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Activation of Phospholipase A by Plant Defense Elicitors[1]

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Participation of phospholipase A (PLase A) in plant signal transduction has been documented for auxin stimulation of growth but not for elicitation of any plant defense response. In this paper, we report two independent assays for monitoring PLase A induction in plant cells and have used these assays to evaluate whether transduction of defense-related signals might require PLase A activation. Oligogalacturonic acid, a potent elicitor of the soybean (*Glycine max*) H_2O_2 burst, was unable to stimulate endogenous PLase A, suggesting that PLase A activation is not an obligate intermediate in the oligogalacturonic acid-induced burst pathway. In contrast, harpin and an extract from the pathogenic fungus *Verticillium dahliae* both stimulated the oxidative burst and promoted a rapid increase in PLase A activity. To evaluate the possible role of this inducible PLase A activity in transducing the oxidative burst, we tested the effect of chlorpromazine-HCl, a PLase A inhibitor on elicitor-stimulated burst activity. Pretreatment with chloropromazine was found to inhibit the H_2O_2 burst triggered by *V. dahliae* extract at the same concentration at which it blocked PLase A activation. In contrast, neither the harpin- nor oligogalacturonic acid-induced burst was altered by addition of chlorpromazine. These data suggest that PLase A stimulation may be important in certain elicitor-induced oxidative bursts (e.g. *V. dahliae*) and that other elicitors such as oligogalacturonic acid and harpin must operate through independent signaling intermediates to activate the same defense response.

PLase A serves as an effector or signaling intermediate in numerous signal transduction pathways in animal cells (reviewed by Axelrod, 1990; Dennis et al., 1991; Liscovitch and Cantley, 1994). Upon activation by G proteins, phosphorylation, or Ca^{2+} binding (Axelrod, 1990; Liscovitch and Cantley, 1994), PLase A hydrolyzes the ester bond at the *sn*-2 position of substrate phospholipids to generate a free fatty acid and the corresponding lysophospholipid. Arachidonic acid, the fatty acid most commonly released in mammalian cells, is a precursor of eicosanoids (i.e. prostaglandins and leukotrienes) that promote localized stress and inflammatory responses in animal cells (Kuehl and Egan, 1980; Smith et al., 1991). Arachidonic acid and its metabolites may also regulate gene transcription and modulate the activity of other second-messenger systems, thus communicating the initial signal to independent pathways in the cell (Axelrod, 1990). In some cells, the lysolipid hydrolysis product may also exhibit potent biological activities, most commonly regulating kinases controlling other signaling pathways (Oishi et al., 1988). Whereas documentation for the involvement of PLase A in animal cell signaling is now extensive, evidence for participation of the enzyme in signal transduction pathways in higher plants is much more limited.

A plant-cell system in which hormone-stimulated PLase A activity has been clearly demonstrated is the auxin-mediated growth response of *Cucurbita pepo* seedlings and soybean (*Glycine max*) cultures (Scherer and André, 1989; Scherer, 1990). In this system, lysophospholipids generated by auxin-stimulated PLase A or exogenous platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) have been shown to activate a protein kinase that phosphorylates an ATPase involved in proton pumping (Scherer et al., 1988; Martiny-Baron and Scherer, 1989). More indirect evidence for PLase A involvement in plant-cell signaling derives from the observed activation of the soybean plasma membrane NADH oxidase by PLase A hydrolysis products (Brightman et al., 1990; Morré and Brightman, 1991) and the release of membrane-bound phosphatidylinositol kinase by treatment of membranes with PLase A (Gross et al., 1992).

Although evidence for the participation of PLase A in plant signal transduction is sparse, substantial support still exists for the role of PLase A products and their metabolites in initiating plant defense responses. First, free fatty acids and lysophosphatidylcholine modulate 1,3-β-D-glucan synthase activity and, hence, callose deposition in suspension-cultured soybean cells (Kauss and Jeblick, 1986). Second, arachidonic acid has been demonstrated to be an elicitor, regulating several defense responses including accumulation of phytoalexins (Bostock and Laine, 1981) and pathogenesis-related gene products in potato (Matton and Brisson, 1989; Choi et al., 1992). Third, jasmonic acid and its fatty acid precursors rapidly increase during elicitation and wounding (Creelman et al., 1992; Gundlach et al., 1992), rendering them likely candidates for systemic stress signal molecules (Enyedi et al., 1992). Thus, independently or via activation of systemin, an 18-amino acid polypeptide (Farmer and Ryan, 1992), jasmonic acid and its cogenors may mediate such host defense responses as in-

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Abbreviations: bis-BODIPY-C_{11}-PC, 1,2-bis-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-undecanoyl)-*sn*-glycero-3-phosphocholine; lysoPI, lysophosphatidylinositol; Mas-17, inactive analog of mastoparan; NBD-C_6-HPC, 2-(6-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino)-hexanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine; OGA, oligogalacturonic acid; PLase, phospholipase; PLase A, phospholipase A_2.

