

# Mobilization of $\text{Ca}^{2+}$ by Cyclic ADP-Ribose from the Endoplasmic Reticulum of Cauliflower Florets<sup>1</sup>

Lorella Navazio<sup>2</sup>, Paola Mariani, and Dale Sanders\*

The Plant Laboratory, Department of Biology, University of York, P.O. Box 373, York YO10 5YW, United Kingdom (L.N., D.S.); and Dipartimento di Biologia, Università di Padova, Via U. Bassi 58/B, 35131 Padova, Italy (P.M.)

The  $\text{NAD}^+$  metabolite cADP-Rib (cADPR) elevates cytosolic free  $\text{Ca}^{2+}$  in plants and thereby plays a central role in signal transduction pathways evoked by the drought and stress hormone abscisic acid. cADPR is known to mobilize  $\text{Ca}^{2+}$  from the large vacuole of mature cells. To determine whether additional sites for cADPR-gated  $\text{Ca}^{2+}$  release reside in plant cells, microsomes from cauliflower (*Brassica oleracea*) inflorescences were subfractionated on sucrose density gradients, and the distribution of cADPR-elicited  $\text{Ca}^{2+}$  release was monitored. cADPR-gated  $\text{Ca}^{2+}$  release was detected in the heavy-density fractions associated with rough endoplasmic reticulum (ER). cADPR-dependent  $\text{Ca}^{2+}$  release co-migrated with two ER markers, calnexin and antimycin A-insensitive NADH-cytochrome *c* reductase activity. To investigate the possibility that contaminating plasma membrane in the ER-rich fractions was responsible for the observed release, plasma membrane vesicles were purified by aqueous two-phase partitioning, everted with Brij-58, and loaded with  $\text{Ca}^{2+}$ : These vesicles failed to respond to cADPR.  $\text{Ca}^{2+}$  release evoked by cADPR at the ER was fully inhibited by ruthenium red and 8-NH<sub>2</sub>-cADPR, a specific antagonist of cADPR-gated  $\text{Ca}^{2+}$  release in animal cells. The presence of a  $\text{Ca}^{2+}$  release pathway activated by cADPR at higher plant ER reinforces the notion that, alongside the vacuole, the ER participates in  $\text{Ca}^{2+}$  signaling.

Multiple and variable environmental signals are sensed by plants and lead to coordination of their growth and development. Stimulus-response coupling for many of these signals is widely accepted to be mediated (at least in the early steps) by modulation of cytosolic free  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_c$ ; Sanders et al., 1999). Intracellular  $\text{Ca}^{2+}$  stores are integral components of  $\text{Ca}^{2+}$ -based signal transduction pathways, operating both as efficient sites for  $\text{Ca}^{2+}$  sequestration and as sources for rapid and localized release of the ion in response to a stimulus (Malhó et al., 1998; Sanders et al., 1999). The imposing presence of a large vacuole, containing millimolar levels of  $\text{Ca}^{2+}$  (Felle, 1988) and occupying up to 90% of the total volume in most mature cell types, has led to the view that the vacuole is quantitatively the most significant intracellular  $\text{Ca}^{2+}$  pool. Identification of several active  $\text{Ca}^{2+}$  transporters and  $\text{Ca}^{2+}$  release channels at the vacuolar membrane (Allen and Sanders, 1997; Evans and Williams, 1998) has further reinforced the appreciation of the vacuole as a major  $\text{Ca}^{2+}$  store with the potential to participate in signaling-related  $\text{Ca}^{2+}$  mobilization. Thus, the vacuolar membrane possesses release pathways for  $\text{Ca}^{2+}$  that are gated by voltage (Johannes et al., 1992; Allen and Sand-

ers, 1994a; Ward and Schroeder, 1994) by inositol 1,4,5-trisphosphate ( $\text{InsP}_3$ ; Alexandre et al., 1990; Allen and Sanders, 1994b) and by the  $\text{NAD}^+$  metabolite cADP-Rib (cADPR; Allen et al., 1995).

In the context of  $\text{Ca}^{2+}$  signaling in plants, limited attention has been paid to the endoplasmic reticulum (ER), despite the clear prominence of the ER in both  $\text{Ca}^{2+}$  homeostasis and signaling in animal cells (Pozzan et al., 1994). Nevertheless, in the last few years, the biochemical characterization and molecular cloning from plants of the ER luminal  $\text{Ca}^{2+}$  buffering protein calreticulin (Chen et al., 1994; Navazio et al., 1995) and of P-type  $\text{Ca}^{2+}$ -ATPase pumps located at ER membranes (Thomson et al., 1993; Liang et al., 1997; Harper et al., 1998; Hong et al., 1999), have confirmed the potential importance of the ER in cellular  $\text{Ca}^{2+}$  relations. The discovery of a voltage-gated  $\text{Ca}^{2+}$  channel that mediates  $\text{Ca}^{2+}$  release from the ER of tendrils of *Bryonia dioica* (Klüsener et al., 1995) has served to reinforce the possibility of a role for the ER in higher plant  $\text{Ca}^{2+}$  signaling. Furthermore, microinjection studies in pollen tubes have revealed the presence of a non-vacuolar  $\text{InsP}_3$ -releasable  $\text{Ca}^{2+}$  store that is possibly associated with the ER (Franklin-Tong et al., 1996), and membrane fractionation studies on cauliflower (*Brassica oleracea*) florets have led tentatively to similar conclusions (Muir and Sanders, 1997). An additional class of  $\text{Ca}^{2+}$  release pathway residing exclusively on the ER is activated by the NADP metabolite nicotinic acid adenine dinucleotide phosphate (NAADP; Navazio et al., 2000). The ER has also been hypothesized to be responsible, through  $\text{Ca}^{2+}$  release, for the generation of the Cha-

<sup>1</sup> This work was supported by the European Molecular Biology Organization (award of a long-term fellowship to L.N.), by the Ministero Università Ricerca Scientifica e Tecnologica (to P.M.), and by the Biotechnology and Biological Sciences Research Council (to D.S.).

<sup>2</sup> Present address: Dipartimento di Biologia, Università di Padova, Via U. Bassi 58/B, 35131 Padova, Italy.

\* Corresponding author: email ds10@york.ac.uk; fax 44-1904-434317.

rophyte action potential (Plieth et al., 1998) and of repetitive  $\text{Ca}^{2+}$  spikes in the freshwater green alga *Eremosphaera viridis* (Bauer et al., 1998). In this latter case, the pharmacology of  $\text{Ca}^{2+}$  release suggested the involvement of a ryanodine receptor-like  $\text{Ca}^{2+}$  channel, possibly activated by cADPR.

cADP-Rib mobilizes  $\text{Ca}^{2+}$  in a range of plant and animal cell-types (Lee, 1997). Firm evidence for a second messenger role for cADPR has been established through measurement of changes in cADPR concentration, which parallel those in  $[\text{Ca}^{2+}]_c$  (Guse et al., 1999). In plants, a physiological role for cADPR in signaling by the drought and stress hormone abscisic acid has been demonstrated (Wu et al., 1997; Leckie et al., 1998). cADPR-mediated induction of abscisic acid-responsive gene expression was shown to be exerted by means of mobilization of internal  $\text{Ca}^{2+}$  stores, although the cytological identity of the  $\text{Ca}^{2+}$  pools on which cADPR acts still remains to be fully defined (Wu et al., 1997).

