Plant Physiol. (1993) 102: 101–105

Inositol Trisphosphate Metabolism in Subcellular Fractions of Barley (*Hordeum vulgare* L.) Mesophyll Cells[1]

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Phosphatases in cytosolic fractions, vacuoles, and vacuolar membranes from barley (*Hordeum vulgare* L.) leaves were found to dephosphorylate inositol 1,4,5-trisphosphate (IP_3). 1,4-inositol bisphosphate (1,4-IP_2) is the main product of IP_3 dephosphorylation by the cytosolic fraction. The activity was strictly Mg^{2+} dependent. In contrast, IP_3 dephosphorylation activity of both the soluble vacuolar and the tonoplast fractions was inhibited up to 50% by Mg^{2+}. When vacuolar membranes were incubated with IP_3, 1,4-IP_2 was produced only under neutral and slightly alkaline conditions. Under acidic conditions, however, dephosphorylation yielded putative 4,5-inositol bisphosphate. Li^+ (20 mM) and Ca^{2+} (100 μM) strongly inhibited activity in the soluble vacuolar fraction but had only a slight effect on the activities of the cytosolic and tonoplast fractions.

In plant and animal cells, cytosolic free Ca plays a central role in signal transduction (Berridge and Irvine, 1989). Intracellular Ca^{2+} storage pools are responsible for the modulation of cytosolic free Ca^{2+} levels during signaling. In animal cells, the primary site of Ca^{2+} release is at the ER, whereas in plants, Ca transport activities have been detected at the plasma membrane, the ER, and the tonoplast (Schumaker and Sze, 1985; Gräf and Weiler, 1990). Several studies have shown that IP_3 causes a specific and saturable transient release of Ca^{2+} from intact vacuoles or tonoplast vesicles (Schumaker and Sze, 1987). Inhibition of the IP_3-dependent Ca^{2+} release by TMB 8 or heparin in animal as well as in plant systems (Brosnan and Sanders, 1990) suggests a similarity between the mechanisms involved, even though the channels are localized in different membranes. Metabolites of the inositol pathway have been detected in plant cells (Boss and Massel, 1985; Heim and Wagner, 1986; Ettlinger and Lehle, 1988), and it has been shown that the cytoplasmic release of IP_3 from a caged form can initiate stomatal closure (Gilroy et al., 1990). These results suggest that IP_3 acts as an intracellular signal not only in animal cells but also in plant cells. Intracellular signals are expected to be transient, which implies that the signal must be either metabolized or removed. Indeed, in animal cells an Mg^{2+}-dependent IP_3 5-phosphatase activity has been described for different cell types (Berridge and Irvine, 1989; Shears, 1989). 1,4-IP_2 is not able to mobilize Ca^{2+}, and the 5-phosphatase, therefore, plays a pivotal role in terminating the release of Ca^{2+} into the cytosol.

Until now, there have been few reports concerning the breakdown of IP_3 in plants (Joseph et al., 1989; Memon et al., 1989; Drøbak et al., 1991). Furthermore, the results differ with respect to the reported initial breakdown product, Ca^{2+} sensitivity, and Li^+ inhibition. These contradictory data may be due to the action of nonspecific vacuolar phosphatases. Therefore, we have used evacuolated barley (*Hordeum vulgare* L.) mesophyll protoplasts to obtain vacuolar free cytosolic extracts for the investigation of IP_3 dephosphorylation. Furthermore, we have compared the abilities of the cytosolic fraction and of the soluble as well as the membrane-bound vacuolar fractions to dephosphorylate IP_3.

MATERIALS AND METHODS

Barley (*Hordeum vulgare* L. cv Gerbel) was grown in a growth cabinet with 12 h of fluorescent light (45 μmol $m^{-2}s^{-1}$) at 22°C and 12 h of dark at 18°C; RH was 75%.

