Phosphoinositide Breakdown During the K^+/H^+ Exchange Response of Tobacco to *Pseudomonas syringae* pv. *syringae*

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The hypersensitive response (HR) of tobacco to *Pseudomonas syringae* pv. *syringae* involves activation of a K^+/net H^+ exchange response (XR). Dependence of this response on calcium influx suggests that it is triggered by a signal transduction pathway. To test this hypothesis, we studied *in vivo* phospholipid metabolism during the XR. Suspension-cultured tobacco cells were incubated with ^3H- or ^{14}C-labeled fatty acids to radiolabel endogenous lipids. Cells were then inoculated with *P. s.* pv. *syringae* or with a *hrp* mutant that does not induce the HR or the XR in tobacco. In XR-induced cells, radioactivity in phosphatidylinositol decreased 20–30% within 4 hr, relative to cells inoculated with the *hrp* mutant. In contrast, radioactivity in other phospholipids remained constant or increased over this time period. When tobacco cells were pulse-labeled with ^{32}Pi during the XR, radioactivity in phosphatidylinositol and phosphatidylinositol 4-phosphate was reduced while radioactivity in other phospholipids was markedly increased. Phosphatidylinositol breakdown, calcium influx, and the XR were inhibited by bromophenacylbromide, a phospholipase inhibitor, and by neomycin, which blocks metabolism of polyphosphoinositides. These results suggest that increased activity of phosphoinositide-specific enzymes, possibly phospholipase C and/or phosphoinositide kinases, play a role in XR activation.

Disease resistance of higher plants is frequently associated with a hypersensitive response (HR). This response causes the rapid death of individual plant cells that interact with the pathogen and is accompanied by the transcriptional activation of an array of defense genes (Lamb *et al.* 1989; Graham and Graham 1991). These events prevent the pathogen from infecting additional plant cells. It is widely postulated that disease resistance and HR induction in plants are triggered by the specific binding of pathogen elicitors to plant cell receptors (Gabriel and Rolfe 1990). The purification of HR elicitors from bacteria expressing an avirulence gene, *avrD,* from *Pseudomonas syringae* pv. *tomato* (Keen *et al.* 1990) and a *hrp* gene cluster from *Erwinia amylovora* (Wei *et al.* 1992) supports this hypothesis. However, plant receptors for these elicitors have not been identified.

Induction of the hypersensitive response in various plant species by phytopathogenic bacteria has been extensively studied (Klement 1986). Hypersensitive cell death of cultured tobacco cells inoculated with incompatible *P. syringae* pathovars is preceded by a K^+ efflux/net H^+ uptake exchange response (XR) (Atkinson *et al.* 1985; Baker *et al.* 1987). This response is not induced by saprophytic bacteria, by *Escherichia coli,* or by *hrp* mutants of *P. syringae* pathovars or of *Erwinia amylovora* (Baker *et al.* 1987; Wei *et al.* 1992). It is induced weakly or not at all by compatible pathogens (Baker *et al.* 1991). Cultivar-specific hypersensitivity (controlled by known resistance and avirulence genes) of soybean to *P. s.* pv. *glycinea* involves XR activation (Orlandi *et al.* 1992) as does the HR of tobacco to harpin, an elicitor from *E. amylovora* (Wei *et al.* 1992). The XR may contribute to hypersensitive plant cell death by causing massive K^+ loss and destruction of the plasma membrane H^+ gradient in plant cells. The XR is closely linked to active oxygen generation, which may independently contribute to cell death (Baker *et al.* 1991).

Several characteristics of the XR suggest that it is mediated by an ion channel or other transporter. For example, the specificity of K^+ efflux and its tight coupling to net H^+ uptake are inconsistent with electrolyte loss via nonspecific membrane damage. In addition, the XR appears to be regulated by calcium. Its initiation in tobacco cells coincides with an increase in calcium influx (Atkinson *et al.* 1990). Calcium influx, the XR, and the HR can be prevented by La^{3+}, Co^{2+}, and Cd^{2+} ions, which block non-voltage-regulated calcium channels (Hille 1984). These data suggest that the XR is activated by a signal transduction pathway that includes calcium influx. Since phospholipases A_1, A_2, C, and D, are all implicated in eukaryotic signal transduction (Berridge 1986), we asked whether one or more of them plays a role in XR initiation. To answer this question, we have conducted studies of *in vivo* phospholipid metabolism during XR activation. We show here that increased phosphoinositide breakdown accompanies the XR and calcium influx responses of tobacco to *P. s.* pv. *syringae.* These results are consistent with involvement of phosphoinositide-specific phospholipase C in a signal transduction pathway for XR activation.