duction of proteinase inhibitors (Farmer and Ryan, 1990), initiation of Phe ammonia lyase gene transcription (Gundlach et al., 1992), and regulation of wound-responsive genes for chalcone synthase, vegetative storage proteins, and Pro-rich cell-wall proteins (Creelman et al., 1992). Although activation of PLase A during induction of a defense or wounding response has been frequently hypothesized (Farmer and Ryan, 1990; Hamberg and Gardner, 1992), to our knowledge the signaling event has never been directly observed or measured. In this study, we have used three distinct techniques, TLC and two fluorescence assays, to demonstrate the involvement of PLase A activation in the elicitation of soybean cell suspensions by several elicitors. We postulate that this activation event may generate fatty acids involved in regulation of localized defense responses during elicitation.

MATERIALS AND METHODS

Materials

Synthetic mastoparan, a peptide originally derived from the venom of *Vespula lewisii*, and Mas-17, its inactive analog (two amino acid substitutions), were obtained from Peninsula Laboratories (Belmont, CA). Chlorpromazine-HCl and aristolochic acid (i.e. inhibitors that block PLase A activation or displace the enzyme from the membrane, respectively) were purchased from Calbiochem. All other chemicals were reagent grade or of higher purity and were obtained from major chemical suppliers.

Plant-Cell Culture

Cell-suspension cultures of soybean (*Glycine max* Merr. cv Kent) were maintained in W-38 medium (Hasegawa et al., 1980) as described earlier (Legendre et al., 1993b). When an elicitation study was to be conducted, 9 cm^3 of filtered cells were transferred to 100 mL of fresh medium and allowed to grow for approximately 36 h before use, unless stated otherwise in the figure legends.

Elicitors

An OGA that elicits H_2O_2 production in soybean cell-suspension cultures was purified as described previously (Legendre et al., 1993b). The preparation used in this study contained 0.36 mg/mL galacturonic acid equivalents, as determined by the method of Blumenkrantz and Asboe-Hansen (1973). A *Verticillium dahliae* 277 elicitor containing 1.15 mg/mL protein was prepared as described earlier (Davis et al., 1992). Harpin, an elicitor of the hypersensitive response from the pathogenic bacterium *Erwinia amylovora*, was expressed in *Escherichia coli* and obtained as a generous gift from Dr. Steven V. Beer (Cornell University, Ithaca, NY). The synthetic hepta-β-glucan elicitor was kindly provided by Dr. Barbara Helpap (Bayer Chemical Co., Leverkusen, Germany).

Spectrofluorometric Determination of PLase A Activity

Two spectrofluorometric methods were developed for assaying PLase A activity in vivo. PLase activity was first monitored using NBD-C_6-HPC (excitation wavelength 470 nm, emission wavelength 540 nm; Molecular Probes, Eugene, OR) as the fluorescent substrate by following the increase in fluorescence on release of nitrobenzoxadiazole-hexanoic acid from NBD-C_6-HPC (Moreau, 1989). Ten microliters of a 1-mg/mL stock of NBD-C_6-HPC in 1:1 ethanol:water was added to 3 mL of a cell suspension (approximately 1.5×10^5 cells/mL) and equilibrated for 10 min prior to transfer to the spectrofluorometer for analysis. Cells (1.5 mL) were maintained in suspension in the fluorometer by mild stirring. Elicitors were added 3 min after the start of stirring, and the fluorescence change was recorded continuously with time.

Monitoring PLase A activity using bis-BODIPY-C_{11}-PC (excitation wavelength 485 nm, emission wavelength 517 nm; Molecular Probes) was done essentially as described for NBD-C_6-HPC, except 10 μL of a 1 mg/mL stock in ethanol was added to 1.5 mL of cells prior to transfer to a fluorometer.

Confocal Microscopy

Cells were treated with NBD-C_6-HPC or bis-BODIPY-C_{11}-PC and mastoparan as described in the section above. The cells were then examined under a Bio-Rad MRC-600 confocal imaging system as described by Lee and Low (1995), except that the excitation light was provided by the 488 nm line of a krypton-argon mixed-gas ion laser. A green emission filter 522DF35 and a pin hole diameter setting of 2 mm were also used. Images were collected using a ×60 objective.