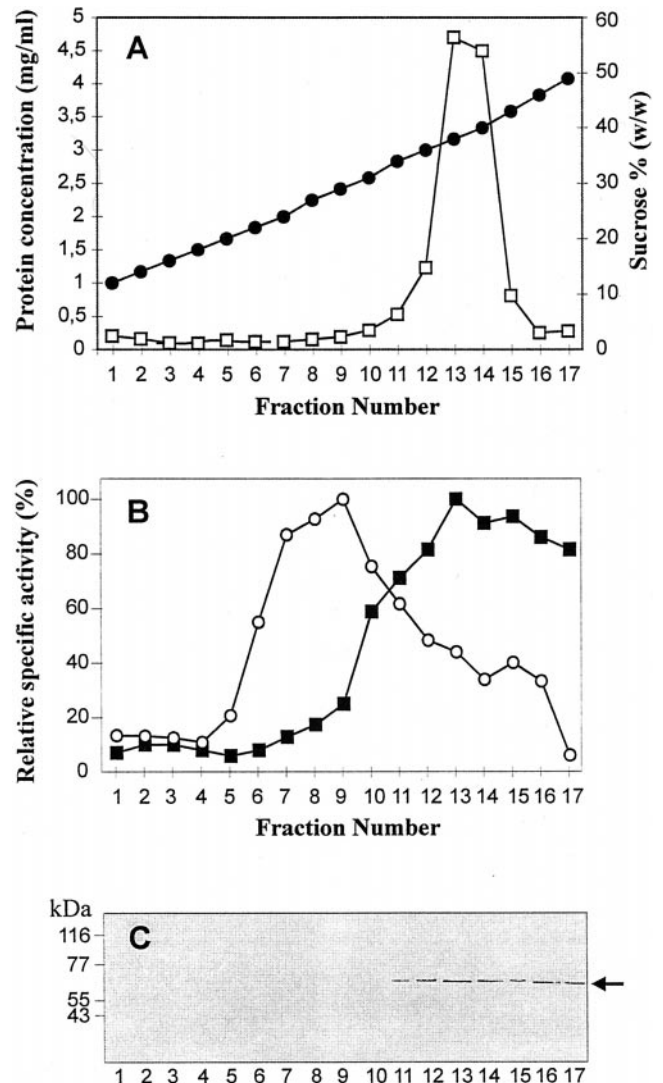
In this study we have investigated the localization of cADPR-sensitive  $\text{Ca}^{2+}$  stores in cauliflower inflorescences. This plant tissue represents an ideal system to investigate the nature of  $\text{Ca}^{2+}$  channels in the ER since the rapidly dividing cells of the inflorescence possess an extensive ER network but relatively small vacuoles (Muir and Sanders, 1997). By cell fractionation and subsequent  $\text{Ca}^{2+}$  transport analysis of the microsomal subfractions we show here that cADPR is effective at releasing  $\text{Ca}^{2+}$  not only from the vacuole but also from the ER membrane pool. The occurrence of agonist-mobilizable  $\text{Ca}^{2+}$  discharge from the ER suggests that the role of this compartment in origination of cytosolic  $\text{Ca}^{2+}$  signals in plant cell stimulus-response coupling is more crucial than has hitherto been envisaged.

## RESULTS

### Distribution of ER and Vacuolar Membrane Markers after Continuous Gradient Centrifugation of Cauliflower Microsomes

Figure 1A shows the distribution of total protein in the subfractions obtained by isopycnic centrifugation of cauliflower microsomes on a linear 10% to 45% (w/w) Suc density gradient made up over a 50% Suc cushion. To facilitate the separation of the ER from the vacuolar membrane vesicles, 3 mM  $\text{MgSO}_4$  was included in both homogenization medium and Suc solutions (Liang and Sze, 1998). Most of the protein was recovered in fractions 13 to 14 collected at high buoyant density (38%–40% [w/w] Suc), resembling the pattern previously reported by Muir and Sanders (1997) in the same experimental system, although we observed a narrower peak here.

The distribution of the ER marker enzyme antimycin A-insensitive NADH-Cyt *c* reductase among the microsomal subfractions is illustrated in Figure 1B. The presence of  $\text{Mg}^{2+}$  in all preparative media re-

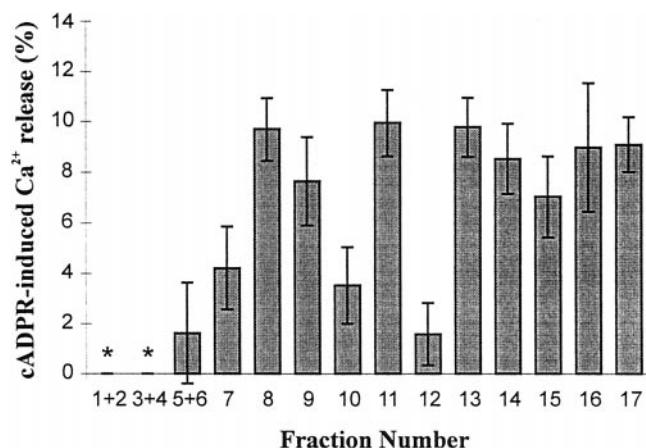


**Figure 1.** Subfractionation of cauliflower microsomes by Suc gradient centrifugation. A, Suc concentrations (●) determined by refractometry, and distribution of protein (□) among microsomal subfractions. B, Distribution of ER and vacuolar marker enzyme activities. ○, Bafilomycin  $\text{A}_1$ -sensitive  $\text{H}^+$ -ATPase (100% = 99.4 nmol  $\text{mg}^{-1}$   $\text{min}^{-1}$ ); ■, antimycin A-insensitive NADH-Cyt *c* reductase (100% = 59.5 nmol  $\text{mg}^{-1}$   $\text{min}^{-1}$ ). C, Western analysis of microsomal subfractions (10  $\mu\text{g}$  protein per lane) decorated with antibodies against calnexin (1:1,000 diluted). The arrow indicates 65 kDa cauliflower calnexin.

sulted in ER residing exclusively at densities higher than 30% (w/w) Suc, as anticipated for ER membrane vesicles bearing attached ribosomes (rough ER) (Robinson et al., 1994).

Vacuolar membranes, identified by bafilomycin-sensitive  $\text{H}^+$ -ATPase activity, peaked in the low-density region of the gradient (Fig. 1B, fractions 7–9, 24%–29% [w/w] Suc), in agreement with previous results obtained by Askerlund (1997) in cauliflower.

Antibodies against ER marker proteins were also used to study the distribution of ER membranes after continuous Suc gradient centrifugation. Figure 1C



**Figure 2.** Distribution of cADPR-induced Ca<sup>2+</sup> release among cauliflower microsomal subfractions. Vesicles from each fraction were preloaded with Ca<sup>2+</sup> and potential further uptake following Ca<sup>2+</sup> release subsequently inhibited as detailed in "Materials and Methods." An aliquot was removed for radioactivity counting and cADPR (1  $\mu$ M) was then added. Three aliquots were removed successively over the ensuing 2 min to measure changes in accumulated Ca<sup>2+</sup>. Results are the means  $\pm$  SE of three to six replicates from two different membrane preparations. Stars indicate non-detectable uptake.

shows western blots of cauliflower microsomal subfractions decorated with antibodies against the integral ER membrane protein calnexin against the Arabidopsis (Huang et al., 1993). A polypeptide of around 65 kD was detected in agreement with the  $M_r$  of plant calnexin. The presence of calnexin only in the heavier Suc fractions (fractions 11–17) gives further support to the results obtained by enzyme assay, confirming the distribution of ER membranes in this region of the gradient.

