Plant Physiol. (1994) 106: 1403–1412

A 42-Kilodalton Annexin-Like Protein Is Associated with Plant Vacuoles[1]

Darren F. Seals, Mark L. Parrish, and Stephen K. Randall*

Biology Department, Indiana University-Purdue University at Indianapolis, 723 West Michigan Street, Indianapolis, Indiana 46202–5132

A 42-kD, calcium-dependent, membrane-binding protein (VCaB42) was associated with partially purified vacuole membranes. Membrane-dissociation assays indicated that VCaB42 binding to vacuole membranes was selective for calcium over other cations and that 50% of VCaB42 remained membrane bound at 61 ± 11 nM free calcium. A 13-amino acid sequence obtained from VCaB42 showed 85% similarity with the endonexin fold, a sequence found in the annexin family of proteins that is thought to be essential for calcium and lipid binding. The greatest similarity in amino acid sequence was observed with annexin VIII (VAC-β). The calcium-binding properties and sequence similarities suggest that VCaB42 is a member of the annexin family of calcium-dependent, membrane-binding proteins. Functional assays for VCaB42 on vacuole membrane transport processes indicated that it did not significantly affect the initial rate of calcium uptake into vacuole membrane vesicles. Because VCaB42 is vacuole localized (likely on the cytosolic surface of the vacuole) and is 50% dissociated within the physiological range of cytosolic free calcium, we hypothesize that this protein is a sensor that monitors cytosolic calcium levels and transmits that information to the vacuole.

The vacuole of mature plant cells dominates in size and is functionally versatile, making it an important organelle for cellular homeostasis (for reviews, see Wiebe, 1978; Taiz, 1992; Wink, 1993). Among its many functions, the vacuole accumulates cations such as sodium, potassium, magnesium, and calcium, as well as various inorganic anions, organic acids, and other metabolites. The vacuole serves accordingly as a storage and retrieval compartment. One consequence of the vacuolar accumulation of solutes is the osmotic influx of water resulting in the generation and maintenance of cell turgor.

Calcium, a well-known component of signal transduction pathways, can activate and regulate numerous cellular processes in eukaryotes. Small changes in intracellular calcium concentrations regulate such activities as cell growth, metabolism, transport, and secretion. Levels of cytosolic calcium are tightly controlled through homeostatic mechanisms. Generally under "resting" conditions, cytosolic calcium is maintained at submicromolar levels (Hepler and Wayne, 1985; Felle, 1988; Miller et al., 1990; Bush, 1993), whereas extracytosolic levels may be several orders of magnitude greater (Hepler and Wayne, 1985). Cytosolic calcium levels can be quickly regulated by the sarcoplasmic reticulum in muscle and skeletal tissues, the smooth ER and/or calciosome in nonmuscle tissues, and the plasma membrane in all eukaryotic systems (Pietrobon et al., 1990). Because plant cell vacuoles contain free calcium concentrations ranging from 50 μM to 3 mM (Williamson, 1981; Kinzel, 1989; DuPont et al., 1990), this organelle may also be involved in calcium homeostasis (Schumaker and Sze, 1985; Miller et al., 1990).

For the plant vacuole to play an active role in calcium homeostasis, it must have a calcium uptake system and a calcium buffering system, as well as a means to regulate the release of calcium (Pietrobon et al., 1990). There is considerable evidence that the vacuole membrane, or tonoplast, has mechanisms for the accumulation and release of calcium. A calcium-proton exchanger, indirectly driven by the vacuolar, proton-pumping ATPase, was shown to drive calcium accumulation into the vacuole (Schumaker and Sze, 1985; Blumwald and Poole, 1986). This may also be driven in part by a pH gradient generated by the vacuolar, Pi-dependent proton pump (Rea and Sanders, 1987). A calcium-selective channel of unknown physiological function may also allow entry of calcium from the cytoplasm into the vacuole at very negative vacuole membrane potentials (Pantoja et al., 1992). An IP_3-responsive channel capable of releasing calcium into the cytosol may be operational on vacuole membranes (Schumaker and Sze, 1987; Alexandre et al., 1990). Indeed, the vacuole may contain the largest store of IP_3-releasable calcium (Canut et al., 1993).

Consistent with IP_3-releasable calcium in the vacuole, Brosnan and Sanders (1993) have demonstrated IP_3-binding activity on the vacuole membrane. Other channels that may regulate the release of vacuolar calcium have also been characterized (Hedrich and Schroeder, 1989; Bertl and Slayman, 1992; Gelli and Blumwald, 1993). To date no vacuolar calcium transport proteins have been identified on the molecular level. In terms of proteinaceous calcium buffers, a calsequestrin-related protein (Chou et al., 1989) and a calreticulin-like protein (Menegazzi et al., 1993) have been identified in plants, although the intracellular locations are not yet known. No vacuolar calcium buffers have been

[1] Supported in part by a Howard Hughes Undergraduate Research Fellowship to M.L.P., an Indiana University-Purdue University at Indianapolis Faculty development grant to S.K.R., and National Science Foundation grant MCB-9205052 to S.K.R.

* Corresponding author; fax 1–317–274–2846.

Abbreviations: IP_3, inositol 1,4,5-triphosphate; r_{max}, maximal radius; VCaB42; the 42-kD, vacuole membrane-associated, calcium-binding protein.

identified, although several candidates are at present being characterized (Randall, 1992).

There are numerous calcium-binding proteins that utilize calcium to regulate cellular functions. Among these are the elongation factor hand family of proteins (which includes calmodulin) that are involved in signal transduction, the low-affinity calcium-binding proteins like calsequestrin and calreticulin that function in a buffering capacity, and the annexins.

The annexins form a major group of calcium-binding proteins that bind to membranes in a calcium-dependent manner (Geisow and Walker, 1986; Klee, 1988; Burgoyne and Geisow, 1989). This family includes a broad range of proteins that were previously classified as calelectrins, calcimedins, calpactins, endonexins, lipocortins, and synexin. The ubiquitously distributed annexins are distinguished from other calcium-binding proteins by sharing a repetitive 17-amino acid sequence termed the endonexin fold (Creutz, 1992). This sequence is contained within each of four domains each consisting of 70 to 80 amino acids (annexin VI has eight domains). A part of each domain is a calcium- and phospholipid-binding region. At present there is no known common function among the annexins; however, annexins have been implicated in such diverse roles as the formation of calcium channels and the inhibition of blood coagulation or inflammation (Römisch and Pâques, 1991). Several annexins may also bind, aggregate, and fuse secretory vesicles during exocytosis (Creutz, 1992).