Phospholipid Separation and Quantitation of PLase A Hydrolysis Products

A soybean cell suspension (1.5 mL) was labeled for 12 min with 50 μCi of $NaH_2{}^{32}PO_4$ (ICN). Elicitors were added 2 min after commencement of labeling to ensure that both elicitor treatment and cell labeling finished simultaneously. Ten minutes after elicitor addition, cellular activity was stopped by homogenization with 2 volumes of solution A (chloroform:methanol:concentrated HCl, 200:100:2.5, v/v) in a Dounce homogenizer (Wheaton, Millville, NJ). Following phase separation, the upper phase of each sample was re-extracted with 1 volume of solution A and the new lower phase was combined with the one collected previously. The pooled lower phases were further extracted with 3 mL of solution B (chloroform:methanol:1 M HCl, 3:45:45, v/v), and the organic phase was dried under a stream of nitrogen. The dried extract was redissolved in 50 μL of chloroform:methanol (2:1, v/v), and 30 μL of each sample were analyzed by TLC (1% impregnated potassium oxalate silica H plates; Analtech, Newark, DE) with chloroform:methanol:4 N NH_4OH (90:70:20, v/v) as the running solvent. Plates were air dried and developed by iodine vapors and autoradiography. Individual spots were identified with authentic standards (Sigma and GIBCO-BRL) and quantitated by scintillation counting of the scraped areas.

Spectrofluorometric Determination of H_2O_2 Production

H_2O_2 production in cultured soybean cells was detected by monitoring the oxidative quenching of a fluorescent peroxidase substrate, pyranine (8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt, excitation wavelength 405 nm, emission wavelength 512 nm; Molecular Probes) as described previously (Low and Heinstein, 1986; Apostol et al., 1989). Briefly, 1.5 mL of a cell suspension were treated with 7 μL of a 0.2 mg/mL pyranine stock solution in water, transferred to a fluorometer, and maintained in suspension in the spectrofluorometer by mild stirring. Elicitors were added at the start of stirring. All elicitors and pharmacological agents were tested to ensure that they did not alter peroxidase activity (Dwyer et al., 1996).

O_2 Electrode

Production of H_2O_2 was also monitored by the rapid increase in O_2 consumption following elicitor addition using a liquid-phase Clark-type O_2 electrode (Hansatech Instruments Ltd., King's Lynn, UK), as described by Dwyer et al. (1996). Briefly, 1 mL of cell suspension was placed in an O_2 electrode chamber and maintained in suspension by mild stirring. The basal steady-state rate of metabolic O_2 consumption by the cells was recorded as a quasilinear decrease in the O_2 content of the medium. Upon stimulation with elicitors, an accelerated rate of O_2 consumption was recorded. The difference between the stimulated and unstimulated slopes was taken as the O_2 consumption arising from the oxidative burst.

RESULTS

Time Course of Mastoparan-Stimulated PLase A Activity

To facilitate investigation of PLase A activity in transducing the elicitation signal, we developed a simple fluorometric assay to monitor PLase A activity. In animal cells, mastoparan, a generic G-protein activator (Higashijima et al., 1988), has been shown to activate PLase A in a G-protein-dependent manner (Gil et al., 1991). In suspension-cultured soybean cells, Scherer (1992) also demonstrated that mastoparan can stimulate endogenous PLase A activity; however, in this case a cumbersome in vivo radiolabeling protocol was used. Therefore, we have made use of mastoparan as a positive control to validate the application in plants of two fluorometric assays of PLase A activity previously developed for use in animal cells.

Figure 1A reveals the change in fluorescence of soybean cells labeled with the fluorescent PLase A substrate bis-BODIPY-C_{11}-PC and then treated with stimulants or inhibitors of PLase A activity. Hydrolysis of one of the fatty acyl chains of bis-BODIPY-C_{11}-PC releases the attached fluorophore from a self-quenching interaction with the adjacent acyl chain fluorophore and thereby results in a fluorescence increase (Meshulam et al., 1992). As shown in the figure, labeled cell suspensions treated with either buffer (C) or the inactive Mas-17 displayed a continuous increase in fluorescence, suggesting a high level of endogenous unstimulated PLase A activity. This constitutive PLase A