Mesophyll protoplasts and vacuoles were isolated as described previously (Kaiser et al., 1982; Rentsch and Martinoia, 1991). Tonoplasts (vacuolar membranes) were prepared by mixing one part of a packed sediment of vacuoles with three parts of 20 mM Tricine-imidazole (pH 7.2) and 4 mM $MgCl_2$. After a 5-min incubation on ice, the tonoplasts were sedimented for 35 min at 169,000*g*. The membranes were resuspended in a medium containing 50 mM Hepes-KOH (pH 7.9) and 2 mM DTT and centrifuged again. The pellet was resuspended in the same buffer at a concentration of 1 to 1.5 mg of protein mL^{-1}. Soluble vacuolar fractions were obtained by desalting the supernatant on a PD 10 column (Pharmacia, Uppsala, Sweden). Evacuolated protoplasts were isolated and purified as described by Hörtensteiner et al. (1992). From this fraction, cytosolic proteins were extracted by mixing one part of a packed sediment of evacuolated protoplasts with three parts of a buffer containing 20 mM Tricine-imidazole (pH 7.2) and 4 mM $MgCl_2$. After 5 min on ice and subsequent treatment in a Potter Elvehjem homogenizer (Braun), the particulate material was sedimented for 35 min at 169,000*g*.

Unless stated otherwise, hydrolysis of IP_3 was detected by

[1] This work was supported by the Swiss National Foundation, grant No. 31-25196.88.

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Abbreviations: IP_1, 1-inositol monophosphate; IP_2, inositol bisphosphate; IP_3, inositol 1,4,5-trisphosphate; 1,4-IP_2, 1,4-inositol bisphosphate; p4,5-IP_2, putative 4,5-inositol bisphosphate.

incubating tonoplasts (20–30 μg of protein), cytosolic fractions (0.5–2 mg of protein), or desalted soluble vacuolar fractions (0.05–0.1 μg of protein) in 100 μL of a medium containing 150 Bq of [^{3}H]IP_3 (100 MBq mmol^{-1}), 50 mM Hepes-KOH (pH 7.9), 5 mM $MgCl_2$, 2 mM DTT, and 1 mg mL^{-1} of BSA. In some assays leupeptin (10 μg mL^{-1}) and PMSF (1 mM) were included in the incubation medium to protect the enzymes from proteolytic degradation. The reaction was stopped by adding 200 μL of boiling 30 mM EDTA and, after the sample was heated for 5 min at 96°C, the extracts were freeze-dried (Resink et al., 1987). One milliliter of water was added to each sample immediately before the analysis of the breakdown products. Separation of the breakdown products was performed using the HPLC method described by Binder et al. (1985) with slight modifications (Irvine et al., 1985). Samples were loaded onto an anion-exchange column (SAX 10, 0.46 × 25 cm; Whatman). After the samples were washed with water for 3 min, separation of the inositol phosphates was achieved using a 13-min linear gradient from 0 to 0.75 M ammonium formate buffered to pH 3.7 with orthophosphoric acid, followed by a 4-min linear gradient from 0.75 to 1.5 M ammonium formate (pH 3.7) and a 7-min elution with 1.5 M ammonium formate (pH 3.7). The column eluate was mixed continuously with 4 volumes of Hionicfluor scintillator (Canberra Packard, Downers Grove, IL), and radioactivity was determined with an HPLC radioactivity detector (Canberra Packard, Flow-one/Beta Series A-250). Derivates of IP_3 were identified with standard myo-[2-^{3}H]IP_1, myo-[2-^{3}H]1,4-IP_2, and myo-[2-^{3}H]IP_3. p4,5-IP_2 was identified by comparing our chromatograms with published chromatograms, in which a similar separation method was used and inositol 4,5-bisphosphate has been identified (Drøbak et al., 1991). Nonspecific phosphatase activity was measured using methylumbelliferyl phosphate as substrate (Hörtensteiner et al., 1992).

