Phospholipase C Activation during Elicitation of the Oxidative Burst in Cultured Plant Cells*

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Although phospholipase C hydrolysis of polyphosphoinositides constitutes one of the major second messenger pathways in animal cells, its participation in signal transduction in higher plants has not been established. To determine whether activation of phosphatidylinositol-directed phospholipase C might be involved in signaling the elicitor-induced oxidative burst in plants, suspension-cultured soybean cells were treated with two stimulants of the H₂O₂ burst and examined for polyphosphoinositide turnover. Both polygalacturonic acid elicitor and the G protein activator, mastoparan, promoted a transient increase in inositol 1,4,5-trisphosphate (IP₃) content that exceeded basal IP₃ levels (0.9 ± 0.4 pmol of IP₃/10⁶ cells, n = 28) by 2.6- and 7-fold, respectively. In each case, intracellular IP₃ content reached a maximum at 1 min post-stimulation and declined to near basal levels during the subsequent 5-10 min. Neomycin sulfate, an inhibitor of polyphosphoinositide hydrolysis, blocked the IP₃ transient, and Mas-17, an inactive analogue of mastoparan, induced no change in IP₃. Thin layer chromatography of lipid extracts of the soybean cells corroborated the above results by revealing a rapid decrease in phosphatidylinositol monophosphate and phosphatidylinositol 4,5-bisphosphate following polygalacturonic acid elicitor and mastoparan (but not Mas-17) stimulation. Since the rise in IP₃ preceded H₂O₂ production and since neomycin sulfate inhibited the appearance of both, we hypothesize that phospholipase C activation might constitute one pathway by which elicitors trigger the soybean oxidative burst.

Even though polyphosphoinositides are minor cellular membrane components, they constitute critical elements in the transduction of a multitude of signals across animal cell plasma membranes (Berridge, 1993). In animal cells, specific phospholipase C isozymes are activated by G proteins, receptor tyrosine kinases, or as yet undiscovered mechanisms, promoting the cleavage of phosphatidylinositol 4,5-bisphosphate into a water soluble, cytosolic moiety (inositol 1,4,5-trisphosphate or IP₃)¹ and a plasma membrane-associated component (diacylglycerol). IP₃ is thought to bind and stimulate calcium channels associated with internal organelles, thereby transiently increasing cytosolic calcium content. Diacylglycerol, in contrast, may activate a class of protein kinases referred to as protein kinase C. Loss of membrane polyphosphoinositides may also directly alter cytoskeletal associations, providing additional effector pathways for transmitting an initial signal to remote parts of the cell (Yin, 1987; Anderson, 1989; Janney and Stossel, 1989).

Recent advances in the plant sciences have demonstrated the existence of each of the above signal transduction components within photosynthetic organisms (reviewed in Coté and Crain (1993), Einspahr and Thompson (1990), Morse et al. (1989), and Drebak (1991, 1992). Further, enhanced phosphatidylinositol lipid turnover has been demonstrated after challenging plant cells or plant tissues with auxins (Zbell and Walter-Back, 1988; Morre et al., 1984; Ettlinger and Lehle, 1988), light (Memon and Boss, 1990; Morse et al., 1990; Peeler and Thompson, 1990), pathogen-derived substances (Kurosaki et al., 1987), cell wall degrading enzymes (Chen and Boss, 1990), or in the case of the alga, Dunaliella salina, hypoosmotic growth medium (Einspahr et al., 1988), and in the case of the alga, Chlamydomonas reinhardtii, acid (Quarmby et al., 1992). However, except for reports showing inactivation of K⁺ channels of Vicia stomatal guard cells after photolysis of caged IP₃ (Blatt et al., 1990), and inhibition of phosphatidylinositol mono- (and di-) phosphate stimulation of plasma membrane ATPase activity by neomycin sulfate (Chen and Boss, 1991), there is no evidence suggesting a physiological role for IP₃ as a second messenger in higher plants. Only in the green alga, C. reinhardtii, has a causal link between rapid IP₃ accumulation, Ca²⁺ mobilization, and a downstream process, flagellar excision, been recently established (Yueh and Crain, 1993).

One of the more intensely studied signal transduction pathways in plants is that involving induction of a defense response by a pathogen-derived elicitor (Ryan, 1992; Ryan and Farmer, 1991; Dixon and Lamb, 1990; Lamb et al., 1989). Despite an early report by Kurosaki et al. (1987) claiming enhanced production of IP₃ after treatment of carrot cells with a crude pathogen extract, the involvement of IP₃ in elicitor signal transduction has remained controversial. Thus, questions have arisen as to whether a true elicitor or a contaminating phospholipase in the pathogen extract promoted the suggested IP₃ production, and such concerns have

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¹ The abbreviations used are: IP₃, inositol 1,4,5-trisphosphate; PGA, polygalacturonic acid elicitor with a degree of polymerization of ~13; PIP, phosphatidylinositol monophosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate; MAS17, the inactive analogue of mastoparan.

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been amplified by the inability of Strasser et al. (1986) to reproduce the observation either in soybean or in parsley cells. Furthermore, the absence of kinetic data on the anticipated rise and fall in IP3 content common to IP3-mediated pathways has also weakened the claim, leaving the issue of IP3 involvement in higher plant signal transduction largely unresolved.