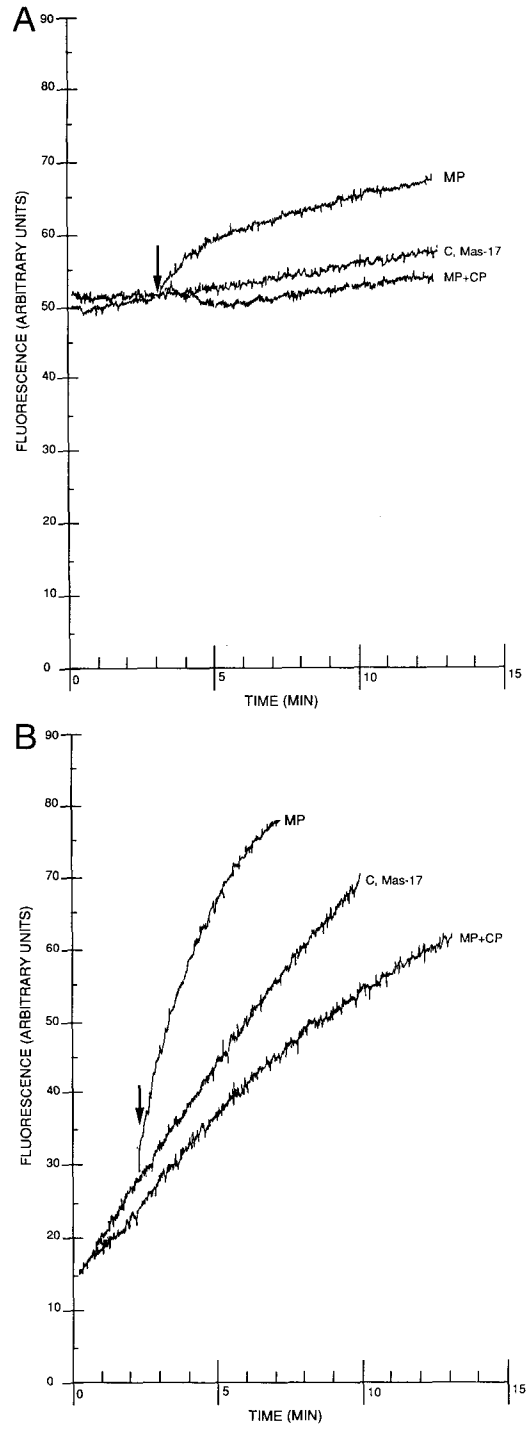

Figure 1. Time course of mastoparan-stimulated PLase A activity in suspension-cultured soybean cells. Soybean cells were equilibrated with 3.3 μg/mL bis-BODIPY-C_{11}-PC (A) or 3.3 μg/mL NBD-C_6-HPC (B), and the relative fluorescence was monitored using a Perkin-Elmer MPF44A spectrofluorometer. The cells were stimulated 3 min after the start of stirring (arrow) with mastoparan (MP, 30 μg/mL final concentration), its inactive analog Mas-17 (30 μg/mL final concentration), or a buffer control (C). PLase A activity was monitored by following the increase in fluorescence upon release of labeled fatty acids. Chlorpromazine-HCl (CP, 0.26 mM final concentration), a PLase A inhibitor, was added when desired at the start of stirring. Similar activity profiles were obtained in three independent experiments.

activity has been observed by others (Scherer and Morré, 1978) and may derive from an elicitation-independent enzyme located in the cell wall or plasma membrane. Importantly, stimulation of bis-BODIPY-C_{11}-PC hydrolysis could be induced by addition of low but eliciting concentrations of mastoparan (Fig. 1A, MP), a wasp venom peptide that activates numerous G proteins (Higashijima et al., 1988, 1990; Legendre et al., 1992). Based on the slopes of the respective fluorescence transitions, the initial rate of bis-BODIPY-C_{11}-PC hydrolysis induced by mastoparan was approximately 2.5 times the basal rate of hydrolysis in the two controls. Chlorpromazine-HCl, a nonspecific inhibitor of PLase A activity that presumably functions by binding calmodulin (Scheuer, 1989), abolished the stimulated increase in bis-BODIPY-C_{11}-PC fluorescence when added prior to mastoparan treatment. Taken together, these results suggest that bis-BODIPY-C_{11}-PC reports on PLase A activity in soybeans and that, if the constitutive activity is subtracted, the probe may be useful for monitoring the induction of PLase A during cell signaling.

Results obtained with a second fluorescent PLase A substrate (NBD-C_6-HPC) were qualitatively similar, except that the constitutive component of the PLase A activity was not as readily detected (Fig. 1B). Thus, only a slow rate of fluorescence increase was observed in control (C) and Mas-17-treated cells, and this basal rate was greatly augmented by mastoparan (MP). Furthermore, chlorpromazine-HCl inhibited the mastoparan-induced PLase A activity. Although either bis-BODIPY-C_{11}-PC or NBD-C_6-HPC could have been used for further studies of the elicitor-stimulated PLase A activity, we selected bis-BODIPY-C_{11}-PC because of its greater sensitivity to PLase A activation.

Confocal Microscopy

Because most PLase A-signaling pathways are believed to occur at the plasma membrane, it was important to examine the subcellular localization of both bis-BODIPY-C_{11}-PC and NBD-C_6-HPC in cultured plant cells. Therefore, we analyzed soybean cell suspensions treated with the two dyes by confocal microscopy. The image in Figure 2A reveals that bis-BODIPY-C_{11}-PC is located primarily in the plasma membrane, with a fraction associated with internal membranes and the nucleus. Meshulam et al. (1992) obtained similar results when staining and fractionating human neutrophils. The staining pattern obtained with NBD-C_6-HPC (Fig. 2B) was identical with that obtained with bis-BODIPY-C_{11}-PC. Importantly, when the above dye-treated cells were subsequently incubated in 3 M NaCl to retract the plasma membranes from the cell walls, the fluorescence was observed to withdraw with the plasma membranes, suggesting that little if any dye was localized in the cell walls (data not shown).