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RESULTS

We used a *hrp* mutant, strain B7, as a control for these experiments because it differs from the wild-type *P. s.* pv. *syringae* strain only by a transposon insertion in its *hrp* gene cluster. This is particularly important since saprophytic bacteria and *hrp* mutants of phytopathogenic bacteria elicit HR-independent responses in plant cells (Baker *et al.* 1991; Jakobek and Lindgren 1993). Since we wanted to screen out such responses, a *hrp* mutant was the best available control for our studies. *P. s.* pv. *tabaci*, which is compatible on tobacco, is also XR$^-$ (Baker *et al.* 1991), but its use as a control would have introduced unknown genetic variables into these experiments.

Lipid breakdown during HR initiation.
We labeled tobacco cells with radiolabeled arachidonic and linolenic acids because both contain *cis*-1,4-pentadiene systems and are thus good substrates for lipoxygenase, which may play a role in HR initiation (Croft *et al.* 1990; Keppler and Novacky 1987). Although linolenic acid is abundant in most plant species, arachidonic acid has not been detected in angiosperms (Hitchcock and Nichols 1971; Anderson 1989). Nevertheless, we included the latter because it is readily incorporated into plant lipids and because extensive information on its metabolism in animal cells is available.

After the 24- to 48-hr radiolabeling period, tobacco cells were washed and inoculated with *P. s.* pv. *syringae* (Hrp$^+$ or Hrp$^-$), and lipids were extracted at hourly intervals. Table 1 lists the average starting (0 hr) radioactivities in the major phospholipid and neutral lipid fractions. The data listed are from tobacco cells inoculated with the Hrp$^-$ strain. When tobacco cells were labeled with [^{3}H]arachidonic acid, approximately 50% of total radioactivity was found in phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), triacylglycerol (TG), and diacylglycerol (DG). This value was 60–65% when cells were labeled with [^{14}C]linolenic acid. Over the 5-hr test period, radioactivities of lipid species in Hrp$^-$-treated cells remained constant or increased up to 10 or 15% (data not shown) due to basal lipid metabolism under these conditions. Up to 50% of total radioactivity in tobacco lipid extracts was not accounted for by the lipid fractions we measured. Other radioactive fractions may have included free fatty acids and monoacylglycerol, neither of which was well resolved by our thin-layer chromatography (TLC) procedure. It is also plausible that some radioactivity may have been incorporated into nonlipid compounds. Thus, increased radioactivity in some lipid species over the test period may reflect their net synthesis from radioactive precursors in tobacco cells.

The 0-hr values for Hrp$^+$ lipid fractions did not vary by more than 5% from those of the Hrp$^-$control. This is indicated by Figure 1, which presents the radioactivities of lipids in the 0- to -5-hr Hrp$^+$ extracts relative to the Hrp$^-$ controls. During the XR, radioactivity in PI and DG decreased between 1 and 5 hr after inoculation, and this was initially accompanied by increased radioactivity in PC, PE, and TG, relative to the Hrp$^-$ controls. We obtained qualitatively similar results when cells were labeled with either [^{3}H]arachidonic or [^{14}C]linolenic acids. In each case, two experiments were conducted and all four experiments exhibited the trends discussed above. The data indicate that PI breakdown began at the time of XR initiation or shortly thereafter. Increased extracellular pH was detected within 1 hr after inoculation, while decreased radioactivity in PI was not detected until 2 hr. However, our methods of lipid analysis were less sensitive than the pH measurements used to monitor the XR. In some experiments, we observed decreased radioactivity in PC and PE in the 5-hr lipid extracts of Hrp$^+$-treated cells.

To further study phospholipid metabolism during the XR, we pulse-labeled tobacco cells with ^{32}P*i* after inoculation with bacteria and extracted their lipids. Total uptake of ^{32}P*i* by Hrp$^+$-treated cells was about one half that of the Hrp$^-$ controls as judged by the amount of radioactivity in the aqueous phase after lipid extraction. For the experiment shown in Table 2, 100-μl aliquots of aqueous fractions from control cells contained 3,433 ± 643 cpm, while equivalent aliquots from XR-induced cell fractions contained only 1,765 ± 86 cpm. Reduction of the plasma membrane H$^+$ gradient by the XR may be responsible for this difference, since P*i* uptake in plant cells is coupled to OH$^-$ efflux or H$^+$ uptake (Lin 1985). When corrected for total ^{32}P*i* uptake as described in the methods section, radioactivity in PE, PC, PG, and PA was increased 1.7- to 4-fold in XR-induced cells, relative to controls. Although radioactivity in phosphatidylserine (PS) was insufficient for scintillation counting, autoradiograms of TLC plates suggested that its labeling was also enhanced in XR-induced cells. In contrast, radioactivity in PI and phosphatidylinositol 4-phosphate (PIP) was only 25–50% that of controls. Radioactivity in LPIP from Hrp$^-$- and Hrp$^+$-inoculated cells was not significantly different. Prolonged exposure of X-ray film to TLC plates revealed a radiolabeled product that comigrated with phosphatidylinositol 4,5-bisphosphate (PIP$_2$) standards. However, the radioactivity in these bands could not be reliably quantified. We also extracted lipids from tobacco cells that were exposed to ^{32}P*i* for 45- and 90-min periods beginning at 2 hr after inoculation. These experiments gave results