Recently, several plant annexins have been identified based on amino acid sequence similarities, antibody cross-reactivities, and functional similarities with animal annexins. Plant annexin-like sequences have been identified in tomato (*Lycopersicon esculentum*) (Boustead et al., 1989), maize (*Zea mays*), *Tradescantia* and tobacco (*Nicotiana tabacum*) (Blackbourn et al., 1991), potato (*Solanum tuberosum*) and barley (*Hordeum vulgare*) (Smallwood et al., 1992), pea (*Pisum sativum*) (Clark et al., 1992), *Lilium* (Blackbourn et al., 1992), and cotton (*Gossypium hirsutum*) (Andrawis et al., 1993). Partial sequences from these proteins show varied degrees of similarity to the endonexin fold with a high of 70% for pea p35 (see Table I; Clark et al., 1992). Tomato p35 and maize p33 and p35 are related to bovine and chicken annexin VI, respectively, as shown by antibody cross-reactivities (Boustead et al., 1989; Blackbourn et al., 1991). Antibody raised against the electric organ of *Torpedo* cross-reacted with tomato p34 (Boustead et al., 1989). Like the annexins discovered in vertebrates, the functional roles proposed for plant annexins are diverse. Consistent with a role in exocytosis, pea p34 has been immunolocalized to tissues specialized for secretion (Clark et al., 1992), whereas maize p33 and p35 bind and aggregate secretory vesicles (Blackbourn et al., 1992; Blackbourn and Battey, 1993). A recently discovered annexin-like protein in cotton fibers inhibits the plasma membrane-localized callose synthase activity (Andrawis et al., 1993).

A long-term goal of this laboratory is to understand the role of the vacuole in the regulation of cytosolic levels of calcium, and conversely, the role of calcium in the regulation of vacuolar processes. Previously we have characterized vacuolar, calcium-binding, lumenal proteins (Randall, 1992). This report describes the properties of a 42-kD, calcium-dependent, vacuole membrane-binding protein (VCaB42) that is likely associated with the cytosolic surface of the vacuole. The calcium-binding affinity and specificity of this protein make it a candidate signal transducer in higher plants.

MATERIALS AND METHODS

Plant Material and Vacuole Membrane Isolation

Celery (*Apium graveolens* L.) was obtained from a local market and petioles were prepared for vacuole membrane isolation as described (Randall and Sze, 1987; Randall, 1992) with some modifications. All procedures were performed at 4°C to reduce proteolysis. Briefly, whole petioles were homogenized in a buffer composed of 50 mM Hepes, pH 7.40, 250 mM mannitol, 1 mM DTT, and 1 mM $CaCl_2$. One millimolar PMSF (0.05% DMSO) and 0.1% (w/v) BSA were present during homogenization to reduce protease activity. The addition of the protease inhibitors 1 mM benzamidine, 10 μg/mL aprotinin, and 1 μg/mL leupeptin had no significant effect on the apparent size or yields of VCaB42 and were generally not included. Homogenization was accomplished in a Waring blender for 15 s at high speed with a ratio of 2.5 mL homogenization buffer per g fresh weight tissue. The homogenate was filtered through six layers of cheesecloth. Microsomal pellets were resuspended in 2.5 mM Hepes, pH 7.20, 250 mM mannitol, 1 mM DTT (resuspension buffer) containing 1 mM $CaCl_2$ and loaded on a dextran step gradient composed of 12, 7, and 4% (w/w) dextran (74,200 average M_r). When 1 mM $CaCl_2$ was included in dextran solutions, there was no significant change in membrane yields or purity. Gradients were centrifuged for 2 h at 72,128*g* at r_{max} (SW 28 rotor). Interfaces were collected and washed by dilution with resuspension buffer containing 1 mM $CaCl_2$ and recentrifugation for 30 min at 121,896*g* at r_{max} (SW 28 rotor). The final pellets were resuspended in resuspension buffer containing 5 mM EGTA and centrifuged for 30 min at 214,200*g* at r_{max} (type 90Ti rotor). This treatment caused proteins, previously bound to the vacuole membrane in a calcium-dependent manner, to dissociate. The supernatant resulting from the EGTA wash (EGTA supernatant) was stored at −80°C. Routinely, vacuolar preparations were obtained after centrifugation on single-step gradients composed of 5% (w/w) dextran. For comparative purposes, some vacuole membranes were isolated in the presence of 3 mM EGTA.

Calcium Green 2 Standard Curves

Fluorimetric assays were used to estimate free calcium concentrations in $CaCl_2$ and EGTA buffers at 8°C. Free calcium standard curves were constructed utilizing Calcium Green 2 (1 μM final concentration) and a calcium calibration buffer kit I (Molecular Probes, Inc., Eugene, OR) according to the manufacturer's recommendations. Fluorescence measurements were taken on a Perkin Elmer fluorimeter model MPF-66 interfaced with a model 7700 computer and a Fisher Scientific Isotemp Refrigerated Circulator model 9000 (Fig. 1).

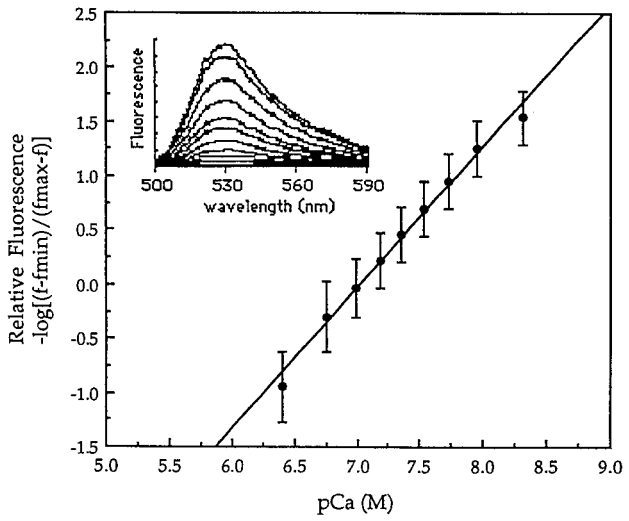

Figure 1. Calcium Green 2 was used to generate a free calcium standard curve in the presence of EGTA at 8°C. Free calcium concentrations were based on a K_d^{EGTA} of 43 ± 11 nM at pH 7.40 and 8°C. Data are the average of five experiments. The inset shows an example of the type of data from which the standard curve was generated. Fluorescence emission of Calcium Green 2 (1 μM final concentration) was measured after excitation at 488 nm. The emission maximum at 530 nm was calculated as the relative fluorescence ($-\log[(f - f_{min})/(f_{max} - f)]$), which was linearly related to the concentration of free calcium. The emission scans shown were obtained at 0, 5, 11, 19, 29, 43, 65, 100, 170, and 390 nM.

Determination of Cation Selectivity

Other cations were tested for their ability to substitute for calcium in causing VCaB42 to associate with vacuole membranes. Vacuole membranes (0/5% interfaces) were isolated in the presence of 1 mM $CaCl_2$, washed, and resuspended in resuspension buffer containing 100 μM $CaCl_2$. Membranes (45 μg of membrane protein; Bradford, 1976) were diluted with resuspension buffer containing 500 μM EGTA. The final concentration of free calcium was 20 nM as measured by Calcium Green 2 fluorescence. Resuspension buffer was supplemented with either 20 or 500 μM magnesium or potassium (sulfate salts), or enough $CaCl_2$ to attain 140 nM free calcium. Since the affinity of EGTA for other cations was several orders of magnitude lower than that for calcium (Sillén and Martell, 1971), any displacement of calcium by other cations was ignored. Samples were gently agitated at 4°C and centrifuged for 30 min at 213,000*g* at r_{max} (TLA-100.3 rotor). All supernatants were immediately precipitated with 12.5% TCA and proteins were analyzed by SDS-PAGE (Laemmli, 1970). Gels were stained with Coomassie brilliant blue R-250.