Quantification of LysoPI Content

Although the results of our fluorometric assays corroborate the claim of Scherer (1992) that mastoparan activates endogenous PLase A enzymes in plants, we chose to confirm the same conclusion by an alternate experimental

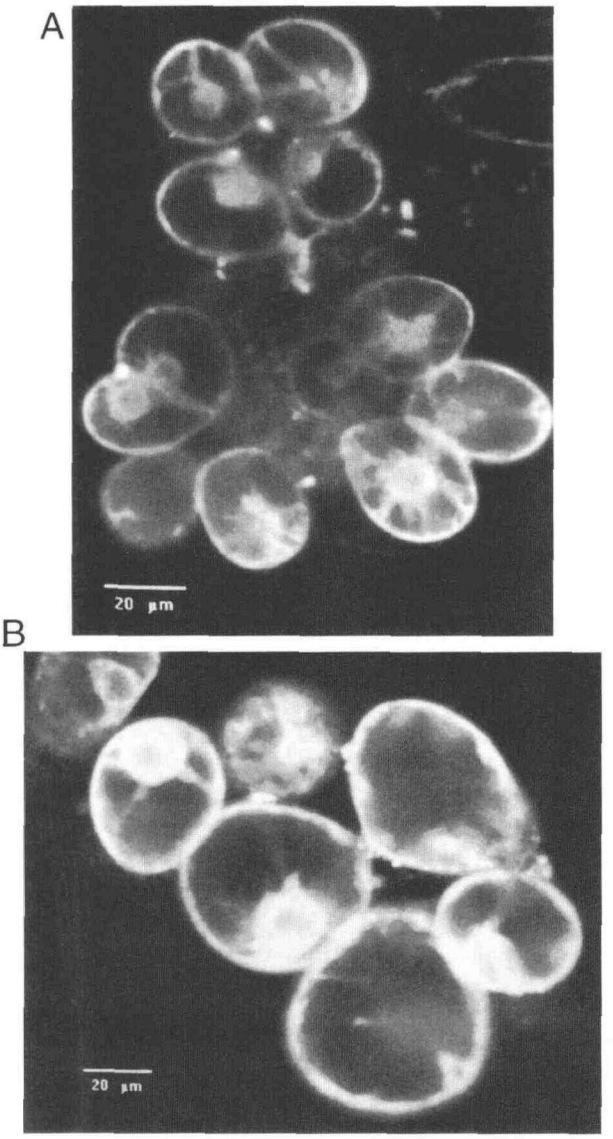

Figure 2. Confocal microscopy. Soybean cells were equilibrated with 3.3 μg/mL bis-BODIPY-C_{11}-PC (A) or 3.3 μg/mL NBD-C_6-HPC (B) and 30 μg/mL mastoparan before viewing under a Bio-Rad MRC-600 confocal imaging system. Images were collected using a ×60 objective.

procedure. Instead of monitoring the release of fatty acids fluorometrically, we assayed the production of lysophospholipids by TLC. The TLC profile obtained on extracted lipids from mastoparan-stimulated cells (data not shown) was in agreement with earlier observations, with R_F values for phosphatidylinositol-4,5-bisphosphate, phosphatidylinositol-4-phosphate, lysoPI, and phosphatidylinositol being 0.15, 0.42, 0.62, and 0.67, respectively (Legendre et al., 1993a). Figure 3 shows that mastoparan treatment elevated lysoPI levels 3-fold over those of untreated control cells. Furthermore, as previously seen in the fluorescence assay, this increase was abolished by addition of chlorpromazine-HCl prior to mastoparan treatment. Also, Mas-17 was ineffective in stimulating an increase in lysoPI content, confirming that the mastoparan-induced stimulation was

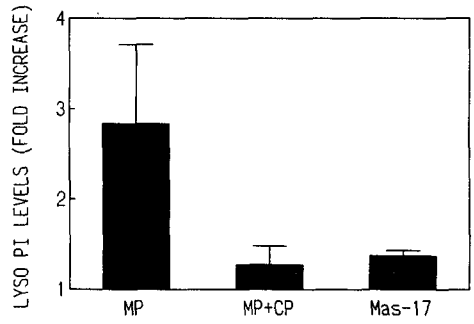

Figure 3. Quantification of lysoPI content. Soybean cells were labeled with $NaH_2{}^{32}PO_4$ alone or $NaH_2{}^{32}PO_4$ plus other additives as follows: MP, mastoparan (30 μg/mL final concentration); MP + CP, mastoparan (30 μg/mL final concentration) in the presence of chlorpromazine-HCl (0.26 mM); Mas-17 (30 μg/mL final concentration). After the required incubation, phospholipids were extracted and separated by TLC as described in "Materials and Methods." The plates were autoradiographed, and the lysoPI content was quantitated by scintillation counting of scraped areas. The measured values are displayed as the fold increase over control. Bars indicate SE.

specific for the active form of the peptide. Since these results correspond closely to those obtained in the fluorescence assays, we conclude that the fluorescence assays accurately report the changes in PLase A activity in vivo. Decreases in phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate content on mastoparan treatment (data not shown) were also similar to those observed earlier (Legendre et al., 1993a).