We have begun to characterize a system that appears well suited for the study of intracellular signaling in plants (Low and Heinsteim, 1986; Apostol et al., 1989; Legendre et al., 1993). The system involves a pure oligogalacturonide elicitor (PGA) that binds to a cell surface receptor (Horn et al., 1989) and triggers, within 2 min, the release of H2O2 from cultured soybean cells. This oxidative burst has been shown to be under control of G proteins (Legendre et al., 1992), citrusate (Apostol et al., 1987), as well as calcium and protein phosphorylation (Schwage and Hager, 1992). Even though, by comparison with the neutrophil oxidative burst in humans, activation of a plant phospholipase C might be expected to link these events, no study has yet addressed a role for this enzyme in the elicitor-induced oxidative burst in plants.

We report here that cellular IP3 levels rise during activation of H2O2 biosynthesis in cultured soybean cells by the elicitor, PGA, and by the G protein stimulator, mastoparan. This IP3 production correlates with a decrease in phosphatidylinositol 4-monophosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP2) content. In addition, the plant oxidative burst can be inhibited by pretreating the plant cells with the phosphate (PIP2) content. In addition, the plant oxidative burst can be inhibited by pretreating the plant cells with the neomycin sulfate. Taken together, these data suggest that polyphosphoinositide turnover may constitute a signal for the stimulation of H2O2 production by elicitor molecules in higher plants.

MATERIALS AND METHODS

Cell Cultures—Soybean cell suspensions (Glycine max var. kent) were maintained in W-38 medium (Hasegawa et al., 1980) and transferred every 10 days at an initial density of 9 cm3 packed cells/100 ml of medium, as described previously (Legendre et al., 1992). The cells were assayed 36 h after transfer, when H2O2 release was non-detectable except upon stimulation with elicitor. H2O2 Detection and Quantitation—The release of H2O2 by plant cells was monitored by following the bleaching of the fluorescent dye, pyranine (Molecular Probes, λex = 405 nm, λem = 512 nm), upon oxidation by H2O2 via naturally occurring external peroxidases (Apostol et al., 1989). Pyranine (1.4 μg) dissolved in water was added to 1.5 ml of cell suspension immediately before transfer to a spectrophotometer cuvette where the cells were maintained in suspension by mild mechanical stirring until elicitor addition. The oligogalacturonide elicitor (PGA) was purified from an acid hydrolysate of sodium oligogalacturonide (PGA) that binds to a cell surface receptor (Horn et al., 1989) to obtain in signal transduction in cultured soybean cells, we treated the cells with mastoparan, a generic activator of animal G proteins (Higashijima et al., 1988), and examined whether phosphatidylinositol-directed phospholipase C activity was enhanced, as it commonly is in animal cells (Norgauer et al., 1992). As shown in Fig. 1, mastoparan is a potent stimulant of IP3 release, a hydrolysis product of phosphatidylinositol 4,5-bisphosphosphate, in cultured soybean cells. A roughly 7-fold increase over basal IP3 levels (0.9 ± 0.4 pmol IP3/106 cells, n = 28) was seen in the first minute following treatment with the peptide. Further, as expected from a signaling event, the rise in IP3 content was transient, returning slowly to unstimulated levels after 10 min after mastoparan addition. These kinetic data are in agreement with kinetics typically observed in animal cells (Berridge, 1993). In addition, concentrations of mastoparan as low as 1 μM (Fig. 2) are capable of stimulating phospholipase C activity, consistent with the ability of this concentration of peptide to stimulate G proteins in animal cells (Higashijima et al., 1988).

The aminoglycoside neomycin binds to phosphatidylinositol 4,5-bisphosphate and prevents its hydrolysis by phospholipase C (Chen and Boss, 1991; Quarmby et al., 1992). Consistent with this effect, addition of 100 μM neomycin sulfate

![Fig. 1. Kinetics of stimulation of IP3 production by mastoparan and its inhibition by neomycin sulfate](image-url)
after treatment with increasing concentrations of mastoparan or Mas-17. The quantity of IP$_3$ released after treatment with different concentrations of mastoparan (○○○) or Mas-17 (□□□) was compared with IP$_3$ levels in untreated cell suspensions. Each point represents the average value (±S.E. of the mean) obtained on three separate cell suspensions. Variations among the data are sometimes smaller than the size of the symbol and therefore do not appear on the graph.

resulted in total inhibition of IP$_3$ accumulation (Fig. 1). Not only were stimulated increases in IP$_3$ content eliminated, but basal IP$_3$ levels also decreased to virtually undetectable levels (10–20% of control values). The inhibition was very rapid and could be readily observed by 30 s after inhibitor addition. Furthermore, addition of Mas-17, an analogue of mastoparan that does not activate G proteins (Higashijima et al., 1990), was similarly ineffective in promoting IP$_3$ release (Fig. 2).

Taken together, these data suggest that soybean cells have the signaling apparatus to respond to an external stimulus and transiently activate hydrolysis of phosphatidylinositol 4,5-bisphosphate to yield the water-soluble second messenger, IP$_3$.