RESULTS

Degradation of IP_3 by Specific Subcellular Fractions

Specific subcellular fractions were incubated with 10 μM IP_3. Soluble vacuolar phosphatases degraded IP_3 very efficiently even at a pH of 7.9 (Fig. 1, a and b). It is interesting that after short periods of incubation similar portions of 1,4-IP_2 and p4,5-IP_2 were detectable, whereas after prolonged incubation 1,4-IP_2 was the predominant IP_2 species. This observation suggests that p4,5-IP_2 is the preferred substrate for vacuolar phosphatases. The products 1,4- and p4,5-IP_2 as well as IP_1 (the different forms could not be distinguished) could be detected. Prolonged incubation resulted in the formation of myoinositol. Cytosolic extracts were also able to degrade IP_3 (Fig. 1c). In addition to the two IP_2 products, a small portion of IP_1 and increasing amounts of myoinositol were present in the assay after incubation with cytosolic extracts. IP_1 was always present, however, at low concentrations, suggesting that this breakdown product is metabolized faster than the inositol bisphosphates. The ratio of 1,4-IP_2 to p4,5-IP_2 remained constant during IP_3 breakdown. Prolonged incubations resulted in an increase in myoinositol. Incubation of vacuolar membranes with IP_3 resulted in the generation

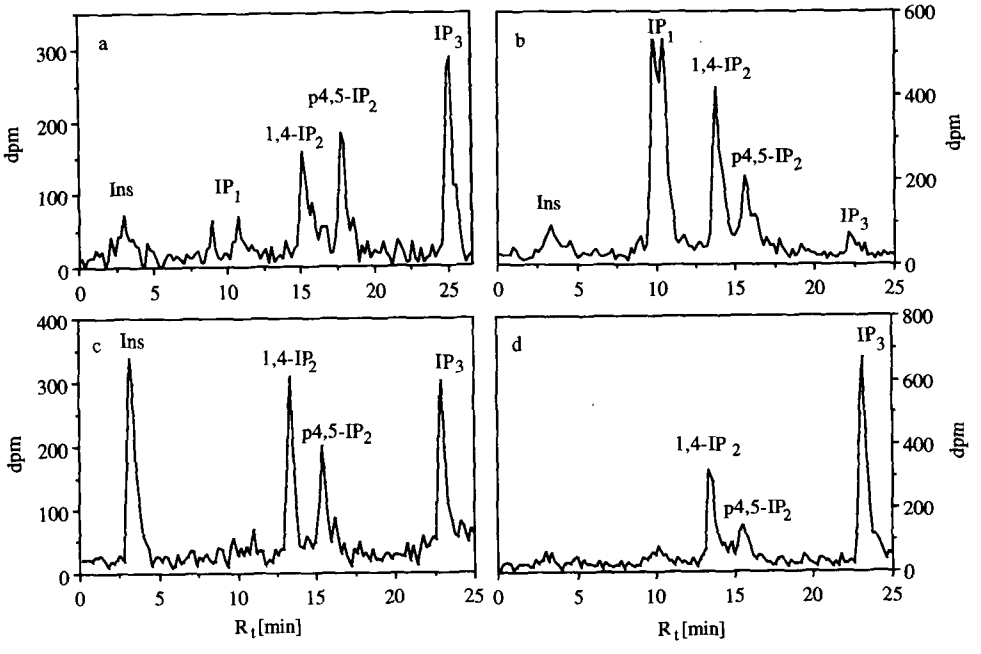

Figure 1. Breakdown of IP_3 by vacuolar sap (a and b; 0.05 μg of protein, corresponding to 5 × 10^3 vacuoles), cytosolic fractions (c; 0.5 mg, corresponding to 5 × 10^5 cells), and vacuolar membranes (d; 30 μg, corresponding to 3 × 10^6 vacuoles). Incubation was for 5 min (a), 30 min (b), or 60 min (c and d) in the presence of 5 mM $MgCl_2$ at pH 7.9. No breakdown products could be detected within this time when IP_3 was incubated without any cell extracts. The comigration of other inositol monophosphates with IP_1 cannot be excluded. Ins, Myoinositol.

Table I. *Mg^{2+} dependence of IP_3 breakdown by cytosolic and tonoplast fractions*

Rates were calculated from the initial breakdown rates. Values are given for a typical experiment; percentages are the means of several experiments (n = number of experiments). One hundred percent corresponds to the maximal activity in the respective assay. In the assay without Mg^{2+}, 5 mM EDTA was included to chelate any free Mg^{2+} present. n.d., Not done.