Antibodies against the intraluminal ER Ca<sup>2+</sup>-binding proteins calreticulin (Navazio et al., 1995) and BiP (Denecke et al., 1991) were also used, but both of these reticuloplasmic proteins were found to be spread throughout the gradient (data not shown). This wide distribution is likely to be due to the release of soluble proteins from the ER lumen during homogenization.

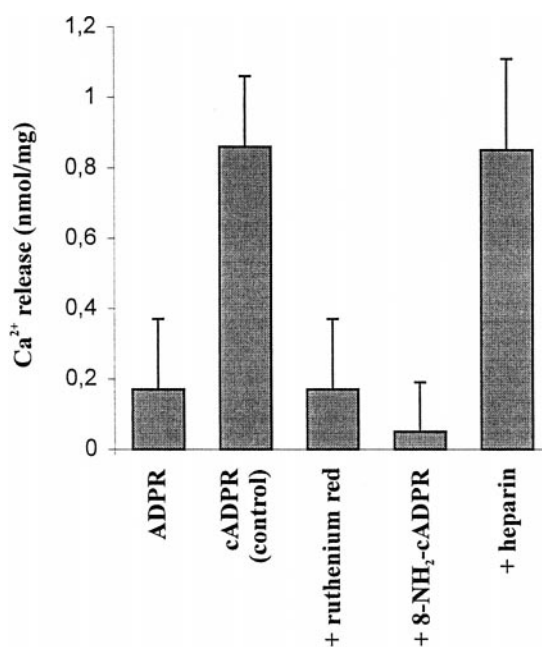
#### Distribution of cADPR-Dependent Ca<sup>2+</sup> Release among Microsomal Subfractions

Membrane vesicles separated on linear Suc gradients were subsequently used for Ca<sup>2+</sup> transport studies. With the exception of fractions 1 to 4, collected at 12% to 18% (w/w) Suc, all other fractions exhibited ATP-dependent Ca<sup>2+</sup> accumulation (data not shown). The transport-incompetence of the lightest fractions is likely to be due to the non-membranous origin of these samples, containing only soluble proteins released during cell disruption and fractionation. Vesicles from each fraction were incubated for 60 min in the uptake medium to obtain steady-state Ca<sup>2+</sup> loading and then analyzed with respect to their ability to release Ca<sup>2+</sup> in response to cADPR. Figure 2 shows

that 1  $\mu$ M cADPR released Ca<sup>2+</sup> from two populations of membrane vesicles in cauliflower. The first peak of cADPR-sensitive membranes was found at 27% to 29% (w/w) Suc (fractions 8 and 9), coinciding with the peak of vacuolar membrane marker enzyme activity. The second population of vesicles responsive to cADPR was located at 34% to 49% (w/w) Suc (fractions 11–17), where most of the ER marker enzyme activity is recovered. It has to be noted that membrane vesicles from fraction 12, collected at 36% (w/w) Suc, although exhibiting a fairly high ER marker enzyme activity could not be discharged by addition of cADPR. Failure of fraction 12 to release Ca<sup>2+</sup> in response to this agonist was apparent in two different membrane preparations, suggesting the occurrence of ER subcompartments where the density of the receptor is too low to be detected. However, given the low signal-to-noise ratio, we cannot be confident that fraction 12 is a cADPR-insensitive fraction.

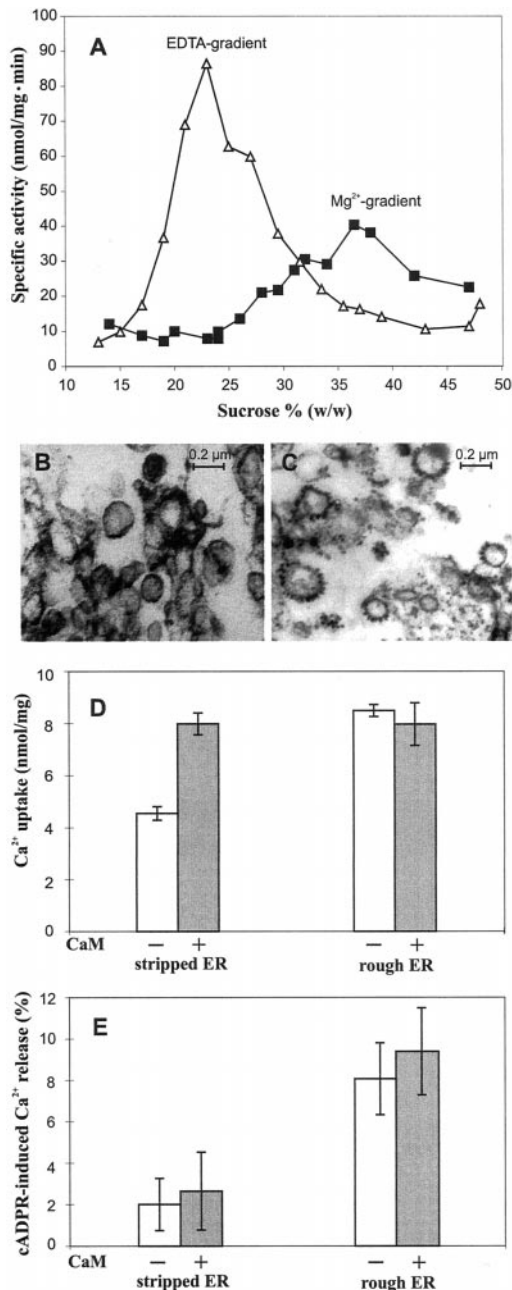
The magnitude of Ca<sup>2+</sup> release was found to be similar in both vacuolar and rough ER membrane populations, ranging from 8% to 10% of the total accumulated A23187-sensitive Ca<sup>2+</sup> (Fig. 2).

The cADPR-elicited Ca<sup>2+</sup> release properties of fraction 13, collected in the middle of the ER marker enzyme activity (38% [w/w] Suc) were further investigated. Figure 3 shows that when 1  $\mu$ M ADPR (the hydrolytic product of cADPR) was admi-



**Figure 3.** Specificity and inhibitor sensitivity of cADPR-induced Ca<sup>2+</sup> release from rough ER-enriched fractions. Cauliflower membrane vesicles collected at 38% (w/w) Suc were allowed to accumulate Ca<sup>2+</sup> for 60 min before addition of either 1  $\mu$ M ADPR (first bar) or 1  $\mu$ M cADPR (remaining bars). Potential inhibitors of Ca<sup>2+</sup> release (30  $\mu$ M ruthenium red, 2.5  $\mu$ M 8-NH<sub>2</sub>-cADPR, and 10  $\mu$ M heparin [ $M_r$  = 4k–6k]) were added 1 min prior to the addition of cADPR. Results are the means  $\pm$  SE of three experiments.





