al., 1996) cofractionates with a marker enzyme of ER. Furthermore, this cofractionation was independent of the physical technique that was used to affect the separation of membranes. We therefore suggest that this InsP_3-binding site is located on the ER.

To determine the subcellular origin of the membrane vesicles that possessed InsP_3-binding activity, the crude microsomal membrane preparation was separated using Suc density gradient centrifugation and by free-flow electrophoresis. A series of stepped Suc density gradients were also used to obtain an ER-enriched membrane preparation.

In the presence of Mg^{2+}, a significant percentage of ER membranes are associated with ribosomes and consequently have a higher buoyant density. If the Mg^{2+} is removed (e.g. by complexing with EDTA) the ribosomes will dissociate from the ER and the buoyant density of the membrane vesicles will be reduced (Ray, 1977; Jones, 1980a, 1980b). This phenomenon results in the density of ER membrane vesicles being Mg^{2+}-dependent and can be used to assist in the identification of ER-derived membrane vesicles.

In Suc density gradient centrifugations carried out in the absence of Mg^{2+} there was a strong correlation between [^{3}H]InsP_3 binding and the ER marker enzyme, antimycin A-insensitive cytochrome c reductase. When similar separations were performed in the presence of Mg^{2+}, both cytochrome c reductase activity and [^{3}H]InsP_3 binding were shifted to higher Suc densities. Unfortunately, in the presence of Mg^{2+} ions, only a very small amount of proteins remained in fraction 1, which corresponds to smooth ER, so that we were not able to perform the measurements of either marker enzyme activities or [^{3}H]InsP_3 binding and calculate which part of the activities was shifted. In spite of this, comparison of Figures 1 and 2 clearly shows a significant shift of both the ER marker and [^{3}H]InsP_3-binding activities to fraction 7 (rough ER). The distribution profile of other marker enzymes was independent of Mg^{2+} concentration. The observed increase in the specific activity of cytochrome c reductase at very high Suc concentrations (fraction 10) that did not correlate with [^{3}H]InsP_3 binding may be due to an antimycin A-insensitive cytochrome c reductase activity associated with vesicles derived from the outer mitochondrial membrane (Moller and Lin, 1986).

The distribution of cellular membranes in the Suc density gradient in the presence or absence of Mg^{2+} ions correlates with results previously published (Robinson et al., 1994). Because of the relatively high content of glycerol (1.1 M) in the suspension buffer, the actual density of the Suc gradients is higher than would be the case if only Suc (without glycerol) were present in the suspension buffer. Smooth ER (fractions 1–4 in the absence of Mg^{2+}) had a density 1.09

to 1.12 g cm^{-3}, whereas rough ER (fractions 5–9 in the presence of Mg^{2+}) had a density 1.13 to 1.16 g cm^{-3}. These densities are in good agreement with data published by Robinson et al. (1994). The density of our tonoplast fraction, which ranged from 1.13 to 1.16 g cm^{-3} (fractions 5–9) is higher than generally reported. Nevertheless, a very broad range for a tonoplast marker was reported by Morré et al. (1987), with high activity of the tonoplast marker a-mannosidase between 24% and 35% (w/w) Suc. Furthermore, Robinson et al. (1994) reported densities of 1.07 to 1.09 g cm^{-3} for tonoplast from mesophyll and root storage tissue and of 1.17 to 1.19 g cm^{-3} for tonoplast from seed storage tissue and maize root and coleoptile.

The separation of membrane vesicles achieved by free-flow electrophoresis was as predicted by theory. The positively charged plasma membrane vesicles migrated toward the cathode and the negatively charged tonoplast vesicles migrated toward the anode. Other intracellular membrane vesicles remained largely unresolved in the middle of the separation field (Morré et al., 1987). The maximum activity of both cytochrome c reductase and [^{3}H]$InsP_3$ binding was found in fraction 3. These activities were both clearly resolved from the plasma membrane and tonoplast markers. Antimycin A-insensitive cytochrome c reductase activity and [^{3}H]$InsP_3$ binding also displayed a broader distribution than cytochrome c oxidase. This may be due to some degree of separation between smooth and rough ER vesicles.