Mg^{2+}	IP_3 Breakdown			
	Cytosol		Tonoplast	
mM	nmol 10^{-6} cells h^{-1}	% (n)	nmol 10^{-6} cells h^{-1}	% (n)
0	≤0.06	≤2 (2)	0.072	100
0.5	1.5	46 (2)	n.d.	n.d.
5	3.2	100	0.039	48.5 (4)

of 1,4-IP_2 and p4,5-IP_2. Further breakdown products were present only in trace amounts (Fig. 1d). Large differences in the degradation rates were observed for the different fractions. Based on the number of cells represented by each fraction, the rate of breakdown of IP_3 was 1 to 2 μmol 10^{-6} cells h^{-1} for the soluble vacuolar phosphatases, 2 to 4 nmol 10^{-6} cells h^{-1} for the cytosolic fraction, and 40 to 80 pmol 10^{-6} cells h^{-1} for the tonoplast fraction. No differences were observed if protease inhibitors were present in the assay mixture.

Mg^{2+}, Ca^{2+}, and Li^+ Dependence of IP_3 Dephosphorylation

Hydrolysis of IP_3 by cytosolic fractions was strongly Mg^{2+} dependent: no 1,4-IP_2 or p4,5-IP_2 was detectable in the absence of Mg^{2+} at pH 7.9 (Table I). The generation of IP_2 by vacuolar membranes exhibited different characteristics (Table I): Mg^{2+} did not stimulate the formation of IP_2 but, rather, inhibited it by approximately 50%. A similar inhibition also could be observed for the vacuolar phosphatases (not shown). Dephosphorylation of IP_3 and IP_2 by cytosolic and tonoplast fractions was inhibited only slightly (10–20%) by 20 mM LiCl or 100 μM $CaCl_2$. In contrast, the formation of IP_1 by soluble vacuolar phosphatases was strongly inhibited by the same concentrations of LiCl and $CaCl_2$ (Table II).

Table II. *Dephosphorylation of IP_3 by soluble vacuolar phosphatases*

Inhibition by Li^+ and Ca^{2+}. Values are percentages of radioactivity added as IP_3 at time 0. Soluble vacuolar protein (1 μg, corresponding to approximately 1 × 10^4 cells) was added to 1 nmol of [^{3}H]IP_3 (150 Bq) (100%). Incubation was stopped after 30 min. Means of two independent experiments are given.

Treatment	Metabolites in Reaction Mixture (1 nmol = 100%)				
	IP_3	p4,5-IP_2	1,4-IP_2	IP_1	Myoinositol
Control	1.9	10.1	15.1	66.4	6.5
20 mM LiCl	26.2	19.7	37.6	12.9	3.6
100 μM $CaCl_2$	32.7	18.5	34.8	9.6	4.5

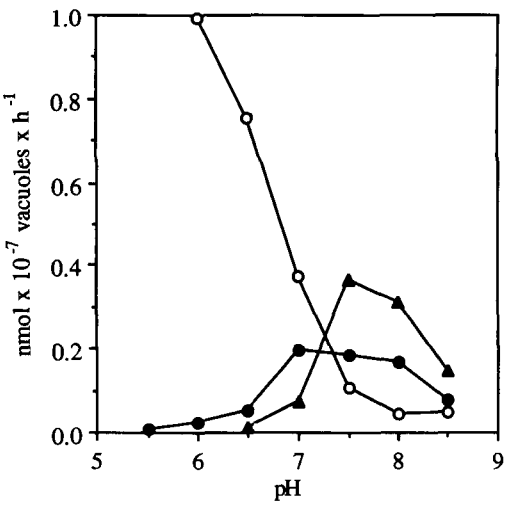

Figure 2. Formation of 1,4-IP_2 (●, ▲) and p4,5-IP_2 (○) from IP_3 by vacuolar membranes as a function of pH in the absence (▲) or presence (●, ○) of 5 mM Mg^{2+}. The amount of protein corresponding to 10^7 vacuoles is approximately 100 μg. Mes-KOH (pH 5.5–6.5) and Hepes-KOH (pH 7.0–8.5) were used as buffers.