Table 1. Incorporation of [^{14}C]linolenic and [^{3}H]arachidonic acids into tobacco lipids

		dpm/50 μl lipid extract[a]			
Lipid	**Experiment**	**dpm [^{14}C]**	**% Total**	**dpm [^{3}H]**	**% Total**
PI	1	3,806	6.8	4,968	7.6
	2	8,668	7.4	9,600	9.8
PC	1	18,955	34.1	11,830	18.1
	2	39,476	33.7	18,767	19.3
PE	1	6,831	12.3	6,392	9.9
	2	14,946	12.8	11,567	12.0
TG	1	4,174	7.5	7,194	11.0
	2	8,130	6.9	13,414	13.8
DG	1	1,101	2.0	1,240	1.9
	2	2,914	2.5	2,479	2.5
Total	1	55,626	100	65,398	100
	2	117,217	100	97,435	100

[a] Tobacco cells were incubated with radiolabeled fatty acids for 24-40 hr. Cells were washed and inoculated with bacteria (Hrp$^-$ strain) as described for Figure 1 and lipids were extracted at 0 hr after inoculation. Data are actual values from four independent experiments and are normalized as described in the text.

that were qualitatively similar to those described above (data not shown).

Effects of inhibitors on tobacco cell responses.

Bromophenacylbromide (BPAB), a nonspecific phospholipase inhibitor (Schibeci and Schacht 1977), and neomycin, a compound that binds polyphosphoinositides and prevents their metabolism (Shen and Winter 1977), abruptly stopped extracellular alkalinization in the XR-induced cells (Fig. 2). Neomycin alone caused some alkalinization in Hrp$^-$ treatments and alkalinization in Hrp$^+$ treatments was reduced only to this level. This suggests an underlying toxicity that perturbs the plasma membrane or perhaps decreases availability of ATP to drive H$^+$ extrusion. The IC$_{50}$ values were 5 and 50 μM, respectively, for BPAB and neomycin (Fig. 3). These compounds were