An ER-enriched fraction prepared according to Jones (1980b) gave the clearest relationship between an ER marker and markers of other membranes. However, the highest antimycin A-insensitive cytochrome c reductase activity was actually found in the fraction collected after the first centrifugation (data not shown; see "Material and Methods"). This can happen due to a high content of mitochondrial membranes in that fraction. Mitochondrial membrane vesicles also contained portion of antimycin A-insensitive cytochrome c reductase activity derived from outer mitochondria membrane (Moller and Lin, 1986). Mitochondrial membrane vesicles contain antimycin A-insensitive cytochrome c reductase activity derived from outer mitochondrial membranes (Moller and Lin, 1986), and there is likely to have been a high concentration of mitochondrial membranes in that first fraction. This could explain why the relative activity of antimycin A-insensitive cytochrome c reductase activity in the enriched fraction was only twice that of the MF, whereas $InsP_3$ binding was about 30 times greater. The presented results indicate a strong correlation between the ER enzyme marker, cytochrome c reductase, and the putative $InsP_3$-R. This supports the conclusion that an $InsP_3$-R may be located on the ER in plant cells. Such a conclusion fits with the well established picture of phosphoinositide signaling in animal cells where the $InsP_3$-R is thought to be almost exclusively located on the ER. The properties and localization of $InsP_3$ -R in plant cells are, by comparison, poorly understood. There have been a few reports of putative $InsP_3$-R from plant cells (Brosnan and Sanders, 1993; Biswas et al., 1995; Dasgupta et al., 1997), but these studies did not specifically address the subcellular localization of the $InsP_3$-binding site. However, membrane fractions enriched for tonoplast exhibited higher specific $InsP_3$ binding than microsomal fractions. Cramer et al. (1998) recently demonstrated cross-reactivity between antibodies raised against a mammalian $InsP_3$-R and proteins from isolated vacuoles. In contrast to vacuoles, purified plasma membrane did not reveal any cross-reactivity.

Another approach to the location of $InsP_3$-R is to study the release of Ca^{2+} from different vesicles in response to a challenge with $InsP_3$. There is an increasing body of evidence that suggests that the vacuole is a source of mobile Ca^{2+} ions sensitive to $InsP_3$ in plant cells. This evidence includes several investigations where authors studied calcium release from intact vacuoles (Lommel and Felle, 1996) or from tonoplast vesicles (Schumaker and Sze, 1987). A further series of investigations (Alexandre et al., 1990; Johannes et al., 1991; Alexandre and Lassalles, 1992; Allen and Sanders, 1994) used patch clamp techniques to study calcium channels sensitive to $InsP_3$. The biochemical characteristics of these channels were similar to channels previously described in animal tissues. However, the patch clamp technique is limited to work with isolated vacuoles or protoplasts and there are, therefore, no results available concerning ER. Canut et al. (1993) reported that membrane fractions derived from free-flow electrophoresis responded to $InsP_3$ by releasing Ca^{2+}. These fractions were characterized as being enriched in vacuolar membrane based upon the activities of marker enzymes. Nevertheless there were not present any Ca^{2+} ions in their experimental buffer and under these conditions the $InsP_3$-binding protein requiring Ca^{2+} for binding would not bind $InsP_3$, and Ca^{2+} release would not occur. Muir and Sanders (1997) adopted a similar approach in combination with the use of an antibody against peptides corresponding to the type 1 mammalian $InsP_3$-R. They found two distinct membrane fractions that were sensitive to $InsP_3$ and cross-reacted with the antibody raised against peptides corresponding to the type 1 mammalian $InsP_3$ receptor. One of these fractions was clearly derived from vacuolar membranes, whereas the second may have originated from plasma membrane or ER. These authors suggested that there may be more than one intracellular store of Ca^{2+} in plant cells. This conclusion is consistent with the fact that young meristematic cells lack central vacuoles, which in older cells may be the major calcium store.