pH Dependence of IP_3 Breakdown

Determination of the hydrolysis of IP_3 by vacuolar membranes as a function of the pH of the incubation mixture showed that p4,5-IP_2 is preferentially released under acidic conditions. The pH profile of this activity is similar to that of vacuolar phosphatases (Boller and Wiemken, 1986). In contrast, 1,4-IP_2 can be detected only after incubation at higher pH values (Fig. 2). Soluble phosphatases show a similar but less pronounced pH-dependent shift in the proportion of

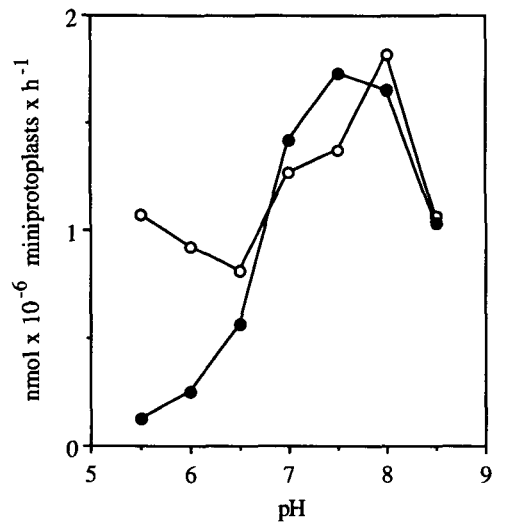

Figure 3. pH-dependent formation of 1,4-IP_2 (●) and p4,5-IP_2 (○) from IP_3 by cytosolic fractions in the presence of 5 mM Mg^{2+}. The amount of protein corresponding to 10^6 miniprotoplasts (evacuolated protoplasts) is approximately 1 mg. Of the total cellular protein, 85 to 90% are present in the miniprotoplasts. Mes-KOH (pH 5.5–6.5) and Hepes-KOH (pH 7.0–8.5) were used as buffers.

breakdown products (not shown). IP_3 hydrolysis in the cytosolic fraction shows that under acidic conditions, as well as for vacuolar membranes, p4,5-IP_2 is released (Fig. 3). The pH dependence of 1,4-IP_2 generation is similar to that shown for vacuolar membranes. In contrast to vacuolar membranes, however, a second peak of p4,5-IP_2 can be found at pH 8. The release of p4,5-IP_2 in neutral or slightly alkaline conditions was strongly Mg^{2+} dependent.

DISCUSSION

Plant vacuoles contain high activities of hydrolytic enzymes such as phosphatases (Matile, 1978; Boller and Wiemken, 1986). As shown in Figure 1a, soluble vacuolar phosphatases are able to degrade IP_3 even at pH 7.9. In barley mesophyll cells, the rate of IP_3 breakdown by these phosphatases is much faster than that in cytosolic extracts. The IP_3-hydrolyzing activity in total cell extracts may, therefore, reflect the predominant activity of soluble vacuolar phosphatases rather than those of specific cytosolic phosphatases. The use of microsomal fractions is also problematic. Vacuolar phosphatases are largely soluble; however, a small but consistent part of the acid phosphatase activity appears to be associated with the tonoplast fraction. In our case, about 1 to 3% of the total activity can be detected in the membrane fraction even after extensive washing with 50 mM Hepes-KOH (pH 7.9) (not shown). These observations may explain the contradictory published results (Memon et al., 1989; Joseph et al., 1989; Drøbak et al., 1991). Drøbak et al. (1991) reported that the major dephosphorylation product of IP_3 was 4,5-IP_2, whereas 1,4-IP_2 was present only in very small quantities. In contrast, Joseph et al. (1989) observed similar amounts of 1,4-IP_2 and 4,5-IP_2. Discrepancies were also reported concerning the effect of Ca^{2+} and Li^+. No investigations of the effect of Mg^{2+} upon IP_3 dephosphorylation have been reported so far.