Effect of Elicitors on PLase A Activity in Soybean Cells

Because mastoparan is not a natural stimulant of plant defense responses, we examined the effects of several pathogen-generated elicitors on the induction of endogenous PLase A activity in soybean cells. OGA, a plant cell-wall component thought to be released during pathogen attack, was chosen because it was previously shown to initiate production of H_2O_2 by soybean cells (Legendre et al., 1992). Hepta-β-glucan, a component of certain pathogenic fungal cell walls (Sharp et al., 1984), and a crude extract from the fungus *V. dahliae* (Low and Heinstein, 1986) were also examined because they elicit phytoalexins in soybean cells. Finally, harpin, a heat-stable protein from the pathogenic bacterium *E. amylovora*, was selected because it elicits the hypersensitive response in tobacco (Wei et al., 1992). It is significant in defining the signaling pathways of these elicitors that all but one of the elicitors were essentially homogeneous: The hepta-β-glucan was synthetic, OGA (degree of polymerization approximately 14) was extensively purified from citrus pectin, and the recombinant harpin contained only minor impurities from its *E. coli* expression host. The final elicitor, a crude extract from *V. dahliae*, was not yet available in pure form. Mastoparan and Mas-17, its inactive analog, were also synthetically pure.

Of the five elicitors examined, only harpin, mastoparan, and the *V. dahliae* extract stimulated an increase in PLase A activity (Fig. 4). Treatment with OGA, Mas-17, and the hepta-β-glucan elicitors yielded bis-BODIPY-C_{11}-PC fluorescence tracings indistinguishable from controls. The most potent stimulant in our hands was recombinant harpin, which increased the slope of the fluorescence transition by a factor of 3. Importantly, the observed increase in fluorescence on treatment with the active elicitors was blocked by chlorpromazine-HCl and aristolochic acid (Fig. 5; harpin data not shown) in a dose-dependent manner, confirming that elicitor-induced PLase A activity was also sensitive to PLase A inhibitors. Similar results were also obtained using NBD-C_6-HPC to assay elicitor induction of PLase A activity (data not shown).

Participation of PLase A in the Soybean Oxidative Burst

To begin to evaluate whether an oxidative burst-signaling pathway might involve PLase A activation, each of the above elicitors was also tested for its ability to induce the oxidative burst in suspension-cultured soybean cells. Figure 6 shows that OGA, the *V. dahliae* extract, and harpin

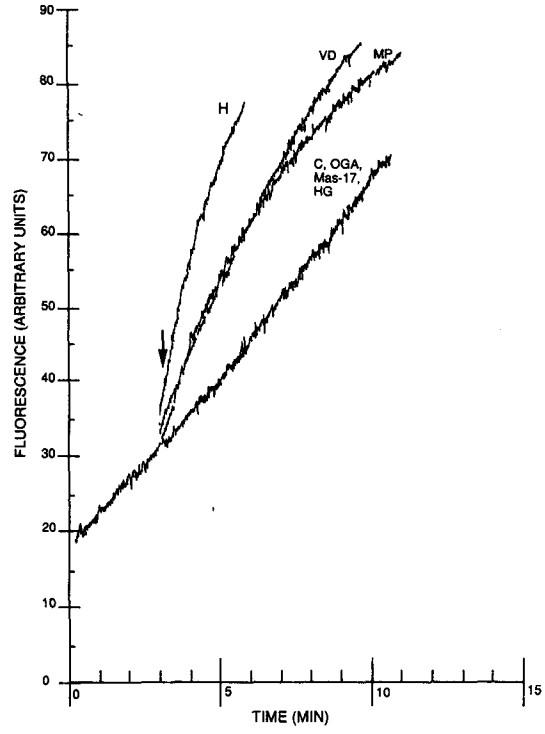

Figure 4. Effects of elicitors on PLase A activity in suspension-cultured soybean cells. Assays were conducted essentially as described in Figure 1A, except in addition to mastoparan (MP) and Mas-17, crude harpin (H, 115 μg/mL final concentration), OGA (4.8 μg/mL final concentration), hepta-β-glucan (HG, 5 μg/mL final concentration), and crude *V. dahliae* extract (VD, 100 μg/mL final concentration) were also tested. In each case, the elicitor was added (arrow) 3 min following commencement of stirring in the fluorescence spectrophotometer. As seen in Figure 1, an increase in fluorescence was observed because of stimulation of PLase A activity by mastoparan; in addition, harpin and the crude *V. dahliae* elicitor also stimulated PLase A activity. Since OGA, Mas-17, and hepta-β-glucan had no effect on PLase A activity, they are represented by a single control tracing (C). Similar stimulatory effects were observed in three independent experiments.