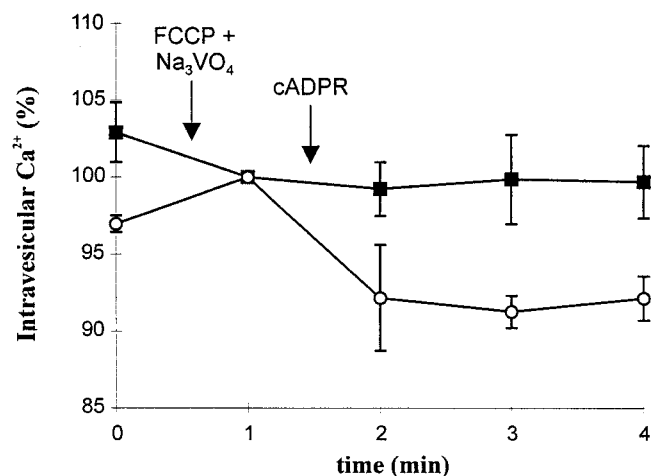
**Figure 4.** Comparison of the  $\text{Ca}^{2+}$  transport properties of cauliflower ER membranes before and after ribosome stripping. A, Distribution of antimycin A-insensitive NADH-Cyt *c* reductase activity among cauliflower microsomal subfractions separated on two subsequent Suc gradients, the first containing 3 mM  $\text{MgSO}_4$  (■), the second 3 mM EDTA (△). Fractions comprising the 37% to 38% (w/w) Suc zone of the  $\text{Mg}^{2+}$ -containing gradient were pooled, treated with EDTA, and recentrifuged isopycnicly into an EDTA-containing gradient. B and C, Electron micrographs of the Suc fractions collected at the peak of NADH-Cyt *c* activity on the EDTA-containing gradient (ribosome-stripped ER) and the  $\text{Mg}^{2+}$ -containing gradient (rough ER), respectively. D and E,  $\text{Ca}^{2+}$  uptake and  $\text{Ca}^{2+}$  release induced by 1  $\mu\text{M}$  cADPR from the same samples analyzed by electron microscopy. Vesicles were incubated for 60 min in  $\text{Ca}^{2+}$  transport medium in the absence (white bars) or presence (gray bars) of spinach calmodulin (6  $\mu\text{g mL}^{-1}$ ; 150 units  $\text{mL}^{-1}$ ). Results are the means  $\pm$  SE of three experiments.

nistrated to the  $\text{Ca}^{2+}$ -loaded vesicles instead of 1  $\mu\text{M}$  cADPR, no  $\text{Ca}^{2+}$  release was detected, demonstrating specificity of release for the cyclic isomer only. A higher concentration of ADPR (10  $\mu\text{M}$ ) was also ineffective in releasing  $\text{Ca}^{2+}$  from a microsomal preparation (data not shown). The cADPR-induced  $\text{Ca}^{2+}$  release was found to be effectively blocked by ruthenium red (83% inhibition at 30  $\mu\text{M}$ , Fig. 3), an antagonist of the cADPR-dependent  $\text{Ca}^{2+}$  release mechanism in both animal (Galione et al., 1991) and plant vacuolar membranes (Allen et al., 1995). However, since ruthenium red has also been shown to inhibit other classes (non-ligand gated) of plant  $\text{Ca}^{2+}$  channel (Marshall et al., 1994), we tested the effect of a more specific antagonist of cADPR action: 8-NH<sub>2</sub>-cADPR (Walseth et al., 1993). This 8-substituted analog of cADPR was found to block  $\text{Ca}^{2+}$  release by cADPR almost completely (92% inhibition at 2.5  $\mu\text{M}$ , Fig. 3). Heparin, a specific inhibitor of the  $\text{InsP}_3$ -mediated pathway in cauliflower microsomes (Muir et al., 1997), was found to be completely ineffective at blocking the  $\text{Ca}^{2+}$  mobilizing action of cADPR on the same ER-enriched membrane vesicles (Fig. 3).

#### $\text{Ca}^{2+}$ Transport Properties of Ribosome-Denuded ER Membrane Vesicles

In an attempt to purify further the ER membranes responsive to cADPR, the  $\text{Mg}^{2+}$ -containing Suc gradient fractions corresponding to the peak activity of antimycin-insensitive NADH-Cyt *c* reductase (37%–38% [w/w] Suc; Fig. 4A) were treated with EDTA to dissociate ribosomes from the ER. The membranes were then recentrifuged isopycnicly onto a second Suc gradient containing EDTA. As a result of stripping, the ribosome-denuded ER membranes shifted to a region of the gradient free of contaminating membranes (21%–27% [w/w] Suc), causing a 2-fold increase in the specific activity of NADH-Cyt *c* reductase (Fig. 4A). This attests to the good degree of purity of ER membranes obtained by this procedure. Electron microscopy of the membrane material collected at the peak of the ER marker enzyme activity before and after the density-shift further supported the biochemical evidence for the removal of ribosomes after EDTA treatment. ER vesicles collected from the  $\text{Mg}^{2+}$ -containing gradient consisted of vesicles heavily studded with ribosomes (Fig. 4C), whereas those collected from the EDTA-containing gradient showed mainly smooth vesicles, suggestive of stripped ER (Fig. 4B).

In  $^{45}\text{Ca}^{2+}$  uptake experiments, the stripped ER membranes exhibited a reduced ability to accumulate  $\text{Ca}^{2+}$  in comparison with the rough ER membranes from which they have derived. The level of  $\text{Ca}^{2+}$  uptake was found to drop from 8.5 to 4.5 nmol  $\text{mg}^{-1}$  (Fig. 4D). However,  $\text{Ca}^{2+}$  uptake by artificially smooth ER vesicles could be restored to levels comparable with those of rough ER vesicles by addition



**Figure 5.** cADPR-sensitivity of the upper and lower phases obtained by aqueous two phase partitioning of cauliflower microsomes. Loading of Ca<sup>2+</sup> into vesicles from the upper (■) and lower (○) phases was terminated by addition of FCCCP (10 μM) and Na<sub>3</sub>VO<sub>4</sub> (200 μM). Curves are standardized to this point (100% Ca<sup>2+</sup> accumulation = 31.9 ± 1.2 ■) and 14.4 ± 0.9 nmol mg<sup>-1</sup> [○]). cADPR (1 μM) was subsequently added and three aliquots removed to estimate Ca<sup>2+</sup> remaining in the vesicles by measuring radioactivity content. Data are the means ± SE of three experiments.

of 6 μg mL<sup>-1</sup> spinach calmodulin. Inclusion in the Ca<sup>2+</sup> transport medium of the same dose of exogenous calmodulin did not have any significant effect on the Ca<sup>2+</sup> accumulation capacity of the rough ER-derived vesicles (Fig. 4C). These results suggest that endogenous calmodulin, which is very tightly associated with plant Ca<sup>2+</sup> pumps *in vivo* (Evans and Williams, 1998) and still present in membrane fractions separated on Mg<sup>2+</sup>-containing gradients, is at least partially lost after continuous gradient centrifugation in the presence of EDTA. In summary, substantial enrichment of these fractions by ER is suggested by the visualization of ribosomes on the vast majority of vesicles, by the strong EDTA-dependent density shift of marker enzyme activity, and by the presence of both calmodulin-independent and -dependent components to Ca<sup>2+</sup> uptake (compare with Hong et al., 1999; Hwang et al., 2000).

It is surprising that the ribosome-denuded ER membrane vesicles were found not to release Ca<sup>2+</sup> in response to cADPR: When fractions corresponding to the NADH-Cyt *c* reductase peak on the EDTA-containing gradient were pooled and challenged with 1 μM cADPR, no significant cADPR-dependent Ca<sup>2+</sup> release was observed (Fig. 4E). On the other hand, no cADPR-elicited Ca<sup>2+</sup> release was detected in any other region of the gradient (data not shown), ruling out the obvious possibility that the responsiveness to cADPR observed in the high-density zone of the Mg<sup>2+</sup>-containing gradient could be due to contaminating vesicles of different membrane origin. Inclusion of calmodulin in the Ca<sup>2+</sup> transport assay was not sufficient to re-establish the cADPR-elicited

Ca<sup>2+</sup> release previously observed in the rough ER preparation (Fig. 4E).