Calcium-Dependent Binding of VCaB42 to Vacuole Membranes

Vacuole membranes were prepared in 100 μM $CaCl_2$ as described above for the cation selectivity experiments. Membranes (45 μg of membrane protein) were diluted with resuspension buffer containing various concentrations of EGTA (0–5 mM) in a final volume of 500 μL, gently agitated at 4°C, and centrifuged for 30 min at 213,000*g* at r_{max} (TLA-100.3 rotor). Aliquots were tested with Calcium Green 2 to determine free calcium concentrations. Supernatants were immediately precipitated in 12.5% TCA and analyzed by SDS-PAGE. Pellets were extracted in 10 mM EGTA and the resultant supernatants were also TCA precipitated and proteins were analyzed by SDS-PAGE. Gels were stained with Coomassie brilliant blue R-250 and were analyzed by densitometry using the NIH Image 1.44 image processing and analysis program in conjunction with an Apple scanner.

Determination of Calcium Transport by Tonoplast Vesicles

Celery was homogenized in 25 mM Hepes/Bis-Tris-propane, pH 7.4, 250 mM mannitol, 1 mM DTT, 3 mM EGTA, and 0.1% (w/v) fatty acid-free BSA. Vacuole membrane vesicles were collected after a 2-h centrifugation at 72,128*g* at r_{max} (SW 28 rotor) at the interface of a 6% (w/w) dextran cushion. The interface was diluted and centrifuged for 30 min at 121,896*g* at r_{max} (SW 28 rotor). The pellet was resuspended in 1 mL of 2.5 mM Hepes/Bis-Tris-propane, pH 7.40, 250 mM mannitol. Since these membranes were isolated in the presence of EGTA, little VCaB42 was present on the vacuole membranes. Calcium uptake by vesicles was measured utilizing a filtration assay (Schumaker and Sze, 1985) in the presence or absence of added VCaB42. A 1-mL reaction generally contained 10 μM $CaCl_2$ (14–44 μCi/μmol ^{45}Ca) and 100 μg of membrane protein. The calcium ionophore A23187 was dissolved in ethanol and utilized at a final concentration of 5 μM.

Amino Acid Sequencing

EGTA supernatants derived from vacuole membranes isolated in 1 mM $CaCl_2$ were precipitated in 12.5% TCA and the proteins were separated by SDS-PAGE in the presence of 0.01% 3-mercapto propionic acid. Polypeptides were electrophoretically transferred to polyvinylidene difluoride paper (Immobilon P, Millipore, Inc., Bedford, MA). For amino-terminal sequencing, blots were stained with Ponceau S and the band of interest was excised and washed in distilled water. To obtain internal amino acid sequences, the first gel was stained with Coomassie brilliant blue R-250 and the band of interest was excised and cleaved with cyanogen bromide in situ according to Nikodem and Fresco (1979). Cleaved polypeptides were separated by SDS-PAGE (16% acrylamide) and electrophoretically transferred to polyvinylidene difluoride paper. The major bands were excised and washed with distilled water. These cyanogen bromide fragments were subsequently digested with trypsin according to Fernandez et al. (1992). Peptides were purified on an Applied Biosystems (Foster City, CA) 130Å C_{18} reverse-phase column and were sequenced by automated Edman degradation on a Beckman 2090 Protein/Peptide Micro Sequencer.

RESULTS

Low-Density Fractions Are Enriched in Vacuole Membranes

A discontinuous dextran step gradient was used to resolve various cellular membranes and to partially purify vacuole

membranes. We have previously shown that the 0/4% dextran interface, when isolated in the presence of EGTA (low calcium), is highly enriched in vacuole membranes (Randall, 1992). We compared the distribution of the vacuole membrane and ER markers to confirm that low-density membranes, when isolated in the presence or absence of calcium, were of similar purity. When membranes were isolated in the presence of calcium, the specific activities of the vacuole and ER markers were similar to those when membranes were isolated in the presence of EGTA (data not shown). There was, however, a 50% decrease in total membrane protein in the 0/4% interface. This resulted in a loss of total ATPase activity (hence vacuole membranes) but only a small change (increase) in specific activity of the nitrate-sensitive ATPase (vacuole marker enzyme), suggesting that the vacuole membranes, when isolated in the presence of calcium, were slightly more purified. The vacuole-enriched fraction (0/4% dextran) had nitrate-sensitive ATPase activity of 6.0 and 8.6 μmol h^{-1} mg^{-1} protein when isolated in the absence or presence of calcium, respectively. The increased purity was most likely due to the shift of the ER to a slightly higher density, possibly due to a calcium-induced stabilization of ribosomes on the RER. The similar purity of these fractions was also observed by SDS-PAGE analysis of these membranes (Fig. 2). In summary, the results indicated that the vacuolar fraction (0/4% dextran interface), whether isolated in the presence or in the absence of calcium, was of similar purity.

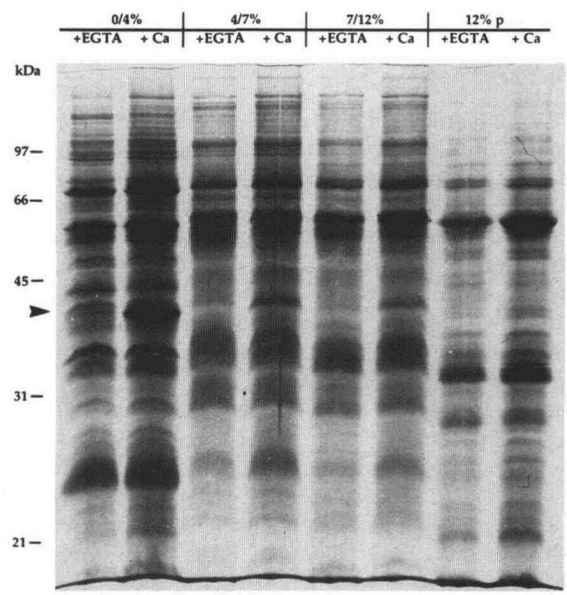

Figure 2. Isolation of membranes in the presence of calcium results in the association of a 42-kD protein with the vacuole membrane-enriched fraction. Membranes isolated in the presence of 1 mM $CaCl_2$ or in the presence of 3 mM EGTA were separated on discontinuous dextran gradients as described in "Materials and Methods." Equivalent amounts of protein were separated by SDS-PAGE (12% acrylamide) and stained with Coomassie brilliant blue R-250. Designations of 0/4%, 4/7%, and 7/12% refer to the dextran interfaces, whereas 12% p refers to the pelleted membranes. The arrowhead designates the position of the 42-kD calcium-dependent, vacuole membrane-binding protein, or VCaB42.