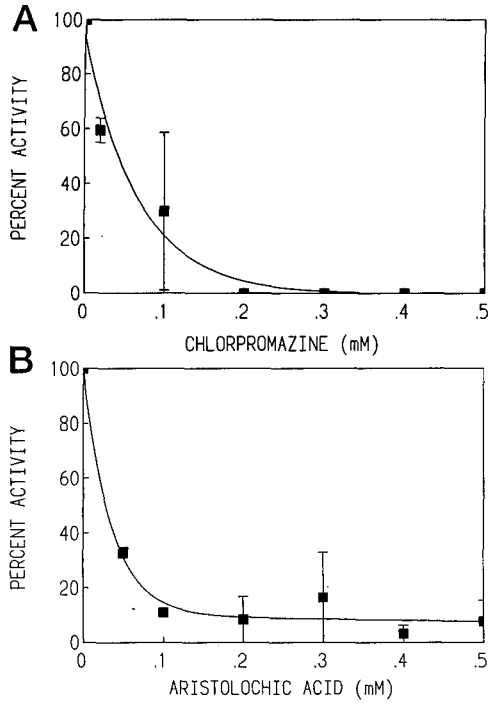

Figure 5. Effect of chlorpromazine-HCl and aristolochic acid on *V. dahliae*-induced PLase A activity. Assays were conducted as described in "Materials and Methods." Approximately 24-h-old cells were treated with varying concentrations of chlorpromazine-HCl (A) or aristolochic acid (B) and their effects on *V. dahliae* extract-induced PLase A activity was monitored by bis-BODIPY-C_{11}-PC fluorescence. Cells not treated with inhibitors served as controls. Data presented here are the averages of two independent experiments. The bars indicate SE.

readily induced a rapid and extensive release of H_2O_2. Production of H_2O_2 by harpin is consistent with the results of Baker et al. (1993) and with its role in eliciting the hypersensitive response. Mastoparan, as noted previously (Legendre et al., 1992), also elicited the H_2O_2 burst but on a much smaller scale. In contrast, hepta-β-glucan, Mas-17, and control buffer exerted no effect on H_2O_2 production, even though the hepta-β-glucan was present at a concentration sufficient to promote maximal phytoalexin biosynthesis (Sharp et al., 1984). Since OGA concentrations that induce a strong oxidative burst do not activate endogenous PLase A, we conclude that stimulation of PLase A is not an obligate step in all elicitor-initiated pathways leading to the oxidative burst.

Because linolenic acid, a common product of PLase A action, can independently induce an oxidative burst in the absence of elicitor (data not shown), it was still conceivable that PLase A activation could participate in signaling either the harpin- or *V. dahliae*-induced burst pathway. To evaluate this possibility, the effect of the PLase A inhibitor chlorpromazine-HCl on the two elicitor-induced bursts was also examined. Since chlorpromazine was found in control studies to directly inhibit the endogenous peroxidases involved in catalyzing H_2O_2-mediated dye quenching, its effect on $O_2^{\cdot-}$/H_2O_2 biosynthesis was measured by quantitating O_2 consumption in soybean cell suspensions with an O_2 electrode, i.e. an oxidative burst assay that is unaffected by the catalytic competence of cell-wall peroxidases. Consistent with the spectrofluorometric assay, OGA, *V. dahliae* extract, and harpin each stimulated a strong increase in O_2 consumption by the soybean cell suspension (data not shown). Importantly, however, only the *V. dahliae*-induced burst was quantitatively inhibited by chloropromazine; the OGA- and harpin-stimulated bursts remained largely unchanged upon inhibitor addition (Table I). These data confirm that the OGA-induced oxidative burst requires no participation of PLase A and that the harpin-triggered burst is transduced by a similarly unrelated pathway. In contrast, the *V. dahliae*-stimulated burst may involve PLase A activation, since both PLase A activation and the oxidative burst were blocked by chlorpromazine-HCl. Further studies with more specific PLase A inhibitors, as they become available, will obviously be necessary to confirm this hypothesis.

DISCUSSION

By three independent methods we have demonstrated that pathogen-generated elicitors can stimulate activation