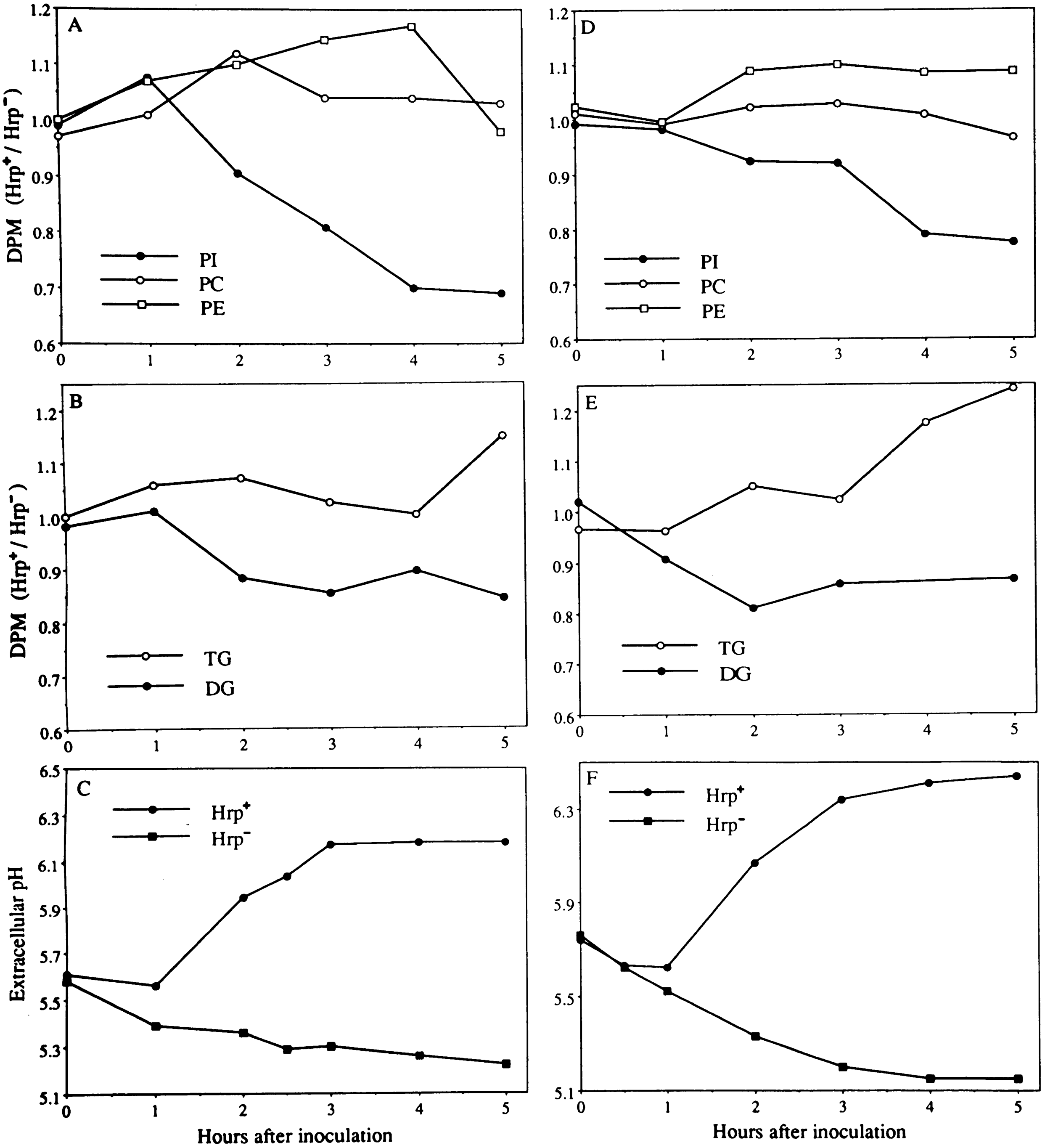

Fig. 1. Radioactivity in **A**, phospholipids; **B**, neutral lipids; and **C**, extracellular pH during the XR of tobacco cells to *Pseudomonas syringae* pv. *syringae*. Suspension-cultured tobacco cells were labeled with [^{3}H]arachidonic acid and then inoculated with *P. s.* pv. *syringae* (Hrp$^+$) or with a *hrp* mutant (Hrp$^-$) that does not induce the XR or the HR in tobacco. Tobacco lipids were extracted at hourly intervals and were purified by TLC. **D-F** same as **A-C**, except that tobacco cells were labeled with [^{14}C]linolenic acid. Data in each graph are means of two experiments that yielded qualitatively similar results.

inhibitory even when added after extracellular alkalinization was well under way. Although neomycin inhibits protein synthesis, another protein synthesis inhibitor, cycloheximide at 40 μg/ml, inhibited extracellular alkalinization only weakly and only after 1 hr. In contrast, 20 μg/ml rapidly stopped the incorporation of ^{35}S-methionine into TCA-precipitable material (data not shown). Thus, the rapid effects of neomycin on the XR were not likely caused by inhibition of protein synthesis. Neomycin, BPAB, and La^{3+} inhibited PI breakdown in XR-induced tobacco cells (Table 3). Calcium influx, previously shown to be blocked by La^{3+} (Atkinson *et al.* 1990) was also inhibited by neomycin and BPAB (Fig. 4).

DISCUSSION

Our results show that activation of the XR in tobacco by *P. s.* pv. *syringae* is accompanied by *in vivo* breakdown of phosphoinositides. Equally important, breakdown of PC and PE is not increased during the same time period. These results are consistent with the stimulation of phosphoinositide-specific PLC during the XR. Concurrent stimulation of PLA_1, PLA_2, or PLD is unlikely since phosphoinositide-specific forms of these activities have not been encountered in eukaryotes. There remains the possibility of XR-linked breakdown of PC and PE at 5 or more hours after inoculation. If this occurs, however, it lags behind phosphoinositide breakdown by 2 or 3 hr.

Our results add to many reports that suggest a role for phosphoinositide metabolism in plant cell signaling (Boss 1989; Einspahr and Thompson 1990; Morse *et al.* 1989). Phosphatidylinositol breakdown in carrot cells treated with an elicitor of phytoalexin synthesis (Kurosaki *et al.* 1987) is particularly relevant to our findings. Our data clearly showed increased breakdown of PIP as well as PI in XR-induced tobacco cells. Since our methods were not sensitive enough to detect PIP_2, we cannot be certain that its breakdown was also increased. Although known PLC isozymes favor PIP_2 or PIP over PI *in vitro*, they accept all of the phosphoinositides as substrates (Meldrum *et al.* 1991; Sandelius and Sommarin 1990; Wheeler and Boss 1990). In both animal and plant cells, PIP_2 levels are much lower than those of PI or PIP (Boss 1989; Fain 1990). Although PIP_2 is rapidly depleted by PLC activity, it can be replenished by phosphorylation of PI and PIP by their respective kinases. Thus increased PI and PIP breakdown during the XR may reflect the action of either PLC and/or PI and PIP kinases on these substrates.