A 42-kD Polypeptide Is Located on Vacuole Membranes

To identify calcium-dependent, membrane-binding proteins, membranes isolated in the presence or absence of calcium were compared by SDS-PAGE (Fig. 2). Consistent with the specific activities of the vacuolar ATPase, the general pattern of polypeptides was similar in membranes isolated under both conditions. However, in the vacuole membrane-enriched fraction (0/4% dextran interface) isolated in the presence of calcium, proteins of approximately 68 and 42 kD were present in relatively greater amounts than in the absence of calcium. The 42-kD protein, henceforth referred to as VCaB42, was not observed in any membrane fraction when isolated in the absence of calcium and was found to be most highly enriched in the 0/4% dextran fraction when isolated in the presence of calcium. Although VCaB42 is present in the other gradient fractions, the relative distribution of VCaB42 is similar to that of vacuole membrane markers on these gradients (Randall, 1992).

Vacuole Membrane Association of VCaB42 Can Be Reversed by EGTA

To show that VCaB42 binding to membranes was reversible and to determine its physical location on vesicles, vacuole membranes isolated in the presence of calcium were subsequently treated with EGTA either with or without sonication. Membrane and soluble components were separated by centrifugation and equal amounts of protein were analyzed by SDS-PAGE (Fig. 3). When membranes were treated with 1 mM calcium, VCaB42 remained associated with the vacuole membrane (lanes 1). Reduction of calcium to less than 5 nM by the addition of 5 mM EGTA solubilized greater than 95% of VCaB42 (lanes 2). Sonication did not solubilize any additional amount (lanes 3). Together these data suggest that VCaB42 associated with the vacuole membrane in a divalent cation-dependent manner and that the protein was likely associated primarily with the outside of the membrane vesicles.

VCaB42 Binding to Vacuole Membranes Is Selective for Calcium

To determine whether VCaB42 binding to membranes was selective for calcium over other cations, vacuole membranes isolated in 100 μM calcium were washed with varying concentrations of calcium, magnesium, or potassium in the presence of 500 μM EGTA. Calcium at 140 nM provided the highest membrane association, with the other cations (micromolar levels) being ineffective in maintaining vacuole membrane association of VCaB42 (Fig. 4). The association of VCaB42 with vacuole membranes was clearly dependent on calcium concentration (Fig. 5). Vacuole membranes treated with less than 5 nM calcium had little VCaB42 associated, whereas VCaB42 remained associated with membranes treated with greater than 390 nM calcium. This was evident by examining either dissociated VCaB42 (Fig. 5A) or

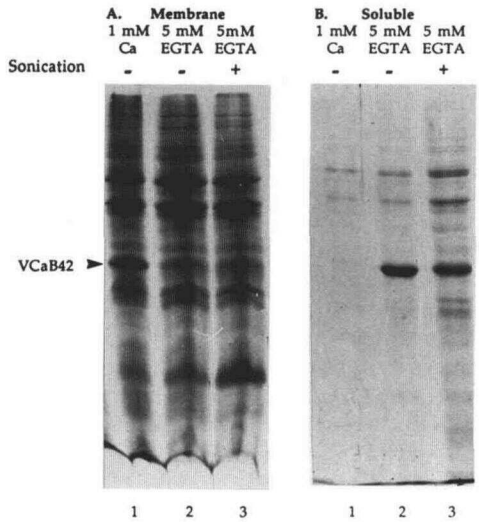

Figure 3. Association of VCaB42 with the vacuole membrane is reversible. Vacuole membranes isolated in the presence of 1 mM $CaCl_2$ were treated with resuspension buffer containing 1 mM $CaCl_2$ (lanes 1), 5 mM EGTA (<5 nM calcium; lanes 2), and 5 mM EGTA (<5 nM calcium) and sonication (lanes 3). Membrane and soluble components were separated by ultracentrifugation for 1 h at 173,502*g* at r_{max} (type 90Ti rotor). Supernatants were precipitated in 12.5% TCA. Equal amounts of protein were analyzed by SDS-PAGE and gels were stained with Coomassie brilliant blue R-250. A, Membrane-associated VCaB42 (pellet); B, soluble VCaB42 (supernatant).

membrane-bound VCaB42 (eluted from membranes with EGTA; Fig. 5B). By both visual inspection (Fig. 5, A and B) and densitometry (Fig. 5C), maximal dissociation of VCaB42 from the vacuole membranes was obtained at less than 5 nM free calcium. The half-maximal dissociation of VCaB42 from the membrane occurred at 61 ± 11 nM free calcium The ratio of calcium concentration required for 90% saturation to the calcium concentration required for 10% saturation (R_s) was estimated to be approximately 20, indicative of a positive cooperative ligand binding (an $R_s < 81$ is considered indicative of positive cooperativity; Kirtley and Koshland, 1967). Hill plot analysis (Fig. 5C, inset) also indicated cooperativity, and assuming simple symmetric cooperativity, predicted an average dissociation constant of 66 nM. The Hill coefficient, estimated by the slope at the 50% VCaB42 bound position (Fig. 5C, inset, dotted line), was 2.02. This suggests that multiple calcium-binding sites are involved in calcium-dependent binding of VCaB42 to the vacuole membrane. The low calcium concentration at which dissociation occurs suggests that VCaB42 binds to vacuole membranes within the expected (physiological) range of cytosolic calcium concentrations.

Calcium Accumulation into Tonoplast Vesicles Is Not Affected by VCaB42

One commonality of function among a variety (although not all) of annexin proteins is the ability to modulate (whether directly or indirectly) calcium transport properties (Pollard and Rojas, 1988; Díaz-Muñoz et al., 1990; Rojas et al., 1990; Pollard et al., 1992). The vacuole's role as a reservoir of calcium suggested that VCaB42 might influence calcium transport across the vacuole. As a first approach to understanding the function of VCaB42, we tested the effect of the presence of VCaB42 on the accumulation of calcium into vacuolar vesicles. Transport of calcium into vacuolar vesicles was ATP dependent, and approximately 40% of the ATP-dependent accumulation was abolished by the presence of the protonophore gramicidin (not shown). These properties suggest that 40% of the calcium accumulation was due to a calcium-proton antiport driven by the pH gradient generated by the vacuolar, ATP-dependent proton pump, similar to those systems present on vacuole membranes of other systems (Schumaker and Sze, 1985; Blumwald and Poole, 1986; DuPont et al., 1990). Calcium accumulation reached a maximum of 12 to 15 nmol calcium/mg membrane protein after approximately 40 min (Fig. 6). The average maximum gramicidin-sensitive calcium accumulation for celery was 5.4 nmol calcium/mg protein. The calcium ionophore A23187 released most of the calcium from the vesicles, indicating that calcium was reversibly bound (Fig. 6). The calcium transport capacity in celery membranes was comparable to that in oat root vacuole, where maximal accumulations attained were approximately 6.5 nmol calcium/mg protein (Schumaker and Sze, 1985).

The initial rate of calcium uptake by vacuolar vesicles was not significantly affected by the presence of VCaB42 (Fig. 6). Although we consistently saw a slightly greater accumulation of calcium in the presence of VCaB42, this difference was not statistically significant. We have also directly tested the effect of VCaB42 on the initial rate and the extent of ATP-dependent proton pumping by examining acridine orange fluorescent quenching and have observed no significant effect.