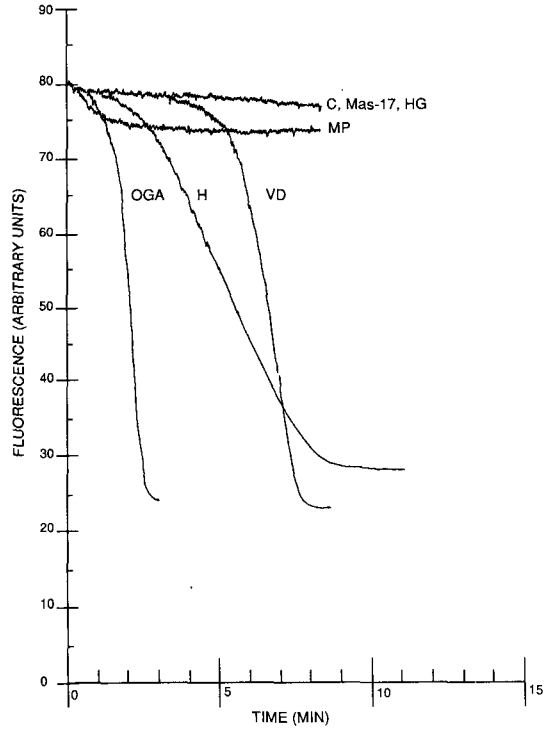

Figure 6. Elicitation of the rapid oxidative burst in suspension-cultured soybean cells. Assays were conducted as described in "Materials and Methods." Approximately 24-h-old cells were treated with the following elicitors: OGA (4.8 μg/mL final concentration), mastoparan (MP, 30 μg/mL final concentration), Mas-17 (30 μg/mL final concentration), crude harpin (H, 115 μg/mL final concentration), crude *V. dahliae* extract (VD, 100 μg/mL final concentration), and hepta-β-glucan (HG, 5 μg/mL). A fluorescence transition was observed because of H_2O_2-catalyzed oxidation of the fluorescent dye pyranine. In the absence of an elicitor (C), no decrease in fluorescence was observed. Similar stimulatory effects were seen in three independent experiments.

Table I. *Effect of the PLase A inhibitor chlorpromazine on elicitor-induced oxidative bursts*

Assays were conducted as described in "Materials and Methods," and percentage inhibition was evaluated from the reduction in the elicitor-induced rate of O_2 consumption (oxidative burst assay) or elicitor-stimulated rate of fluorescent phospholipid hydrolysis (PLase A assay) in the presence of PLase A inhibitors. The data are the averages of two independent experiments performed on separate days.

Elicitor	Percentage Inhibition	
	O_2 consumption	PLase A
V. dahliae[a]	99.7 ± 0.3	100
Harpin[b]	2.6 ± 1.4	100
OGA[c]	5.1 ± 5.1	0

[a] Added to a final concentration of 100 μg protein mL^{-1}. [b] Added to a final concentration of 100 μg protein mL^{-1}. [c] Added to a final concentration of 10 μg oligogalacturonide mL^{-1}.

of an A-type PLase in soybean cells. Because the macromolecular elicitors used are impermeable to the soybean cell, and since the fluorescent dyes reporting on PLase A activity are localized largely to the plasma membrane, we assume that the activated fraction of PLase A activity may be associated with the plasma membrane. Such a location would be consistent with its possible role in signaling a defense response.

To the best of our knowledge, this is the first demonstration of induction of PLase A activity by any plant defense elicitor. Although some evidence was offered that this induction may be important to the *V. dahliae*-stimulated oxidative burst, the possible role of the phospholipid hydrolysis products in promoting other defense responses such as phytoalexin biosynthesis, the hypersensitive response, and the induction of pathogenesis-related gene expression should not be overlooked. An especially interesting question that remains to be addressed is whether the activation of PLase A results in the production of jasmonic acid and the consequent stimulation of its downstream defense products.

Since chlorpromazine, an inhibitor of PLase A, was found to block *V. dahliae* extract stimulation of PLase A and oxidant generation by soybean cells, it is conceivable that an activated PLase A participates in signaling the oxidative burst induced by this elicitor. In contrast, the complete absence of PLase A activation by OGA together with the insensitivity of the OGA-induced burst to chlorpromazine argues equally strongly that the OGA-triggered pathway does not involve PLase A. One OGA-stimulated pathway has already been demonstrated to involve hetereotrimeric G proteins (Legendre et al., 1992) and activation of PLase C (Legendre et al., 1993a). Thus, reconciliation of these data requires that at least two independent pathways be capable of signaling the soybean oxidative burst. A minimum of three independent routes for activation of the soybean oxidase complex must now exist, considering previous evidence for both PLase C-dependent and -independent pathways for the OGA-induced burst.

In view of the similarities between the plant and neutrophil oxidative bursts (Low and Dwyer, 1994), the multiplicity of signaling pathways found in the plant kingdom should not seem surprising. For example, immune complexes but not chemotactic peptides require PLase A activation to trigger the neutrophil oxidative burst (Meshulam et al., 1992). Similarly, insoluble immune complexes but not soluble immune complexes require activation of Tyr kinases in their signaling pathway (Morel et al., 1991). Since stimulants of the neutrophil burst also differ in their requirements for PLase C activation (Morel et al., 1991), the existence of several signaling pathways that can independently promote assembly of the neutrophil oxidase complex is now well accepted. Our recent observation that homologous oxidase components exist in plants (Dwyer et al., 1996) reinforces the expectation that multiple pathways could also lead to plant oxidase activation.

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