Although $InsP_3$-sensitive calcium channels have to date only been detected on the tonoplast, there is

indirect evidence that the ER might also serve as an intracellular calcium store, and certainly an alternative source of mobile calcium within the same cell might broaden the available spectrum of calcium responses. Within plant cells there are three membranes with steep electrochemical gradients for Ca^{2+}—plasma membrane, tonoplast, and ER (Bush, 1995). For example, Ca^{2+} concentrations within isolated ER vesicles from aleurone cells have been measured to be at least 3 μM (Bush et al., 1989). Plant ER also contains low affinity calcium-binding proteins that can serve as a Ca^{2+} storage mechanism. There is also rapid calcium exchange from and into ER vesicles (Bush, 1995), whereas Franklin-Tong et al. (1996) showed that Ca^{2+} waves in pollen tubes that were triggered by photolysis of caged $InsP_3$ were initiated primarily in the nuclear-rough ER cellular locale. There is therefore some evidence that calcium release may occur from the ER in response to $InsP_3$, and we are therefore planning to investigate whether this occurs from the ER-enriched fractions shown here to contain a putative $InsP_3$-R.

MATERIALS AND METHODS

Chemicals

[^{3}H]$InsP_3$ (specific activity 0.77–1.15 TBq mmol^{-1}; TRK999), uridindiphospho-D-[U-^{14}C]Glc ([^{14}C]UDPG; specific activity 11.1 GBq mmol^{-1}; CFB102) were purchased from Amersham Pharmacia Biotech (Uppsala), $InsP_3$ (I 9766) was obtained from Sigma (St. Louis), and Suc for density gradient ultracentrifugation was obtained from Merck (Germany). All other chemicals were of analytical grade.

Plant Material

Chenopodium rubrum L. (ecotype 374) seeds were sown on moist compost and germinated under a 36-h regime consisting of 12 h of light at 30°C, 12 h of darkness at 8°C, and 12 h of light at 30°C. After 3 d at 20°C under constant fluorescent white light (100 μmol m^{-2} s^{-1}) seedlings were transferred to new pots and maintained under the same conditions until harvest. Leaves were harvested after 3 weeks of growth.

Preparation of a Microsomal Fraction

Membrane preparations were carried out at 4°C either in the presence or absence of Mg^{2+}. For preparations in the absence of Mg^{2+}, leaves were ground in homogenization buffer (0.25 M Suc, 3 mM EDTA, 0.2% (w/v) bovie serum albumin, 5 mM dithiothreitol, 10 mM ascorbic acid, and 70 mM Tris [Tris(hydroxymethyl)-aminomethane] adjusted to pH 8.0 with MES [2-(*N*-morpholino)-ethanesulfonic acid]) using a homogenizer (X620, Zipperer, Germany) for 5 min at 20,000 rpm. The ratio of homogenization buffer to tissue was 2 mL g^{-1} fresh weight. The homogenate was filtered through nylon tissue to remove cell debris and the filtrate centrifuged at 6,000*g* (Rav) for 10 min (JS-13.1 rotor, Beckman, Fullerton, CA). The supernatant was decanted and centrifuged at 150,000*g* (Rav) for 45 min (70 Ti rotor, Beckman). The resulting pellet (MF) was resuspended in suspension buffer (1.1 M glycerol, 5 mM dithiothreitol, and 10 mM Tris adjusted to pH 8.0 with MES) using a soft brush, giving a protein concentration of about 20 mg mL^{-1}. Protease inhibitors were present in homogenization and suspension buffer at the following concentrations: 0.23 mM phenyl methyl sulfonyl chloride, 0.83 mM benzamidine, 0.7 μM pepstatin A, 1.1 nM leupeptin, and 77 nM aprotinin.

For preparations in the presence of Mg^{2+}, the concentration of EDTA in the homogenization buffer was 1 mM. Homogenization and suspension buffers contained $MgCl_2$ at final a concentration of 4 mM.