Treatment with 15 mM pyrophosphate, an alternative Mg<sup>2+</sup> chelator that detaches ribosomes from rough ER fractions as effectively as EDTA (Amar-Costesec et al., 1974) resulted in the same loss of sensitivity of the uncoated ER vesicles to the Ca<sup>2+</sup>-mobilizing effect of cADPR (data not shown).

#### The Ca<sup>2+</sup>-Mobilizing Action of cADPR Is Exerted on Internal Ca<sup>2+</sup> Stores Only

Plant cell plasma membranes exhibit equilibrium densities (30%–40%) on Suc gradients that overlap significantly with those of rough ER membranes (Robinson et al., 1994). To investigate whether the cADPR-responsiveness of the high density Suc fractions and in particular that of fraction 11, collected at 34% (w/w) Suc (Fig. 2), could be due to contamination of rough ER with plasma membrane vesicles, cauliflower microsomes were separated by aqueous two phase partitioning and the resulting upper and lower phases analyzed with the <sup>45</sup>Ca<sup>2+</sup> filtration assay. Plasma membrane fractions obtained by phase partitioning are superior in terms of purity to those obtained by Suc density centrifugation (Robinson et al., 1994). This was confirmed by a glucan synthase II enzyme assay, which demonstrated a higher specific activity of this plasma membrane marker in the upper phase obtained by two phase partitioning than in any of the Suc gradient fractions (Table I).

Vesicles from the upper phase were treated with Brij 58 to produce a uniform population of cytosol-side-out plasma membrane vesicles (Johansson et al., 1995) and were then Ca<sup>2+</sup>-loaded. Challenge with saturating doses (1 μM) of cADPR failed to evoke Ca<sup>2+</sup> release (Fig. 5). By contrast, administration of the same dose of cADPR resulted in significant Ca<sup>2+</sup> release from the lower phase (Fig. 5), which contains the bulk of endomembranes. These data demonstrate that Ca<sup>2+</sup> influx across the plasma membrane is not involved in cADPR-elicited Ca<sup>2+</sup> release in cauliflower.

#### DISCUSSION

The ability of cADPR to mobilize intracellular Ca<sup>2+</sup> from storage pools has long been known in animal cells (Clapper et al., 1987; Lee et al., 1989) and has more recently been documented in plant cells (Allen

**Table I.** Analysis of glucan synthase II activity in plasma membrane-enriched fractions obtained by two-phase partitioning and Suc density centrifugation

Membrane Fraction	Specific Activity nmol mg <sup>-1</sup> min <sup>-1</sup>	Enrichment
Microsomes	242.5	1
Upper phase	760.6	3.1
Gradient fraction (34% [w/w] Suc)	288.5	1.2

et al., 1995; Leckie et al., 1998). Plant  $\text{Ca}^{2+}$  release channels gated open by cADPR have been reported at the vacuolar membrane of plants (Allen et al., 1995; Leckie et al., 1998), where their biochemical and pharmacological properties are very similar to those of animal ryanodine receptors (Muir and Sanders, 1996; Muir et al., 1997). However, the occurrence of a cADPR-gated  $\text{Ca}^{2+}$  release pathway at the vacuolar membrane does not preclude, in principle, the possibility that channels belonging to the same class could also be located at plant cell compartments other than the vacuole.

In this study we have investigated in more detail the distribution of plant cADPR-sensitive  $\text{Ca}^{2+}$  stores, using membrane vesicles prepared from inflorescences of cauliflower. We show here that, in addition to the vacuole, the ER can be regarded as a bona fide cADPR-sensitive intracellular  $\text{Ca}^{2+}$  store, at least in cauliflower. cADPR-elicited  $\text{Ca}^{2+}$  release at the ER membrane was found to share the same pharmacological features as the cADPR-gated mechanism located at the vacuolar membrane (Muir and Sanders, 1996; Leckie et al., 1998), i.e. insensitivity to the  $\text{InsP}_3$ -receptor antagonist heparin and effective inhibition by the cADPR-receptor blockers ruthenium red and 8- $\text{NH}_2$ -cADPR. Moreover, the release pathway at the ER is selectively activated by cADPR, ADPR being ineffective in triggering  $\text{Ca}^{2+}$  release.

The magnitude of the  $\text{Ca}^{2+}$  release induced by cADPR did not exceed 10% of the total  $\text{Ca}^{2+}$  accumulated by vesicles from both intracellular membrane locations (ER and vacuole), suggesting that in cauliflower the cADPR-gated channels are present at a lower density than in red beet vesicles (Allen et al., 1995). This could be attributed to species-specific variations (Muir et al., 1997) or, alternatively, to a level of  $\text{Ca}^{2+}$  channel expression that depends on the state of cell differentiation (Gollasch et al., 1998).

The use of high  $\text{Mg}^{2+}$  conditions for the isolation and separation of membrane vesicles on Suc gradients will have reduced to a minimum the risk of contamination of rough ER by vacuolar membranes. Indeed, bafilomycin-sensitive  $\text{H}^+$ -ATPase (vacuolar marker) and antimycin-insensitive NADH-Cyt *c* reductase (ER marker) showed good separation between the two membrane populations. The ER location of cADPR-sensitive  $\text{Ca}^{2+}$  release detected in the high density region of the gradients was further confirmed by immunoblot analysis with antibodies against the ER integral membrane protein calnexin. Antibodies against the ER luminal reticuloplasmic calreticulin and BiP were also used in this study, but the leakage of soluble proteins from the ER lumen during homogenization and the possibility of their subsequent trapping in the forming vesicles, even of different origin, warn against the use of non-membrane-anchored proteins as reliable ER markers in cell fractionation studies.

The possibility that cADPR-induced  $\text{Ca}^{2+}$  mobilization in the ER-containing fraction of the Suc gradient was actually mediated by contaminating plasma membrane vesicles was tested with highly purified plasma membrane vesicles obtained by two-phase partitioning of cauliflower microsomes. Failure of inside-out (cytosol-side-out) plasma membrane vesicles to release the accumulated  $\text{Ca}^{2+}$  in response to cADPR indicates that the plasma membrane is not involved in cADPR-dependent  $\text{Ca}^{2+}$  release, in contrast to the two-phase fraction that was enriched in endomembranes. A similar conclusion has emerged from studies on sea urchin eggs, which established that external  $\text{Ca}^{2+}$  was not required for the increase of cytosolic  $\text{Ca}^{2+}$  generated by cADPR (Lee et al., 1994).

Further purification of ER membranes on Suc gradients in the presence of EDTA or pyrophosphate was found to affect the functional integrity of the cADPR-gated  $\text{Ca}^{2+}$  channels located on these membranes. The "artificially smooth" ER vesicles produced in this way lost their ability to be discharged by cADPR and were severely impaired in their  $\text{Ca}^{2+}$  accumulation properties as well. The effect of both of these  $\text{Mg}^{2+}$ -chelating agents is suspected not to be restricted to the detachment of ribosomes from ER membranes but to involve the removal of some soluble proteins adsorbed on microsomes (such as calmodulin; see "Results") and peripheral membrane proteins as well (Amar-Costesec et al., 1974; Fujiki et al., 1982). Photoaffinity-labeling experiments of cADPR-binding sites in sea urchin egg microsomes have led to the proposal that cADPR does not interact directly with the channel itself but may rather exert its effect through intermediate proteins of 140 and 100 kD (Walseth et al., 1993). We can speculate that these, or some other accessory proteins, required for functional cADPR-gated  $\text{Ca}^{2+}$  channels, are lost during the EDTA/pyrophosphate-dependent density shift of rough ER membranes.