Since there was no significant difference in calcium transport with VCaB42, we believe that VCaB42 has a negligible influence on the calcium transporter and the vacuolar, proton-pumping ATPase. However, the small yet consistent increase in net accumulation in the presence of VCaB42 implies that the calcium transport properties of the vacuole may have been changed.

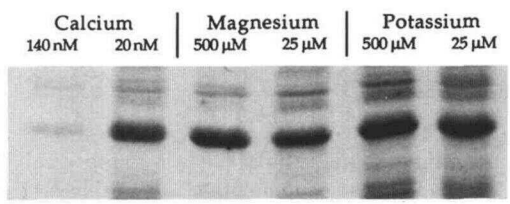

Figure 4. VCaB42 binding to membranes is selective for calcium over other cations tested. Vacuole membranes in 100 µM calcium were diluted with resuspension buffer containing 500 µM EGTA and the various concentrations of cations shown. Free calcium was 20 nM, except where noted. Samples were gently agitated and spun for 30 min at 213,000*g* at r_{max} (TLA-100.3 rotor). Supernatants (representing unbound VCaB42) were treated and analyzed as described (Fig. 3).

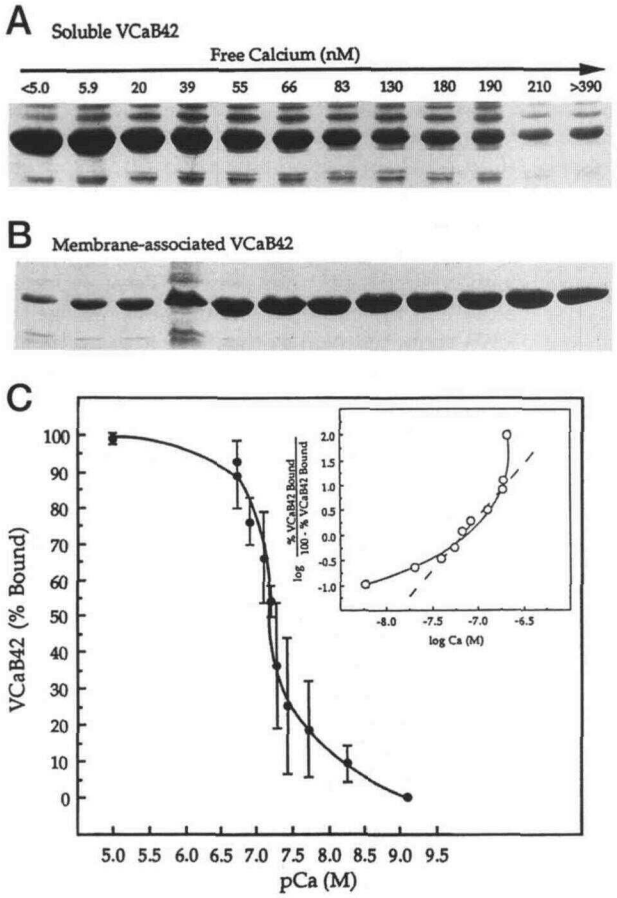

Figure 5. Fifty percent dissociation of VCaB42 from vacuole membranes occurs at nanomolar levels of free calcium. Vacuole membranes isolated in the presence of 1 mM calcium were resuspended in resuspension buffer with varying concentrations of EGTA (see "Materials and Methods"). Membrane and soluble components were separated by ultracentrifugation for 30 min at 213,000g at r_{max} (TLA-100.3 rotor) and proteins were resolved by 12% SDS-PAGE. Free calcium concentrations (up to 390 nM) were determined by Calcium Green 2 (Fig. 1). A, Soluble VCaB42 (supernatants); B, membrane-associated VCaB42 (pellets). Membrane-associated VCaB42 was solubilized with 10 mM EGTA. C, VCaB42, electrophoresed on 12% gels and stained with Coomassie brilliant blue R-250, was quantitated by densitometry. The inset in C is a Hill plot showing a Hill coefficient (slope of dotted line) equal to 2.02 (estimated at the 50% bound point). Data are the average of three independent membrane isolations.

Partial Amino Acid Sequences of VCaB42

Amino-terminal sequencing of the full-length VCaB42 produced a pentapeptide sequence, QVPAV (Table I, sequence 1.1). Because this sequence lacked a Met, it is likely that the amino terminus was either endogenously processed or was proteolytically cleaved during membrane isolation. To obtain internal sequences, VCaB42 was digested with cyanogen bromide followed by trypsin. Amino-terminal sequencing was conducted on tryptic fragments to produce three distinct, internal sequences (Table I, sequences 2.1, 2.2, and 2.3). Sequence 2.2 was highly similar to the consensus sequence of the endonexin fold (a sequence diagnostic for the annexin family of calcium-binding proteins). A comparison of 13 of the 17 amino acid residues in the consensus annexin fold (KGhGTDEpphhphhhoR; Creutz, 1992) showed that 10 are identical matches and 3 others (residues 25, 28, and 34) are conservative replacements found in one or more of the annexins (Table I; Barton et al., 1991). Sequence 2.2 thus shows 77% identity and, considering conservative replacements, 85% similarity at the amino acid level with the endonexin fold consensus sequence. This partial amino acid sequence has the highest similarities across the entire endonexin fold of any putative plant annexin. Sequence 2.3 also shows a marked similarity to amino acids downstream from endonexin fold sequences, especially those in repeat 2 of annexins IV, V, and VIII (Barton et al., 1991). Sequences 2.2 and 2.3 of VCaB42 showed 85 and 78% similarity with corresponding sequences in both annexins IV and VIII. Other significant similarities were observed with the second repeat of annexins I, III, and IX. In general, VCaB42 showed less similarity to the plant annexin sequences than to animal sequences. Sequence 2.2 was 67% similar to pea p35 and sequence 2.3 was 78% similar to maize p35 and tomato p35. Sequences 1.1 and 2.1 showed little similarity with annexin sequences; however, the lack of similarities observed in sequence 1.1 is not surprising, since the amino-terminal regions of the annexins are highly variable. It was interesting that when partially purified VCaB42 was tested for cross-reactivity (by western analysis) with monoclonal antibodies (Biodesigns International, Kennebunkport, ME) raised against annexins I, II, II (light chain), IV, and VI, or to a polyclonal antibody raised against human annexin VIII (Hauptmann et al., 1989),

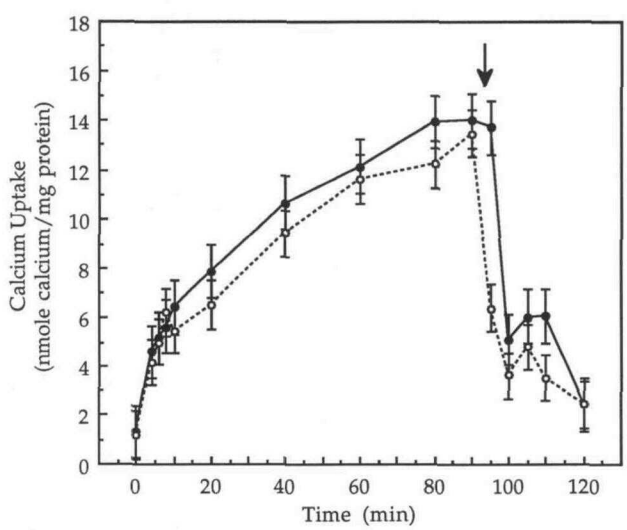

Figure 6. VCaB42 does not significantly influence the uptake of calcium across the vacuole membrane. Membranes were incubated with 3 mM ATP and 10 μM total calcium (containing 0.25 μCi ^{45}Ca/mL) in either the presence (●) or absence (○) of VCaB42 (generally from 0.1–1.0 μg of protein). EGTA was removed from VCaB42 with Sephadex G-25 equilibrated in 250 mM mannitol, 2.5 mM Hepes/Bis-Tris-propane, pH 7.00, and 20 mM KCl. The calcium ionophore A23187 was added at 90 min (arrow). Data are the average of four experiments.