Our results demonstrate that phosphatases localized in different compartments show distinct properties (Tables I and II). Centrifugation of barley protoplasts in a Percoll gradient results in their evacuolation (Hörtensteiner et al., 1992). Cytosolic extracts from these miniprotoplasts (evacuolated protoplasts) contain only about 0.5% of the total acidic phosphatase activity. Dephosphorylation of IP_3 to 1,4-IP_2 by these cytosolic fractions was strongly pH dependent, with an optimum at pH 7.5. It is interesting that the formation of p4,5-IP_2 was also Mg^{2+} dependent at pH 7.9 but not at the lower pH. Li^+ and Ca^{2+} had only a very slight inhibitory effect on IP_3 metabolism (not shown). The observation that the presence of protease inhibitors had no effect on the rate of IP_3 degradation even in long-term experiments is probably due to the fact that evacuolated protoplasts contain less than 2% of the total detectable protease activity (Hörtensteiner et al., 1992). If we assume that IP_3 is released at a concentration of 10 μM during a signal event, the duration of the signal may be estimated as follows: 10^6 cells have an approximate volume of 40 μL; about 3 μL can be attributed to the cytosol (Pfanz et al., 1987). In this case about 30 pmol of IP_3 would be released into the cytosol. If we take 3 nmol 10^{-6} cells h^{-1} as an intermediate cytosolic IP_3 phosphatase activity, the released IP_3 would be hydrolyzed within about 30 s.

In contrast to the cytosolic phosphatase(s), the tonoplast-associated IP_3 phosphatase was inhibited by Mg^{2+}. The pH profile of IP_3 dephosphorylation may reveal whether this phosphatase activity is also responsible for IP_3 degradation in vivo. Because IP_3 is supposed to act on the outer side of the vacuole, one would assume that IP_3 dephosphorylation occurs on the cytosolic side of the tonoplast as well. Therefore, the pH optimum of the tonoplast-bound IP_3 phosphatase activity is expected to be comparable with that of other cytosolic enzymes (pH 7–8). As shown in Figure 2, p4,5-IP_2 is released mainly under acidic conditions. The pH profile of this activity is similar to that of vacuolar phosphatases, and it is, therefore, this product that is formed by the membrane-associated acidic phosphatase activity. In contrast, 1,4-IP_2, the first breakdown product of IP_3 in animal cells (Berridge and Irvine, 1989; Shears, 1989), can be detected only at higher pH values. The pH profile is indeed similar to that of many cytosolic enzymes (Fig. 2) and may suggest that the observed IP_3-phosphatase activity is due to an enzyme localized at the cytosolic face of the membrane. However, it should be mentioned that a less pronounced, but similar, shift of the dephosphorylation products can also be observed when soluble vacuolar phosphatases are incubated at different pH values. The direct demonstration that an IP_3-5-phosphatase is localized on the cytosolic face of the vacuolar membrane was not possible. Because of their large size, isolated vacuoles are quite labile and are disrupted easily. Disruption of only a small fraction of a vacuolar preparation results in the release of high phosphatase activities into the medium. This fact prevented the direct demonstration that the 5-phosphatase activity is localized on the cytosolic face of the tonoplast. However, comparison of the breakdown rates for IP_3 suggests that the tonoplast-bound IP_3 phosphatase is too slow to efficiently stop an IP_3-mediated signal. The cytosolic Mg^{2+}-dependent IP_3 phosphatase is approximately 100-fold more active.

CONCLUSIONS

Using different subcellular fractions, we were able to show that a cytosolic, Mg^{2+}-dependent IP_3 phosphatase is present in plant cells. Crude plant extracts proved to be unsuitable for the investigation of IP_3 metabolism in plants because vacuolar phosphatases are able to degrade IP_3 even at pH 7.9 at rates that may be several times higher than those observed for equivalent cytosolic extracts. Li^+ or Ca^{2+} inhibition are not appropriate markers to attribute the activity to a specific phosphatase. However, the differential Mg^{2+} dependence of vacuolar and cytosolic IP_3 phosphatase activity might be a valid criterion for discriminating between the two enzymes in plant extracts.