Our finding that higher plant ER is mobilized by the endogenous activator of ryanodine receptors, cADPR, reinforces and extends the recent results of Bauer et al. (1998), who postulated the existence of ryanodine-like receptors at an algal ER on the basis of the inhibitory effect of ruthenium red and ryanodine on  $\text{Ca}^{2+}$ -spiking in these cells.

The cADPR-dependent mechanism of  $\text{Ca}^{2+}$  mobilization from the ER appears to be widespread and well conserved throughout evolution, being present with the same characteristics in species that are phylogenetically very distant, including plant, invertebrate, and mammalian cells. Furthermore, the signaling role of this molecule seems to have an ancient origin, the early branching photosynthetic protist *Euglena gracilis* already possessing the cADPR-release pathway (Masuda et al., 1997). Since the same organism has also been demonstrated to express the ER  $\text{Ca}^{2+}$  buffering protein calreticulin (Navazio et al.,



1998), it seems that both these components of the internal Ca<sup>2+</sup> homeostat appeared very early during eukaryote evolution.

At least two classes of Ca<sup>2+</sup>-permeable channel are present at plant ER membranes, i.e. voltage-gated (Klüsener et al., 1995) and ligand-gated by NAADP (Navazio et al., 2000). Our present findings demonstrate the presence of an additional class of ligand-gated channel at the ER. In animals, NAADP and cADPR are regarded a sibling messengers, both being produced by a single enzyme from different pyridine nucleotide substrates (Lee, 1999).

The presence of a variety of Ca<sup>2+</sup> channel types on different plant membranes might give clues to a long-standing problem in Ca<sup>2+</sup> signaling, which is how (given the wide array of stimulus-response pathways in which Ca<sup>2+</sup> is involved) stimulus specificity can be encoded. The specificity of the Ca<sup>2+</sup> signal (the so-called "Ca<sup>2+</sup> signature"; Webb et al., 1996) is likely to rely on the identity of the particular intracellular Ca<sup>2+</sup> pool from which Ca<sup>2+</sup> release is triggered, thereby encoding spatial information, as well as on the dynamic properties of the channels through which release occurs (McAinsh and Hetherington, 1998). Further work will be required to establish whether cADPR and NAADP are differentially used in alternative signaling pathways, or whether they participate jointly in individual Ca<sup>2+</sup> signaling events.

## MATERIALS AND METHODS

### Preparation of Cauliflower Microsomal Subfractions

Cauliflowers (*Brassica oleracea*) were purchased from a local market, and the outermost part (top 0.5 cm) of inflorescences was used. A crude microsomal fraction was isolated as previously described (Muir and Sanders, 1997) except that EDTA was replaced by 3 mM MgSO<sub>4</sub> in all buffers and NaCl was omitted from the buffer used to wash the microsomal pellet. The final pellet was resuspended in gradient basal medium A {25 mM Tris-MES [2-(*N*-morpholino)ethanesulfonic acid], pH 7.5, 3 mM MgSO<sub>4</sub>, 2 μg mL<sup>-1</sup> leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride} containing 8% (w/w) Suc, at a protein concentration of 10 to 15 mg mL<sup>-1</sup>.

Subfractionation of crude microsomes (2 mL) was carried out on a linear 10% to 45% (w/w) Suc gradient (32 mL) over a 50% (w/w) Suc cushion (2 mL). This was centrifuged at 100,000g for 3 h and fractions (2 mL) collected from the top of the centrifuge tube. Centrifugations for 6 and 16 h showed the same distribution of protein and marker enzymes as for 3 h, so the gradient is known to be isopycnic at that time.

Stripping of ribosomes was done by pooling fractions corresponding to the peak of rough ER membranes from six Mg<sup>2+</sup>-gradients prepared as above and diluting with twice their volume of gradient basal medium B (25 mM Tris-MES, pH 7.5, 3 mM EDTA, 2 μg mL<sup>-1</sup> leupeptin, 0.5 mM phenylmethylsulfonyl fluoride). After centrifugation at

150,000g for 1 h, the pellet was resuspended in 2 mL of the same buffer containing 8% (w/w) Suc and layered over a second Suc gradient identical to the first one but made up in gradient medium B. The EDTA-containing gradient was centrifuged at 100,000g for 3 h and fractions collected as above. All operations were performed at 4°C. Suc concentration was measured with a refractometer.

Suc fractions were either used directly for marker enzyme assays and immunoblot analysis, or diluted 3-fold, pelleted (at 100,000g for 1 h), and resuspended in 400 mM glycerol, 5 mM bis-tris propane (BTP)-MES, pH 7.4, 25 mM KCl, 3 mM MgSO<sub>4</sub>, 0.3 mM NaN<sub>3</sub> for Ca<sup>2+</sup> transport assays. Samples were frozen in liquid nitrogen and stored at -80°C until use.

Aqueous two-phase partitioned plasma membrane was prepared from cauliflower microsomes exactly as described by Thomson et al. (1993), using 6.5% (w/w) Dextran T500 and 6.5% (w/w) polyethyleneglycol 3350, and performing three successive steps of partitioning. For Ca<sup>2+</sup> transport assays, upper phase membrane vesicles were treated with 0.05% (w/v) Brij 58 according to Johansson et al. (1995).

Protein concentration was determined using an assay kit (Bio-Rad Laboratories, Hercules, CA) based on the method of Bradford (1976); bovine serum albumin was used as a standard.

### Marker Enzyme Assays

Antimycin A-insensitive NADH-Cyt *c* reductase (ER marker) activity was measured as described by Hodges and Leonard (1974). Bafilomycin A<sub>1</sub>-sensitive H<sup>+</sup>-ATPase (vacuolar marker) determinations were carried out according to Muir and Sanders (1997). Glucan synthase II (plasma membrane marker) was determined as described previously (Navazio et al., 2000).

Marker enzyme analyses were carried out on at least two different membrane preparations and the assays were conducted in duplicates. The results shown are from representative experiments.

### SDS-PAGE and Immunoblot Analysis

SDS-slab gel electrophoresis was carried out according to Laemmli (1970), using 12% (w/v) polyacrylamide minigels. For immunoblot analysis, proteins were electrophoretically transferred onto nitrocellulose membranes and blots were incubated with antibodies against the following: (a) Arabidopsis calnexin (Huang et al., 1993; a gift from N.E. Hoffman, Stanford, CA), (b) spinach calreticulin (Navazio et al., 1995), and (c) tobacco binding protein (BiP; Denecke et al., 1991; a gift from J. Denecke, Leeds, UK). Antibody binding was detected with alkaline phosphatase-conjugated anti-rabbit secondary antibodies (Boehringer Mannheim, Basel) and color development by reaction with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium tablets (Sigma, St. Louis).