Table I. *Sequence similarities of VCaB42 with the annexin super-gene family*

A comparison is made between VCaB42 and the most similar amino acid sequences found in animal (Barton et al., 1991) and putative plant annexins (Smallwood et al., 1990; Blackbourn et al., 1992). The conserved endonexin fold sequence is shown (Creutz, 1992). The endonexin fold in general corresponds to amino acids 19 to 36 of each repeat unit. Asterisks indicate invariant residues in the conserved repeat No. 2 of the annexin super-gene family (Barton et al., 1991). Other similarities correspond to downstream residues 55 through 66 in repeat No. 2 (Barton et al., 1991). Columns indicating VCaB42 identity and similarity are expressed as percent similarity of VCaB42 with the respective sequences (residues 21–34). Endonexin fold similarities, based on available sequences, are expressed as percent similarity to the endonexin fold. When VCaB42 amino acids were in doubt, the amino acid in boldface was used for comparison. Amino acid similarities were calculated according to the following groups: ILMV, KHR, DENQ, ST, AG, and FWY. Amino acid abbreviations are as follows: h, hydrophobic; p, polar; o, hydroxylated; –, unknown. Abbreviations for sequences are: hAVIII(2), human annexin VIII repeat 2 (VAC-β); hAIV(2), human annexin IV repeat 2; hAIII(2), human annexin III repeat 2; dAIX(2), *Drosophila* annexin IX repeat 2. Gaps introduced at residues 20 and 63 indicate that there are no corresponding amino acids in an alignment of repeat 2 with the other annexin repeats (Barton et al., 1991). Other VCaB42 sequences obtained were: OVPAC (seq. 1.1) and TYAETYGED (seq. 2.1).

	Similarities to Endonexin Fold																			Similarities to Downstream Sequence										
	Residue Number (VCaB42, seq. 2.2)																			Residue Number (VCaB42, seq. 2.3)										
	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	VCaB42 Identity	VCaB42 Similarity	Endo. Fold Similarity	55	56	57	58	59	60	61	62	63	64	65	66
Endonexin fold	K		G	h	G	T	D	E	p	p	h	h	p	h	h	h	O	R														
Invariant			*		*	*		*					*	*									*								*	*
VCaB42	–		D/G	–	G	T	N	E	G	L	I	I	S/G	I	L	G	–	–			85	D/S	L	E	E	D	V	A	Y	–	–	T
hAVIII(2)	K		G	L	G	T	K	E	G	V	I	I	E	I	L	A	S	R	69	85	82	S	L	E	E	D	I	Q	A	D	T	S
hAIV(2)	K		G	A	G	T	D	E	G	C	L	I	E	I	L	A	S	R	62	85	94	S	L	E	D	D	I	R	S	D	T	S
hAIII(2)	K		G	A	G	T	N	E	D	A	L	I	E	I	L	T	T	R	62	69	76	S	L	G	D	D	I	S	S	E	T	S
dAIX(2)	S		G	L	G	T	D	E	E	A	I	I	E	I	L	C	T	L	62	69	65	S	L	E	S	D	L	K	G	D	T	S
Pea p35	–		–	L	A	Q	S	E	A	H	I	I	–	I	–	–	–	–	44	67	70											
Tomato p35	S		D	–	A	Y	N	D	E	E	I	I	R	I	L	S	T	–	38	54	53	S	L	H	I	D	V	A	Y	H	T	S
Maize p35	H		K	K	A	Y	S	D	E	E	I	I	R	I	L	T	T	R	31	46	53	S	L	L	E	D	V	A	A	A	–	T
Tomato p34	S		D	K	A	Y	S	D	D	E	V	I	R	I	L	A	T	R	23	54	53	D	I	L	–	Q	L	–	D	E	D	–
Maize p33	R		K	W	K	P	G	N	R	V	L	V	E	I	–	G	T	Q	17	50	44	S	L	G	D	D	I	–	–	–	–	–

no positive reactions were obtained (data not shown). The sequences and immunological data suggest that VCaB42 is a novel annexin.

DISCUSSION

The vacuole is an organelle that contains high levels of calcium. Accumulation of calcium can be driven by a proton pump (ATP and PPi dependent; Sze, 1984; Rea and Sanders, 1987) that creates an electrochemical gradient that drives calcium uptake via a Ca^{2+}/H^+ antiporter (Schumaker and Sze, 1985; Blumwald and Poole, 1986). Because of relatively low cytosolic calcium concentrations (Hepler and Wayne, 1985; Felle, 1988; Miller et al., 1990; Bush, 1993), the large calcium gradient across the vacuole membrane is postulated to serve as one mechanism for the mediation of calcium-activated cellular processes. We have identified a calcium-dependent, vacuole membrane-binding protein called VCaB42. This protein is thought to reside in the cytosol, where it could interact with calcium (at nanomolar calcium levels) and bind to the vacuole membrane. The calcium-binding properties and sequence similarities strongly suggest membership of VCaB42 in the annexin family of calcium-binding proteins. We propose that VCaB42 may play a role in the vacuolar regulation of calcium signaling or conversely in the calcium regulation of vacuolar functions.

VCaB42 binds to the vacuole membrane in a calcium-dependent manner. It is selective for calcium over other monovalent and divalent cations and shows membrane-binding activity at nanomolar calcium levels, indicating its high affinity for calcium. The concentration of calcium at which 50% of VCaB42 is bound to the vacuole membrane is 61 ± 11 nM. The narrow range of calcium concentration over which VCaB42 remains membrane bound (R_s = 20) and the Hill coefficient (2.02) suggest multiple, cooperative calcium-binding sites. The presence of multiple calcium-binding sites identified here is consistent with the identification of three calcium sites in repeats I, II, and IV located by x-ray crystallography of human annexin V (Huber et al., 1992). Since VCaB42 association with vacuole membranes occurs within the range of cytosolic calcium concentrations, it is likely that VCaB42 could respond to fluctuating cytosolic calcium levels by binding or dissociating from the vacuole membrane. Furthermore, the narrow range in which VCaB42 is converted from a membrane-bound to a soluble form suggests that it is a potential sensor for calcium fluctuations between 10 nM free calcium (90% of VCaB42 is soluble) and 190 nM free calcium (90% of VCaB42 is membrane associated).