Table 2. ^{32}P-labeling of phospholipids in exchange response-induced tobacco cells[a]

Phospholipid	dpm/Hrp$^-$	dpm-Hrp$^+$	Hrp$^+$/Hrp$^-$
PI	651 ± 28	318 ± 28	0.49
PIP	1,100 ± 210	300 ± 79	0.27
LPIP	59 ± 14	61 ± 34	1.03
PE	1,713 ± 375	4,831 ± 416	2.82
PC	108 ± 19	291 ± 12	2.69
PG	626 ± 115	2,594 ± 263	4.14
PA	117 ± 20	203 ± 39	1.74

[a] Tobacco cells were inoculated with bacteria as described in the legend to Figure 1. ^{32}Pi was added to tobacco cell suspensions at 2 hr after inoculation and lipids were extracted 20 min later. Phospholipids were purified by thin-layer chromatography. Data are means ± SD of three replicates from an experiment representative of two.

Confirmation of PLC involvement in XR activation will require evidence that DG and inositol phosphates, the products of PLC activity, are generated. We did not observe increased radioactivity in DG, but this would be expected only if DG had been released by PLC but not further metabolized. Furthermore, DG released from a phosphoinositide pool of reduced radioactivity would also

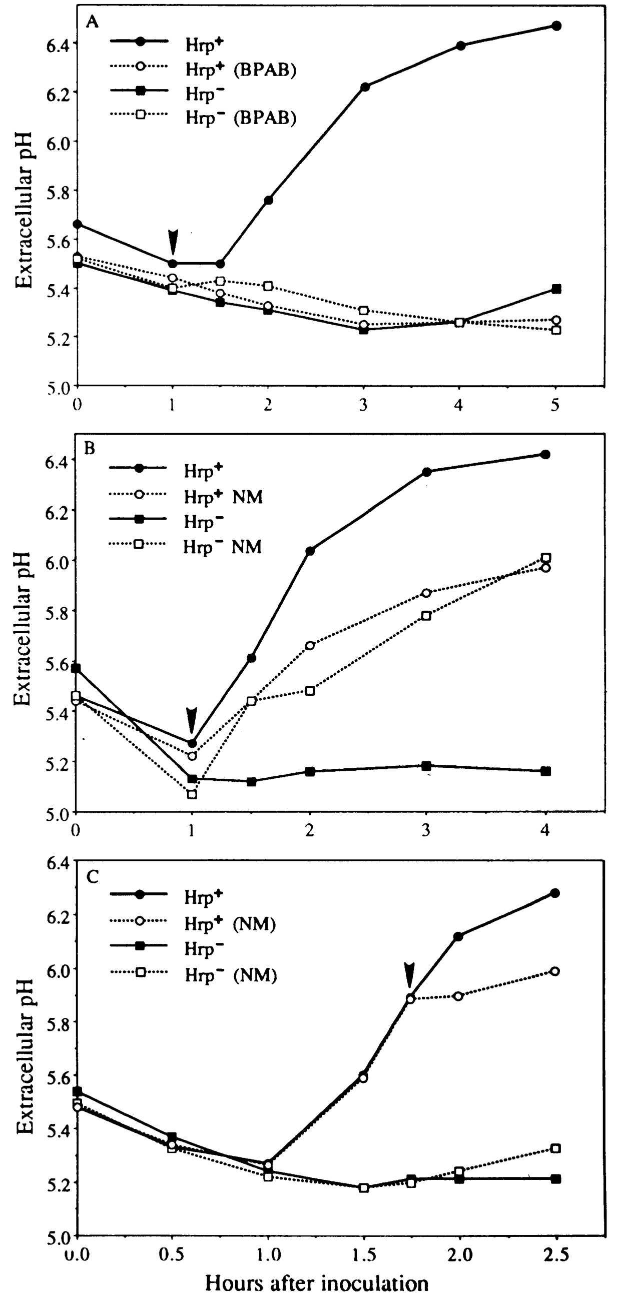

Fig. 2. Effect of inhibitors on the XR of tobacco cells to *P. s.* pv. *syringae*. **A,** Effect of 10 μM BPAB added 1 hr after inoculation as indicated by arrow; **B, C,** Effect of 100 μM neomycin (NM) added after 1 hr or 1.75 hr. Tobacco cells were inoculated with bacteria as described for Figure 1. Data are means of two replicates from experiments representative of three.