## Ca<sup>2+</sup> Transport Assay

Ca<sup>2+</sup> transport by membrane vesicles was measured by a <sup>45</sup>Ca<sup>2+</sup> filtration method (Muir and Sanders, 1996). The assay was carried out at room temperature in 500 μL of a medium containing 400 mM glycerol, 5 mM BTP-MES, pH 7.4, 25 mM KCl, 3 mM MgSO<sub>4</sub>, 0.3 mM NaN<sub>3</sub>, 3 mM ATP-BTP, 50 μg of protein, 10 μM CaCl<sub>2</sub>, together with 5.92 kBq <sup>45</sup>Ca<sup>2+</sup> (Amersham, Buckinghamshire, UK, original specific activity 63 GBq mmol<sup>-1</sup>). Vesicles were incubated in the Ca<sup>2+</sup> transport medium for 60 min to allow steady-state intravesicular levels of Ca<sup>2+</sup> to be obtained. Potential re-loading following Ca<sup>2+</sup> release was abolished by addition of 10 μM carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP) and 200 μM Na<sub>3</sub>VO<sub>4</sub>, inhibitors of Ca<sup>2+</sup>/H<sup>+</sup> antiport and Ca<sup>2+</sup>-pumping ATPases, respectively. Subsequently, 1 μM cADPR (Molecular Probes, Eugene, OR) was added. Potential inhibitors of Ca<sup>2+</sup> release (ruthenium red, 8-NH<sub>2</sub>-cADPR, heparin) were added 1 min prior to the addition of cADPR. Finally, administration of the Ca<sup>2+</sup> ionophore A23187 (10 μM) was used to collapse the remaining Ca<sup>2+</sup> gradient. No additions contained more than 1% of the total assay volume. At defined time intervals, 50-μL aliquots were removed, filtered on pretreated nitrocellulose filters (0.45-μm pore size, type WCN, Whatman, Clifton, NJ), and rapidly washed once with 5 mL of ice-cold wash medium (400 mM glycerol, 5 mM BTP-MES, pH 7.4, 0.2 mM CaCl<sub>2</sub>), using a filtration unit under vacuum (Amicon, Beverly, MA). Intravesicular content of <sup>45</sup>Ca<sup>2+</sup> was determined by liquid scintillation counting. Radioactivity remaining on the filters after the addition of A23187 is defined as nonaccumulated Ca<sup>2+</sup> and was subtracted from all the data points; typically, this correction amounted to approximately 25% of the overall maximum Ca<sup>2+</sup> accumulation.

Cauliflower membrane vesicles do not show any significant "leak" of Ca<sup>2+</sup> in the absence of effectors over the time course of the release experiment (6 min).

## Electron Microscopy

Membrane samples were fixed in 1% (w/v) cacodylate-buffered glutaraldehyde and post-fixed in 1% (w/v) OsO<sub>4</sub> in 0.1 M cacodylate buffer. After dehydration in an ethanol series, samples were embedded in Araldite resin. After lead citrate staining, thin sections were observed with a 300 electron microscope (Hitachi, Tokyo) operating at 75 kV.

## ACKNOWLEDGMENTS

We thank Tim Walseth for the kind gift of 8-NH<sub>2</sub>-cADPR, and Neil E. Hoffman and Jurgen Denecke for the anti-calnexin and anti-BiP antibodies, respectively. We are grateful to Michael Bewell, Lorraine Williams, and Barbara Baldan for helpful advice concerning vesicular <sup>45</sup>Ca<sup>2+</sup> flux assay, phase partitioning, and electron microscopy, respectively.

Received November 28, 2000; accepted January 10, 2001.

## LITERATURE CITED

- Alexandre J, Lassalles JP, Kado RT (1990) Opening of Ca<sup>2+</sup> channels in isolated red beet root vacuole membrane by inositol 1,4,5-trisphosphate. *Nature* **343**: 567–570
- Allen GJ, Muir SR, Sanders D (1995) Release of Ca<sup>2+</sup> from individual plant vacuoles by both InsP<sub>3</sub> and cyclic ADP-ribose. *Science* **268**: 735–737
- Allen GJ, Sanders D (1994a) Two voltage-gated calcium release channels co-reside in the vacuolar membrane of broad bean guard cells. *Plant Cell* **6**: 685–694
- Allen GJ, Sanders D (1994b) Osmotic stress enhances the competence of *Beta vulgaris* vacuoles to respond to inositol 1,4,5-trisphosphate. *Plant J* **6**: 687–695
- Allen GJ, Sanders D (1997) Vacuolar ion channels of higher plants. *Adv Bot Res* **25**: 217–252
- Amar-Costesec A, Wibó M, Thinès-Sempoux D, Beaufay H, Berthet J (1974) Analytical study of microsomes and isolated subcellular membranes from rat liver. *J Cell Biol* **62**: 717–745
- Askerlund P (1997) Calmodulin-stimulated Ca<sup>2+</sup>-ATPases in the vacuolar and plasma membranes in cauliflower. *Plant Physiol* **114**: 999–1007
- Bauer CS, Plieth C, Bethmann B, Popescu O, Hansen UP, Simonis W, Schönknecht G (1998) Strontium-induced repetitive calcium spikes in a unicellular green alga. *Plant Physiol* **117**: 545–557
- 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
- Chen F, Hayes PM, Mulrooney DM, Pan A (1994) Identification and characterization of cDNA clones encoding plant calreticulin in barley. *Plant Cell* **6**: 835–843
- Clapper DL, Walseth TF, Dargie PJ, Lee HC (1987) Pyridine-nucleotide metabolites stimulate calcium release from sea urchin egg microsomes desensitized to inositol trisphosphate. *J Biol Chem* **262**: 9561–9568
- Denecke J, Goldman MHS, Demolder J, Seurinck J, Boterman J (1991) The tobacco luminal binding protein is encoded by a multigene family. *Plant Cell* **3**: 1025–1035
- Evans DE, Williams LE (1998) P-type calcium ATPases in higher plants: biochemical, molecular and functional properties. *Biochim Biophys Acta* **1376**: 1–25
- Felle H (1988) Auxin causes oscillations of cytosolic free calcium and pH in *Zea mays* coleoptiles. *Planta* **176**: 248–255
- Franklin-Tong VE, Drobak BK, Allan AC, Watkins PAC, Trewavas AJ (1996) Growth of pollen tubes of *Papaver rhoeas* is regulated by a slow-moving calcium wave propagated by inositol 1,4,5-trisphosphate. *Plant Cell* **8**: 1305–1321
- Fujiki Y, Hubbard AL, Fowler S, Lazarow PB (1982) Isolation of intracellular membranes by means of sodium-carbonate treatment: application to endoplasmic reticulum. *J Cell Biol* **93**: 97–102
- Galione A, Lee HC, Busa WB (1991) Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release in sea urchin egg homogenates: modulation by cyclic ADP-ribose. *Science* **253**: 1143–1146