The high binding affinity of VCaB42 for calcium is on the same order of magnitude as the high-affinity binding sites of calmodulin (K_d of 310 nM; Beckingham, 1991). Affinities of elongation factor hand calcium-binding proteins are generally higher than the annexins. Reports from in vitro calcium-dependent, membrane-binding aggregation and fusion activities show calcium affinities ranging from submicromolar levels to as high as 1 mM. An exception is annexin II, which binds pure phosphotidylserine vesicles in the presence of less than 10 nM calcium (Powell and Glenney, 1987). The high affinity of VCaB42 for calcium-dependent vacuole membrane binding suggests either that this protein may have a novel calcium-binding domain of high affinity or that the vacuole membrane is an ideal substrate for annexin binding.

The primary sequence of the calcium-binding domain of annexins, the endonexin fold, is distinct from the elongation factor hand calcium-binding domain of calmodulin. Although the annexins and calmodulin-like proteins share similar calcium-binding secondary structures, a helix-loop-helix, the coordinating ligands for calcium differ. The elongation factor hand utilizes acidic amino acids to coordinate binding to calcium, whereas the calcium-binding site of annexins involves the carbonyl oxygens of a Gly-rich domain in the endonexin fold (GhGT; residues 21–24; see Table I) and a conserved acidic residue 39 amino acids downstream (residue 64; see Table I). Also thought to contribute to calcium binding by the annexins is a solvent water molecule and a phospholipid head group (Huber et al., 1990, 1992). Consistent with a secondary structure required for calcium-binding, VCaB42 will not bind ^{45}Ca on ligand blots, nor will it stain blue by Stains-all (data not shown).

The calcium-dependent association of VCaB42 with the vacuole membrane suggests possible localization in the cytosol of plant cells, on the cytosolic side of the vacuole membrane, or in the vacuolar lumen. We favor the hypothesis that this protein is located within the cytosol under "resting" calcium levels and is associated with the outer leaflet of the vacuole membrane as cytosolic calcium levels increase. The observation that sonication did not solubilize any more VCaB42 from EGTA-treated membranes than was solubilized by EGTA alone (Fig. 3) indicates that VCaB42 is on the outside of the vacuolar vesicles as they are isolated. The calcium transport and proton-pumping assays on isolated vesicles indicate that a significant portion of vesicles are right-side-out (relative to vacuoles). It follows that the binding of VCaB42 is likely to the cytosolic surface of the vacuole. Use of antibodies raised against VCaB42 will more definitively answer the question of intracellular location.

Based on VCaB42's calcium-binding affinity and vacuole membrane association, several possible functions are hypothesized. VCaB42 could be involved in maintaining the structural integrity of the vacuole, regulating or forming membrane channels, or regulating calcium levels or vacuolar processes (e.g. catalysis of membrane fusion). VCaB42 has been tested for its effect on two well-known biochemical processes associated with the vacuole membrane. VCaB42 has no significant effect on either the acidification of the vacuole (catalyzed by the proton-pumping ATPase) or on the sequestration of calcium into the vacuolar lumen (catalyzed by the Ca^{2+}/H^+ antiporter).

Although it is clear from Figure 5C that the majority of VCaB42 is removed by the EGTA treatment, a small amount of VCaB42 remains on the membrane. We cannot eliminate the possibility that this remaining amount is sufficient to fully activate vacuolar activities. An alternative explanation for the lack of activation by VCaB42 is that the EGTA supernatant does not contain the requisite factors to allow functional reassociation of VCaB42 with the vacuole. Our work at present is focused on ascertaining the biological function of this protein.

Understanding the involvement of this novel calcium-

dependent, membrane-binding protein in vacuoles may offer new and exciting clues as to how vacuoles function and how calcium is regulated in plant cells. Furthermore, this understanding is likely to have implications for the role of homologous organelles in other cells such as the lysosome and various endosomal compartments. We suggest that it is likely that VCaB42 plays a role in the transduction of calcium signals, either to or from the vacuole.

ACKNOWLEDGMENTS

We would like to extend our thanks to Dr. William Stillwell of the Department of Biology, Indiana University-Purdue University at Indianapolis, for use of the MPF-66 fluorimeter and Millipore water purification system. Special thanks to David Wild for conducting numerous vacuole membrane preparations and to Susan Eichholtz and Kathryn Frazer for western blot analyses. Dr. Blake Pepinsky of the Department of Protein Chemistry, Biogen Inc., graciously provided human annexin VIII as a positive control for antibodies against annexin VIII, which was kindly donated by Dr. Rudolf Hauptmann and Dr. G.R. Adolf of the Ernst Boehringer Institut (Vienna, Austria). Amino acid sequencing was conducted by the Biochemistry Biotechnology Facility of the Indiana University School of Medicine.

Received April 25, 1994; accepted August 10, 1994.
Copyright Clearance Center: 0032-0889/94/106/1403/10.

LITERATURE CITED

Alexandre J, Lassalles JP, Kado RT (1990) Opening of Ca^{2+} channels in isolated red beet root vacuole membrane by inositol 1,4,5-trisphosphate. Nature **343:** 567–570

Andrawis A, Solomon M, Delmer DP (1993) Cotton fiber annexins: a potential role in the regulation of callose synthase. Plant J **3:** 763–772

Barton GJ, Newman RH, Freemont PS, Crumpton MJ (1991) Amino acid sequence analysis of the annexin super-gene family of proteins. Eur J Biochem **198:** 749–760

Beckingham K (1991) Use of site-directed mutations in the individual Ca^{2+}-binding sites of calmodulin to examine Ca^{2+}-induced conformational changes. J Biol Chem **266:** 6027–6030

Bertl A, Slayman CL (1992) Complex modulation of cation channels in the tonoplast and plasma membrane of *Saccharomyces cerevisiae*: single-channel studies. J Exp Biol **172:** 271–287

Blackbourn HD, Barker PJ, Huskisson NS, Battey, NH (1992) Properties and partial protein sequence of plant annexins. Plant Physiol **99:** 864–871

Blackbourn HD, Battey NH (1993) Annexin-mediated secretory vesicle aggregation in plants. Physiol Plant **89:** 27–32

Blackbourn HD, Walker JH, Battey NH (1991) Calcium-dependent phospholipid-binding proteins in plants. Planta **184:** 67–73

Blumwald E, Poole RJ (1986) Kinetics of Ca^{2+}/H^{+} antiport in isolated tonoplast vesicles from storage tissue of *Beta vulgaris* L. Plant Physiol **80:** 727–731

Boustead CM, Smallwood M, Small H, Bowles DJ, Walker JH (1989) Identification of calcium-dependent phospholipid-binding proteins in higher plant cells. FEBS Lett **244:** 456–460

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Brosnan JM, Sanders D (1993) Identification and characterization of high-affinity binding sites for inositol trisphosphate in red beet. Plant Cell **5:** 931–940

Burgoyne RD, Geisow MJ (1989) The annexin family of calcium-binding proteins. Cell Calcium **10:** 1–10

Bush DS (1993) Regulation of cytosolic calcium in plants. Plant Physiol **103:** 7–13