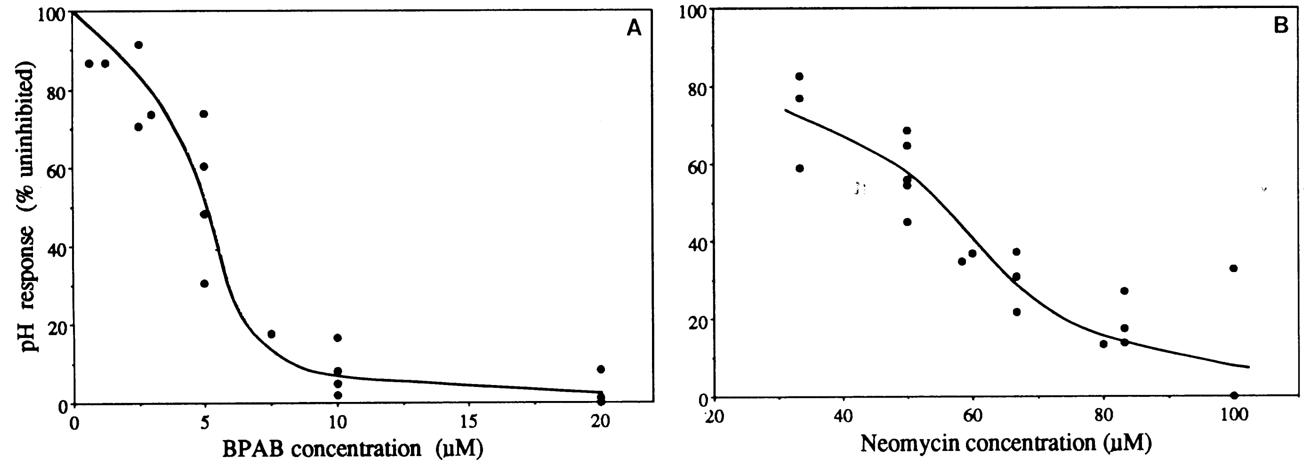

Fig. 3. Dependence of XR on inhibitor concentrations. **A,** BPAB; **B,** neomycin. Tobacco cells were inoculated with bacteria as described in Figure 1. Inhibitors were added at 1 hr and the change in extracellular pH between 1.5 and 3 hr was determined for each sample. The pH response values were obtained by subtracting Hrp$^-$ values from those of Hrp$^+$-treated cells. Each graph shows combined data from three to five experiments.

Table 3. Effect of inhibitors on phosphatidylinositol radioactivity during the exchange response of tobacco cells

Sample	dpm in PI[a]	Average dpm	Hrp$^+$/Hrp$^-$
Hrp$^-$	3881-4114	3997	
Hrp$^+$	2860-2898	2879	0.72
Hrp$^-$ + $LaCl_3$	3571-3844	3707	
Hrp$^+$ + $LaCl_3$	3630-3784	3707	1.00
Hrp$^-$ + BPAB	3813-3974	3894	
Hrp$^-$ + BPAB	3589-3775	3682	0.95
Hrp$^-$ + Neomycin	3300-3640	3470	
Hrp$^+$ + Neomycin	3472-3526	3499	1.01

[a] Tobacco cells were prelabeled with [^{14}C]linolenic acid and inoculated with bacteria as described in Figure 1. Lanthanum (250 µM $LaCl_3$), BPAB (20 µM), and neomycin (100 µM) were added to tobacco cell suspensions at 0.75 hr after inoculation. Lipids were extracted from tobacco cells at 5 hr and purified by TLC. Data are actual values and means from an experiment representative of two.

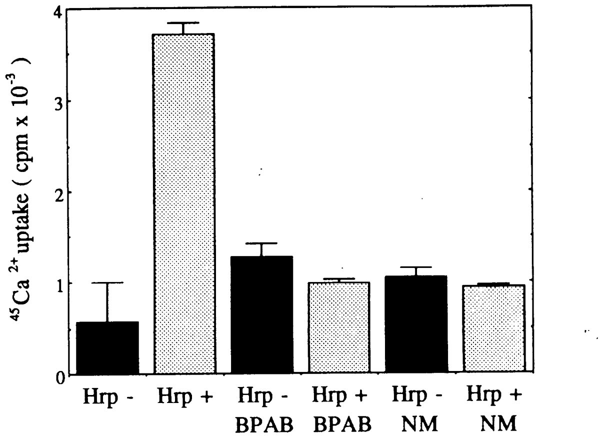

Fig. 4. Effect of inhibitors on calcium influx during the XR of tobacco to *P. s.* pv. *syringae.* Tobacco cells were inoculated with bacteria as described in Figure 1. BPAB (20 µM) and neomycin (NM, 100 µM) were added to tobacco cell suspensions 1.25 hr after inoculation. ^{45}Calcium uptake was measured between 1.5 and 2.5 hr after inoculation. Data are means ± SD from an experiment representative of two.