- Gollasch M, Haase H, Ried C, Lindschau C, Morano I, Luft FC, Haller H** (1998) L-Type calcium channel expression depends on the differentiated state of vascular smooth muscle cells. *FASEB J* **12**: 593–601
- Guse AH, daSilva CP, Berg I, Skapenko AL, Weber K, Heyer P, Hohenegger M, Ashamu GA, Schulze-Koops H, Potter BVL et al.** (1999) Regulation of calcium signaling in T lymphocytes by the second messenger cyclic ADP-ribose. *Nature* **398**: 70–73
- Harper JF, Hong B, Hwang I, Guo HQ, Stoddard R, Huang JF, Palmgren MG, Sze H** (1998) A novel calmodulin-regulated Ca<sup>2+</sup>-ATPases (ACA2) from *Arabidopsis* with an N-terminal autoinhibitory domain. *J Biol Chem* **273**: 1099–1106
- Hodges TK, Leonard RT** (1974) Plasma membrane ATPases. *Methods Enzymol* **32**: 397–398
- Hong BA, Ichida S, Wang Y, Gens JS, Pickard BG, Harper JF** (1999) Identification of a calmodulin-regulated Ca-ATPase in the ER. *Plant Physiol* **119**: 1165–1176
- Huang L, Franklin AE, Hoffman NE** (1993) Primary structure and characterization of an *Arabidopsis thaliana* calnexin-like protein. *J Biol Chem* **268**: 6560–6566
- Hwang I, Sze H, Harper JF** (2000) A calcium-dependent protein kinase can inhibit a calmodulin-stimulated Ca<sup>2+</sup> pump (ACA2) located in the endoplasmic reticulum of *Arabidopsis*. *Proc Natl Acad Sci USA* **97**: 6224–6229
- Johannes E, Brosnan JM, Sanders D** (1992) Parallel pathways for intracellular Ca<sup>2+</sup> release from the vacuole of higher plants. *Plant J* **2**: 97–102
- Johansson F, Olbe M, Sommarin M, Larsson C** (1995) Brij-58, a polyoxyethylene acyl ether, creates membrane vesicles of uniform sidedness: a new tool to obtain inside-out (cytoplasmic side-out) plasma membrane vesicles. *Plant J* **7**: 165–173
- Klüsener B, Boheim G, Liss H, Engelberth J, Weiler EW** (1995) Gadolinium-sensitive, voltage-dependent calcium release channels in the endoplasmic reticulum of a higher plant mechanoreceptor organ. *EMBO J* **14**: 2708–2714
- Laemmli UK** (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**: 680–685
- Leckie CP, McAinsh MR, Allen GJ, Sanders D, Hetherington AM** (1998) Abscisic acid induced stomatal closure mediated by cyclic ADP-ribose. *Proc Natl Acad Sci USA* **95**: 15837–15842
- Lee HC** (1997) Mechanisms of calcium signaling by cyclic ADP-ribose and NAADP. *Physiol Rev* **77**: 1133–1164
- Lee HC** (1999) A unified mechanism of enzymatic synthesis of two calcium messengers: cyclic ADP-ribose and NAADP. *Biol Chem* **380**: 785–793
- Lee HC, Galione A, Walseth TF** (1994) Cyclic ADP-ribose: metabolism and calcium-mobilizing function. *Vitam Horm* **48**: 199–257
- Lee HC, Walseth TF, Bratt GT, Hayes RN, Clapper DL** (1989) Structural determination of a cyclic metabolite of NAD<sup>+</sup> with intracellular Ca<sup>2+</sup> mobilizing activity. *J Biol Chem* **264**: 1608–1615
- Liang F, Cunningham KW, Harper JF, Sze H** (1997) ECA1 complements yeast mutants defective in Ca<sup>2+</sup> pumps and encodes an endoplasmic reticulum-type Ca<sup>2+</sup>-ATPase in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* **94**: 8579–8584
- Liang F, Sze H** (1998) A high affinity Ca<sup>2+</sup> pump, ECA1, from the endoplasmic reticulum is inhibited by cyclopiazonic acid but not by thapsigargin. *Plant Physiol* **118**: 817–825
- Malhó R, Moutinho A, van der Luit A, Trewavas AJ** (1998) Spatial characteristics of calcium signalling: the calcium wave as a basic unit in plant cell calcium signalling. *Philos Trans R Soc Lond B* **353**: 1463–1473
- Marshall J, Corzo A, Leigh RA, Sanders D** (1994) Membrane potential-dependent calcium transport in right-side-out plasma membrane vesicles from *Zea mays* L. roots. *Plant J* **5**: 683–694
- Masuda W, Takenaka S, Tsuyama S, Tokunaga M, Yamaji R, Inui H, Miyatake K, Nakano Y** (1997) Inositol 1,4,5-trisphosphate and cyclic ADP-ribose mobilize Ca<sup>2+</sup> in a protist, *Euglena gracilis*. *Comp Biochem Physiol* **118C**: 279–283
- McAinsh MR, Hetherington AM** (1998) Encoding specificity in Ca<sup>2+</sup> signalling systems. *Trends Plant Sci* **3**: 32–36
- Muir SR, Bewell MA, Sanders D, Allen GJ** (1997) Ligand-gated Ca<sup>2+</sup> channels and Ca<sup>2+</sup> signalling in higher plants. *J Exp Bot* **48**: 589–597
- Muir SR, Sanders D** (1996) Pharmacology of Ca<sup>2+</sup> release from red beet microsomes suggests the presence of ryanodine receptor homologs in higher plants. *FEBS Lett* **395**: 39–42
- Muir SR, Sanders D** (1997) Inositol 1,4,5-trisphosphate-sensitive Ca<sup>2+</sup> release across nonvacuolar membranes in cauliflower. *Plant Physiol* **11**: 1511–1521
- Navazio L, Baldan B, Dainese P, James P, Damiani E, Margreth A, Mariani P** (1995) Evidence that spinach leaves express calreticulin but not calsequestrin. *Plant Physiol* **109**: 983–990
- Navazio L, Bewell MA, Siddiqua A, Dickinson GD, Galione A, Sanders D** (2000) Calcium release from the endoplasmic reticulum of higher plants elicited by the NADP metabolite nicotinic acid adenine dinucleotide phosphate. *Proc Natl Acad Sci USA* **97**: 8693–8698
- Navazio L, Nardi MC, Pancaldi S, Dainese P, Baldan B, Fitchette-Lainé A-C, Faye L, Meggio F, Martin W, Mariani P** (1998) Functional conservation of calreticulin in *Euglena gracilis*. *J Eukaryot Microbiol* **45**: 307–313
- Plieth C, Sattelmacher B, Hansen U-P, Thiel G** (1998) The action potential in *Chara*: Ca<sup>2+</sup> release from internal stores visualised by Mn<sup>2+</sup>-induced quenching of fura-dextran. *Plant J* **13**: 167–175
- Pozzan T, Rizzuto R, Volpe P, Meldolesi J** (1994) Molecular and cellular physiology of intracellular calcium stores. *Physiol Rev* **74**: 595–636
- Robinson DG, Hinz G, Oberbeck K** (1994) Isolation of endo- and plasma membranes. *In* N Harris, KJ Oparka, eds, *Plant Cell Biology, A Practical Approach*. IRL Press, Oxford, pp 245–272
- Sanders D, Brownlee C, Harper JF** (1999) Communicating with calcium. *Plant Cell* **11**: 691–706
- Thomson LJ, Xing T, Hall JL, Williams LE** (1993) Investigation of the calcium-transporting ATPases at the endo-

- plasmic reticulum and plasma membrane of red beet. *Plant Physiol* **102**: 553–564
- Walseth TF, Aarhus R, Kerr JA, Lee HC** (1993) Identification of cyclic ADP-ribose-binding proteins by photoaffinity labeling. *J Biol Chem* **268**: 26686–26691
- Ward JM, Schroeder JI** (1994) Calcium-activated K<sup>+</sup> channels and calcium-induced calcium release by slow vacuolar ion channels in guard cells vacuoles implicated in the control of stomatal closure. *Plant Cell* **6**: 669–683
- Webb AAR, McAinsh MR, Taylor JE, Hetherington AM** (1996) Calcium ions as intracellular second messenger in higher plants. *Adv Bot Res* **22**: 45–96
- Wu Y, Kuzma J, Maréchal E, Graeff R, Lee HC, Foster R, Chua NH** (1997) Abscisic acid signaling through cyclic ADP-ribose in plants. *Science* **278**: 2126–2130