Suc Density Gradient Centrifugation

Further fractionation of the MF was achieved by Suc density gradient centrifugation. Routinely, the MF was layered onto a linear continuous Suc gradient (18%–38% [w/w] Suc in suspension buffer) and centrifuged at 30,000*g* (Rav) overnight (Ti70 rotor, Beckman). The gradient was prepared using an automated gradient sampler (Auto Densi-flow II C, Haakebuchler, Saddlebrook, NJ). After centrifugation, membrane vesicles were removed using the gradient maker, diluted in a ratio of 1:5 with suspension buffer and centrifuged at 220,000g (Rav) for 45 min (Ti rotor, Beckman 70). Pellets were resuspended in a minimal volume of suspension buffer and kept (maximum 4 d) at 0°C to 4°C for immediate use or stored at −70°C.

Free-Flow Electrophoresis

Free flow electrophoresis was performed according to Crespi (1991) using an Elphor VAP 22 (Bender and Hobein, Munich). The chamber buffer consisted of 15 mM triethanolamine, 4 mM potassium acetate, 10 mM Glc, 30 mM Suc, and 240 mM Gly-acetic acid (pH 7.5); the electrode buffer contained 45 mM ethanolamine, 12 mM potassium acetate, and 720 mM Gly-acetic acid (pH 7.5). The conditions for separation were as follows: constant current 120 mA (voltage about 1,200V), chamber buffer flow 4.5 mL fraction^{-1} h^{-1}, injection flow 3 mL h^{-1}, constant temperature 4°C. The distribution of separated membranes was monitored by UV A_{280}. Separated membranes were collected into 100 original fractions. These were then pooled into nine fractions (Fig. 3A), centrifuged, and the resulting pellets resuspended in suspension buffer.

Preparation of ER

Preparation of purified ER was based on the Mg^{2+}-dependent shift in density that occurs when ribosomes dissociate from rough ER. ER was prepared according to Lis and Weiler (1994) with slight modifications. The homogenization buffer contained 6 mM $MgCl_2$. All solutions

containing 6 mM $MgCl_2$ are referred to as "high Mg^{2+}"; solutions without $MgCl_2$ or containing 6 mM EDTA are referred to as "low Mg^{2+}." MF prepared using homogenization buffer containing 6 mM $MgCl_2$ and no EDTA was resuspended in high Mg^{2+} suspension buffer and was layered onto a high Mg^{2+} step gradient consisting of 20%, 30%, and 40% (w/w) Suc in suspension buffer. The gradient was centrifuged for 3 h at 223,000*g* (Rav; 70 Ti rotor, Beckman). Material from the 30% to 40% interface and the 40% layer was collected, diluted 4 to 5 times in high Mg^{2+} suspension buffer and centrifuged at 223,000*g* for 45 min. The resulting pellet was resuspended in low Mg^{2+} suspension buffer and layered onto a low Mg^{2+} step Suc gradient (gradient as above). After centrifugation, material from the 20% layer was loaded on 20% (w/w) Suc. After further centrifugation, smooth ER remained in solution, whereas all other contaminants were pelleted. Supernatant was diluted in low Mg^{2+} suspension buffer and centrifuged. The resulting pellet was resuspended in low Mg^{2+} suspension buffer and is referred as the "ER enriched fraction."