be weakly labeled. However, two observations suggest that DG was continuously generated and metabolized in XR-induced tobacco cells. A major metabolic pathway for DG involves its phosphorylation to phosphatidic acid, via DG kinase, which can then be converted to PC, PE, PI, and other phospholipids (Kanoh *et al.* 1990). In cells labeled with [^{14}C]fatty acids, increased radioactivity in PC and PE that accompanies loss of radioactivity in PI is consistent with incorporation of radiolabeled DG into phospholipids. Similarly, increased incorporation of ^{32}Pi into phosphatidic acid and phospholipids during the XR suggests rapid phosphorylation of diacylglycerol to phosphatidic acid. Similar results are observed in PLC-stimulated mammalian cells given exogenous ^{32}Pi (Sekar and Hokin 1986 and references therein). A second pathway for DG metabolism involves lipase-catalyzed release of free fatty acids (Bishop and Bell 1986). Although we did not measure radioactivity in free fatty acids, involvement of this pathway in XR activation should not be discounted. Acyl-lipid hydrolase activity is stimulated in bean leaves during the HR induced by *P. s.* pv. *phaseolicola* (Croft *et al.* 1990), and the fatty acid composition of phospholipids is altered during the HR of cucumber and tobacco to *P. syringae* pathovars (Ádám *et al.* 1989; Keppler and Novacky 1989).

Although increased phosphoinositide breakdown has not been previously associated with HR initiation, there are reports of increased breakdown of other lipids. For example, phospholipase D activity is stimulated in tobacco leaves during the HR induced by *Erwinia amylovora* (Huang and Goodman 1970). It is not clear how these results relate to ours. In some of our experiments, radioactivity in PE and PC from XR-induced cells decreased between 4 and 5 hr after inoculation. This could reflect delayed stimulation of phospholipase A_1, A_2, or D activities. It is also plausible that HR-linked fatty acid turnover in phospholipids (Ádám *et al.* 1989; Keppler and Novacky 1989) and increased lipase activity (Croft *et al.* 1990) reflect metabolism of PLC-generated DG. In summary, our results are not inconsistent with those reported elsewhere, but additional experimentation is warranted.

The association of phosphoinositide breakdown with the XR, as well as the inhibition of this response and calcium influx by neomycin and BPAB, support a causal role for phosphoinositide metabolism in XR activation. Since BPAB and neomycin have nonspecific effects on cells, however, we cannot be certain that inhibition of cell responses is due to the effects of these compounds on phosphoinositide metabolism. This is particularly a concern with neomycin, which increased net H^+ uptake in Hrp$^-$-treated cells. Interestingly, we found that $LaCl_3$ also blocked PI breakdown. In some mammalian signaling pathways, receptor-mediated PLC stimulation and calcium influx are interdependent. This reflects the initial activation of calcium channels by inositol phosphates and subsequent feedback stimulation of PLC activity by calcium (Rhee *et al.* 1989; Fain 1990). Whether this applies to the XR must be further explored.

Because phosphoinositide breakdown and calcium influx are receptor-regulated in other eukaryotes, our results support an elicitor-receptor model for the XR and for HR-associated resistance. They also raise the question of whether transcriptional activation of defense genes is triggered by some part(s) of the apparent XR signal transduction pathway. Such a scenario is consistent with elicitor-induced PI breakdown (Kurosaki *et al.* 1987), sensitivity of gene induction to calcium channel blockers (Stäb and Ebel 1987), and other indications that elicitors-trigger gene activation via signaling pathways (Dixon and Lamb 1990).

MATERIALS AND METHODS

Plant and bacterial cultures.

Tobacco callus cells (*Nicotiana tabacum* 'Hicks') were grown as suspension cultures as previously described (Atkinson *et al.* 1985). Cultures were diluted with equal volumes of fresh medium every 5 days. For experiments, 8–12 g of tobacco cells was collected by filtration from 2- or 3-day old cultures. Cells were rinsed with 100 ml of assay solution (0.5 mM Mes-Tris, pH 6.0, 0.5 mM K_2SO_4, 0.5 mM $CaCl_2$, 0.175 M mannitol) and 0.5-g samples were suspended in 12 ml of this solution. Cell suspensions were immediately placed in a rotary shaker at 28° C. After a 1-hr adaptation period, tobacco cells were inoculated with an avirulent strain (61) of *P. s.* pv. *syringae* (Baker *et al.* 1987), a bean pathogen. This strain induces a strong and rapid HR in tobacco. As controls, tobacco cells were inoculated with a transposon insertion mutant (strain B7) that induces neither the HR nor the XR in tobacco (Baker *et al.* 1987). The Tn*5* insertion in this mutant lies within an *hrp* gene cluster that is required for pathogenicity and HR induction (Huang *et al.* 1988). Other *hrp* mutants of this bacterium have indistinguishable XR$^-$ and HR$^-$ phenotypes on tobacco and the original selection of strain B7 as a control was arbitrary. To inoculate tobacco cells, bacteria were grown overnight on Kings B agar (King *et al.* 1954) and then shaken in assay solution for 3 hr at 28° C. Bacteria were pelleted by centrifugation and resuspended to 10^9 bacteria per milliliter of solution; 180 μl of this suspension was added to each tobacco cell suspension to yield a final population of 1.5 $\times 10^7$ bacteria per milliliter. All experiments were performed at least twice with similar results.

Extraction of radiolabeled lipids from tobacco cells.