ACKNOWLEDGMENTS

We thank Professor N. Amrhein for critical discussions and John Henstrand for reading the manuscript.

Received December 4, 1992; accepted January 8, 1993.
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LITERATURE CITED

Berridge MJ, Irvine RF (1989) Inositol phosphates and cell signalling. Nature **341:** 197–205

Binder H, Weber PC, Siess W (1985) Separation of inositol phosphates and glycerophosphoinositol phosphates by high-performance liquid chromatography. Anal Biochem **148:** 220–227

Boller T, Wiemken A (1986) Dynamics of vacuolar compartmentation. Annu Rev Plant Physiol **37:** 137–164

Boss WF, Massel MO (1985) Polyphosphoinositides are present in plant tissue culture cells. Biochem Biophys Res Commun **132:** 1018–1023

Brosnan JM, Sanders D (1990) Inositol-trisphosphate-mediated Ca^{2+}-release in beet microsomes is inhibited by heparin. FEBS Lett **260:** 70–72

Drøbak BK, Watkins PA, Chattaway JA, Roberts K, Dawson AP (1991) Metabolism of inositol (1,4,5)trisphosphate by a soluble enzyme fraction from pea (*Pisum sativum*) roots. Plant Physiol **95:** 412–419

Ettlinger C, Lehle L (1988) Auxin induces rapid changes in phosphatidylinositol metabolites. Nature **331:** 176–178

Gilroy S, Read ND, Trewawas AJ (1990) Elevation of cytoplasmic calcium by caged calcium or caged inositol trisphosphate initiates stomatal closure. Nature **346:** 769–771

Gräf P, Weiler E (1990) Functional reconstitution of an ATP-driven Ca^{2+}-transport system from the plasma membrane of *Comelina communis*. Plant Physiol **94:** 634–660

Heim S, Wagner KG (1986) Evidence of phosphorylated phosphatidylinositols in the growth cycle of suspension cultured plant cells. Biochem Biophys Res Commun **134:** 1175–1181

Hörtensteiner S, Martinoia E, Amrhein N (1992) Reappearance of hydrolytic activities and tonoplast proteins in the regenerated vacuole of evacuolated protoplasts. Planta **187:** 113–121

Irvine RF, Anggard EE, Letcher AJ, Downes CP (1985) Metabolism of inositol 1,4,5,-trisphosphate and inositol 1,3,4-trisphosphate in rat parotid glands. Biochem J **229:** 505–511

Joseph SK, Esch T, Bonner WD (1989) Hydrolysis of inositol phosphates by plant cell extracts. Biochem J **264:** 851–856

Kaiser G, Martinoia E, Wiemken A (1982) Rapid appearance of photosynthetic products in the vacuoles isolated from barley mesophyll protoplasts by a new fast method. Z Pflanzenphysiol **107:** 103–113

Matile P (1978) Biochemistry and function of vacuoles. Annu Rev Plant Physiol **29:** 193–213

Memon AR, Rincon M, Boss WF (1989) Inositol trisphosphate metabolism in carrot (*Daucus carota* L.) cells. Plant Physiol **91:** 477–480

Pfanz H, Martinoia E, Lange OL, Heber U (1987) Mesophyll resistances to SO_2 fluxes into leaves. Plant Physiol **85:** 922–927

Rentsch D, Martinoia E (1991) Citrate transport into barley mesophyll vacuoles—comparison with malate uptake activity. Planta **184:** 532–537

Resink TJ, Grigorian GY, Moldabaeva AK, Danilov SM, Bühler FR (1987) Histamine-induced phosphoinositide metabolism in cultured human umbilical vein endothelial cells. Association with thromboxane and prostacyclin release. Biochem Biophys Res Commun **144:** 438–446

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-triphosphate releases Ca^{2+} from vacuolar membrane vesicles of oat root. J Biol Chem **262:** 3944–3946

Shears SB (1989) Metabolism of the inositol phosphates produced upon receptor activation. Biochem J **260:** 313–324