Assay of $InsP_3$-Binding Sites

Binding of $InsP_3$ to membrane vesicles was quantified using a radioligand-binding assay based on the principles outlined by Hulme and Birdsall (1992). A working stock of [^{3}H]$InsP_3$ was prepared by drying 100 μL (37 kBq) of the supplied source under N_2 to remove ethanol, and resuspending in 4 mL of water (exact radioactive concentration was verified by scintillation counting). Routine assays (final volume of 100 μL) contained 0.9 to 1.8 nM [^{3}H]$InsP_3$ in accordance with the specific activity of different batches (20 μL of stock [^{3}H]$InsP_3$ [11,000–12,000 dpm]), 20 mM bis-tris propane (adjusted to pH 9.0 with MES), 10 mM $CaCl_2$, and 20 μL of a suitable dilution of the membrane fraction (20 μg of protein). Assays were initiated by addition of membranes. After 20 min of incubation, 20 μL of water was added to the sample and 20 μL of $InsP_3$ (final concentration of 40 μM) was added to blank (determination of non-specific binding). After a further 20-min incubation, bound and free [^{3}H]$InsP_3$ were separated by rapid filtration through 0.4-μm pore diameter nitrocellulose membranes (Pragopor 6, Pragochema, Czech Republic), which were immediately washed with 2 mL of ice-cold assay buffer. Non-specific binding was quantified by parallel experiments that included 40 μM unlabeled $InsP_3$, and it generally represented 20% to 30% of the total binding, but in different membrane preparations varied from less than 10% to more than 50%. Filter discs were transferred to plastic mini-vials, dissolved in 4 mL of scintillant (Filter count, Packard, Meriden, CT) and radioactivity determined by scintillation spectroscopy (LS 5801, Beckman).

Enzyme Marker Assays

Antimycin A-Insensitive NAD(P)H-Dependent Cytochrome c Reductase (ER Marker)

NAD(P)H-dependent cytochrome c reductase activity was determined spectrophotometrically at 550 nm in the presence of Tris-MES buffer (pH 7.5), 1.6 mM KCN, 0.15 μM antimycin A, 0.5 mM NADH, 56 μM cytochrome c, and 30 to 50 μg/mL protein (Briskin et al., 1987). The reaction was initiated by the addition of cytochrome c and followed for 5 min (cytochrome c E = 18.5 mM^{-1} cm^{-1}) at 25°C.

Cytochrome c Oxidase (Mitochondria Marker)

Cytochrome c oxidase activity was measured according to Briskin et al. (1987). One milliliter of assay medium contained 30 mM Tris-MES buffer (pH 7.5), 2 mM digitonin, and 54 μM cytochrome c. The reaction was initiated by the addition of 100 μL of membrane fraction (containing 30–50 μg of protein) and monitored by measuring A_{550} for 5 min at 25°C. The reduced form of cytochrome c was prepared by adding a few crystals of sodium dithionite to the cytochrome c solution.

1,3-β-Glucan Synthase II (Plasma Membrane Marker)

The activity of 1,3-β- glucan synthase II was quantified using the procedure described by Widell and Larsson (1990) with minor modifications. The assay medium (50 μL) contained 50 mM Tris-MES buffer (pH 8.0), 0.1% (v/v) Triton X-100, 20 mM cellobioze, 16% (v/v) glycerol, 0.33 μM digitonin, 100 μM $CaCl_2$, and 0.8 mM UDP-Glc. The radioactive concentration of [^{14}C]UDP-Glc included in the assay was 830 Bq. Membrane protein concentration was 0.5 to 0.9 mg/mL. The assay was initiated by the addition of substrate. Following incubation for 30 to 50 min at 25°C, the reaction was terminated by immersion of the test tubes in boiling water for 5 min. The reaction medium was transferred onto filter discs (3mm, Whatman, Clifton, NJ) and dried. Filter discs were washed three times for 45 min in 0.5 M ammonium acetate in 30% (v/v) ethanol. Washed discs were dried and their radioactivity was measured by liquid scintillation counting.

Pyrophosphatase (Tonoplast Marker)

The activity of pyrophosphate was determined as described by Blumwald and Poole (1987). The reaction medium contained 30 mM Tris-MES buffer (pH 7.8), 50 mM KCl, 0.5 mM $MgSO_4$, 5 μM gramicidin D, and 0.3 mM sodium pyrophosphate. Membrane preparations were added to a final protein concentration of 25 to 50 μg/mL in 1 mL of total assay volume. The reaction was initiated by the addition of substrate. Following a 20-min incubation at 37°C, the reaction was terminated by the addition of 300 μL of freshly prepared Ames reagent. Released inorganic phosphate was determined according to Ames (1966).

Protein Determination

Protein was estimated according to Bradford (1976) using bovine serum albumin as a standard.

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