Canut H, Carrasco A, Rossignol M, Ranjeva R (1993) Is vacuole the richest store of IP_3-mobilizable calcium in plant cells? Plant Sci **90:** 135–143

Chou M, Krause KH, Campbell KP, Jensen KG, Sjolund RD (1989) Antibodies against the calcium-binding protein. Plant Physiol **91:** 1259–1261

Clark GB, Dauwalder M, Roux SJ (1992) Purification and immunolocalization of an annexin-like protein in pea seedlings. Planta **187:** 1–9

Creutz CE (1992) The annexins and exocytosis. Science **258:** 924–931

Díaz-Muñoz M, Hamilton SL, Kaetzel MA, Hazarika P, Dedman JR (1990) *Modulation of Ca^{2+} release channel activity from sarcoplasmic reticulum by annexin VI (67 kDa calcimedin).* J Biol Chem **265:** 15894–15899

DuPont FM, Bush DS, Windle JJ, Jones RL (1990) Calcium and proton transport in membrane vesicles from barley roots. Plant Physiol **94:** 179–188

Felle H (1988) Auxin causes oscillations of cytosolic free calcium and pH in *Zea mays* coleoptiles. Planta **174:** 495–499

Fernandez J, DeMott M, Atherton D, Mische SM (1992) Internal protein sequence analysis: enzymatic digestion for less than 10 μg of protein bound to polyvinylidene difluoride or nitrocellulose membranes. Anal Biochem **201:** 255–264

Geisow MJ, Walker JH (1986) New proteins involved in cell regulation by Ca^{2+} and phospholipids. Trends Biochem Sci **11:** 420–423

Gelli A, Blumwald E (1993) Calcium retrieval from vacuolar pools. Plant Physiol **102:** 1139–1146

Hauptmann R, Maurer-Fogy I, Krystek E, Bodo G, Andree H, Reutelingsperger CPM (1989) Vascular anticoagulant β: a novel human Ca^{2+}/phospholipid binding protein that inhibits coagulation and phospholipase A_2 activity. Eur J Biochem **185:** 63–71

Hedrich R, Schroeder JI (1989) The physiology of ion channels and electrogenic pumps in higher plants. Annu Rev Plant Physiol **40:** 539–569

Hepler PK, Wayne RO (1985) Calcium and plant development. Annu Rev Plant Physiol **36:** 397–439

Huber R, Berendes R, Burger A, Schneider M, Karshikov A, Luecke H, Römisch J, Paques E (1992) Crystal and molecular structure of human annexin V after refinement. J Mol Biol **223:** 683–704

Huber R, Schneider M, Mayr I, Römisch J, Paques EP (1990) The calcium binding sites in human annexin V by crystal structure analysis at 2.0 Å resolution. FEBS Lett **275:** 15–21

Kinzel H (1989) Calcium in the vacuoles and cell walls of plant tissue. Flora **182:** 99–125

Kirtley ME, Koshland DE (1967) Models for cooperative effects in proteins containing subunits. J Biol Chem **242:** 4192–4205

Klee CB (1988) Calcium-dependent phospholipid- (and membrane-) binding proteins. Biochemistry **27:** 6645–6653

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Menegazzi P, Guzzo F, Baldan B, Mariani P, Treves S (1993) Purification of calreticulin-like protein(s) from spinach leaves. Biochem Biophys Res Commun **190:** 1130–1135

Miller AJ, Vogg G, Sanders D (1990) Cytosolic calcium homeostasis in fungi: roles of plasma membrane transport and intracellular sequestration of calcium. Proc Natl Acad Sci USA **87:** 9348–9352

Nikodem V, Fresco JR (1979) Protein fingerprinting by SDS-gel electrophoresis after partial fragmentation with CNBr. Anal Biochem **97:** 382–386

Pantoja O, Gelli A, Blumwald E (1992) Voltage-dependent calcium channels in plant vacuoles. Science **255:** 1567–1570

Pietrobon D, Virgilio FD, Pozzan T (1990) Structural and functional aspects of calcium homeostasis in eukaryotic cells. Eur J Biochem **193:** 599–622

Pollard HB, Guy HR, Arispe N, de la Fuente M, Lee G, Rojas EM, Pollard JR, Srivastava M, Zhang-Keck ZY, Merezhinskaya N, Caohuy H, Burns AL, Rojas E (1992) Calcium channel and membrane fusion activity of synexin and other members of the annexin gene family. Biophys J **62:** 15–18

Pollard HB, Rojas E (1988) Ca^{2+}-activated synexin forms highly

selective, voltage-gated Ca^{2+} channels in phosphatidylserine bilayer membranes. Proc Natl Acad Sci USA **85:** 2974–2978

Powell MA, Glenney JR (1987) Regulation of calpactin I phospholipid binding by calpactin I light-chain binding and phosphorylation by $p60^{v\text{-}src}$. Biochem J **247:** 321–328

Randall SK (1992) Characterization of vacuolar calcium-binding proteins. Plant Physiol **100:** 859–867

Randall SK, Sze H (1987) Purification and characterization of the tonoplast H^+-translocating ATPase. Methods Enzymol **148:** 123–132

Rea PA, Sanders D (1987) Tonoplast energization: two H^+ pumps, one membrane. Physiol Plant **71:** 131–141

Rojas E, Pollard HB, Haigler HT, Parra C, Burns AL (1990) Calcium-activated endonexin II forms calcium channels across acidic phospholipid bilayer membranes. J Biol Chem **265:** 21207–21215

Römisch J, Pâques EP (1991) Annexins: calcium-binding proteins of multi-functional importance? Med Microbiol Immunol **180:** 109–126

Schumaker KS, Sze H (1985) A Ca^{2+}/H^+ antiport system driven by the proton electrochemical gradient of a tonoplast H^+-ATPase from oat roots. Plant Physiol **79:** 1111–1117

Schumaker KS, Sze H (1987) Inositol 1,4,5-trisphosphate releases Ca^{2+} from vacuolar membrane vesicles of oat roots. J Biol Chem **262:** 3944–3946

Sillén LG, Martell AE (1971) Stability Constants, Supplement No 1, Special Publication 25. The Chemical Society, Burlington House, London, pp 733–735

Smallwood MF, Gurr SJ, McPherson MJ, Roberts K, Bowles DJ (1992) The pattern of plant annexin gene expression. Biochem J **281:** 501–505

Smallwood M, Keen KN, Bowles DJ (1990) Purification and partial sequence analysis of plant annexins. Biochem J **270:** 157–161

Sze H (1984) H^+-translocating ATPases of the plasma membrane and tonoplast of plant cells. Physiol Plant **61:** 683–691

Taiz L (1992) The plant vacuole. J Exp Biol **172:** 113–122

Wiebe HH (1978) The significance of plant vacuoles. Bioscience **28:** 327–331

Williamson RE (1981) Free Ca^{2+} concentration in the cytoplasm: a regulator of plant cell function. What's New in Plant Physiology **12:** 45–48

Wink M (1993) The plant vacuole: a multifunctional compartment. J Exp Bot Suppl **44:** 231–246