Radiolabeled fatty acids (either [^{3}H]arachidonic or [^{14}C]linolenic) in ethanol or toluene were added to a sterile silanized flask, and the solvent was evaporated under N_2. Approximately 20 ml of a 1- to 2-day-old tobacco cell culture was added to this flask and to an identical flask but without the radiolabeled fatty acid. Flasks were sealed aseptically and returned to the shaker for an additional 24–40 hr. For XR induction, tobacco cells (both labeled and unlabeled) were collected by filtration, washed with 100 ml of assay solution, resuspended at a density of 10 g (wet weight)/12 ml, and returned to the shaker. After 30 min, cells were collected again by filtration. They were suspended in assay solution and inoculated as described in the previous section, except that the 1-hr adaptation period was reduced to 30 min. Lipids were extracted at hourly intervals after inoculation. For experiments with ^{32}P*i*, tobacco cells were suspended in assay solution and inoculated with bacteria as described in the previous section, except that 0.5 g of cells was suspended in 7 ml rather than 12 ml of assay buffer. At 2 hr after inoculation, each cell suspension received 5–10 μCi of ^{32}P*i*. Lipids were extracted 20 min later.

To extract lipids, tobacco cells (0.5 g) were collected by filtration and washed with 50 ml of either distilled H_2O (^{3}H- and ^{14}C-labeled cells) or assay solution (^{32}P-labeled cells). Washed cells were transferred to glass flasks and extracted with acidified chloroform and methanol (Boss and Massel 1985). Chloroform phases were evaporated under a stream of N_2, and lipids were redissolved in 0.25–1 ml of chloroform. For experiments with ^{32}P*i*, the aqueous phase was saved for quantification of radioactivity. Lipid samples were stored at −20° C.

TLC of lipid extracts.

For analysis, 50- to 100-μl aliquots of radiolabeled lipid extracts were spotted onto TLC plates. Phospholipids and neutral lipids were separated on silica gel 60 as described by Dixon and Hokin (1984). Phospholipids were separated in the first dimension with tetrahydrofuran/acetone/methanol/water (50:20:40:8) and in the second dimension with chloroform/acetone/methanol/acetic acid/water (50:20:10:15:5). Neutral lipids were separated with hexane/diethylether/acetic acid (65:35:4). Phosphoinositides were separated with chloroform/methanol/water/ammonium hydroxide (90/90/22/7) on EDTA/sodium tartrate-treated silica gel 150 plates. Two plates were developed simultaneously in each tank: One was spotted with lipid extract from Hrp$^+$-inoculated tobacco cells and the other with the corresponding Hrp$^-$ extract. After development, lipids were stained with iodine vapor. Radiolabeled lipids were located by comparison with authentic lipid standards and by autoradiography [^{14}C, ^{32}P]. Spots on plates corresponding to PI, PIP, PC, PE, TG, or DG were scraped into scintillation vials and lipids were eluted with 1 ml of methanol. These lipids were well resolved from others on TLC plates. Radioactivity was determined by liquid scintillation counting. Data from each experiment were normalized to the average dpm/sample (Dixon and Hokin 1984) as follows:

Corrected dpm = dpm in spot × ave total dpm from all $CHCl_3$ extracts/total dpm in individual extract.

In experiments with $^{32}\mathrm{P}i$, our results indicated reduced uptake of $^{32}\mathrm{P}i$ by Hrp$^+$-inoculated tobacco cells relative to Hrp$^-$ controls. Radioactivity in phospholipid fractions was therefore corrected for phosphate uptake (Martin 1986) as follows:

Corrected dpm = dpm in spot × ave total dpm in all aqueous fractions/dpm in individual aqueous fraction.

Effects of inhibitors on tobacco cell responses.

Tobacco cell suspensions were prepared and inoculated as described in the section on plant and bacterial cultures. Inhibitors were dissolved in water ($LaCl_3$ and neomycin) or ethanol (*p*-bromophenacylbromide [BPAB]) and 12–48 μl of these solutions was added to cell suspensions at 0.75–1.75 hr after inoculation, as indicated. Untreated cell suspensions received equivalent volumes of solvent and final ethanol concentration did not exceed 0.4%. To measure calcium influx, $[^{45}Ca]Cl_2$ was added to cell suspensions at 1.5 hr after inoculation. Calcium uptake was stopped after 2 hr by adding 10 mM $CaCl_2$ and 0.25 mM $LaCl_3$ to the assay solution. Tobacco cells were then washed and their radioactivity was measured as previously described (Atkinson *et al.* 1990).

Materials.

$[^3H]$arachidonic acid, $^{32}\mathrm{P}i$, and $[^{45}Ca]Cl_2$ were obtained from New England Nuclear. $[^{14}C]$Linolenic acid was obtained from Amersham. Neomycin, BPAB, and lipid standards were obtained from Sigma.

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