Production of IP$_3$ after PGA Addition to Plant Cells—A key element in evaluating whether IP$_3$ production in plant cells is physiologically relevant involves finding a natural stimulator of an intracellular signal transduction pathway that also promotes IP$_3$ release. The recent observation that mastoparan stimulates H$_2$O$_2$ production in soybean cells (Legendre et al., 1992) directed our attention toward PGA, a natural activator of the same oxidative burst. Fig. 3A shows that addition of 1.8 or 9.0 μg/ml of this oligouronide elicitor/ml of cell suspension (i.e. concentrations that give rise to oxidative bursts of intermediate and maximal intensities, respectively (Fig. 3B)) results in the transient release of IP$_3$ with kinetics identical to those observed in Fig. 1. PGA-stimulated IP$_3$ levels are, however, lower than those obtained during mastoparan activation, generally rising only 2.6 times above basal concentrations upon maximal stimulation. This increase, however, is reproducible and comparable with results from Morse et al. (1989) showing a maximum 1.3-fold increase in IP$_3$ content after white light treatment of Samanea pulvini. Such levels of IP$_3$ accumulation are also not unusual in animal systems (Nigam et al., 1992) and may reflect the existence of regulatory mechanisms moderating the production of IP$_3$ or removing it rapidly to prevent sustained stimulation of the cells.

Decrease in Polyphosphoinositide Levels after PGA or Mastoparan Addition to Soybean Cells—Because of the controversy surrounding the involvement of polyphosphoinositide hydrolysis in signal transduction in plants (Côté et al., 1990), we decided to corroborate the above data using another experimental method. In this study, we evaluated the decrease in substrate (PIP and PIP$_2$) concentrations following stimulation of the soybean cells to learn whether they correlated with the increased levels of the metabolite, IP$_3$, observed above. Initially, we incubated soybean cells in [32P]NaH$_2$PO$_4$ to attempt to achieve maximum labeling of cellular phospholipids. Spots on thin layer chromatography plates corresponding to PIP and PIP$_2$ were separated and readily identified with the use of authentic standards (RF values of 0.38 and 0.10, respectively). Quantitative analysis by phosphate determination indicated that PIP and PIP$_2$ represented only 3.6 and 0.2 mol % of the total extracted plant lipid, in agreement with the data of Einspahr et al. (1988). In contrast, a short term (3 min) labeling of plant cells with $^{32}$PO$_4$ showed that most of the incorporated label was found in inositol-containing lipids. This suggests that plant cells, like animal cells, metabolize inositol-containing lipids at a higher rate than other phospholipids (Côté and Crain, 1993). Using this simpler short term labeling protocol, we observed that stimulating plant cells with mastoparan or PGA resulted in a rapid decrease in PIP and PIP$_2$ content (Table I). Mastoparan induced roughly a 2-fold larger change in levels of inositol phospholipids than seen with PGA, as expected from its ability to release larger quantities of IP$_3$. Further, the inactive mastoparan analogue, Mas-17, did not promote a decrease in PIP or PIP$_2$ levels, again implying that the transient activation of polyphosphoinositide hydrolysis requires participation of a G protein.

Neomycin Sulfate Inhibits PGA- and Mastoparan-induced H$_2$O$_2$ Production—To test whether IP$_3$ production by mastoparan or PGA plays a role in their stimulation of the H$_2$O$_2$ burst in plant cells, neomycin sulfate was added to soybean cells prior to elicitor challenge. Fig. 4 shows that such treatment results in a maximal 60% inhibition of the PGA-induced
burst in cultured soybean cells induce transient activation of by addition of 3 pg of PGA production by neomycin sulfate. HzO2 production was initiated to elicitor addition (less than accordance with earlier results showing that neomycin inhibits before elicitor addition did not result in greater inhibition, in in plants.

The ability of mastoparan, a G protein activator in animal cells, to stimulate phospholipase C activity in plants suggests that IP3 production may be under G protein control in plants, as is well established in several animal systems (Berridge, 1993). Activation of phospholipase C by GTP-γ-S in membranes isolated from sycamore (Dillenschneider et al., 1986) and in partially purified membrane fractions from the green algae Dunaliella salina (Einspahr et al., 1989) provides further support for this contention. Unfortunately, the identity of the G proteins involved in these regulatory processes is currently unknown. In animal cells, phospholipase C coupled G proteins are characterized by their inability to be ADP-ribosylated by either pertussis or cholera toxin (Sternweis and Smrcka, 1992; Berridge, 1991). This same unique feature is surprisingly shared by the only two plant G protein sequences reported to date (Ma et al., 1990, 1991), and by a 45-kDa soybean GTP-binding protein (Legendre et al., 1992). Interestingly, the last 45-kDa soybean polypeptide has been shown to be activated by the same oligouronide elicitor (PGA) that was employed in this study to stimulate release of IP3. However, whether the 45-kDa soybean G protein participates in an essential step in signaling the oxidative burst requires considerable further investigation.

REFERENCES

Dunaliella salina

Phospholipase C Activation During Plant Signal Transduction