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Transient Activation of Plasmalemma K^+ Efflux and H^+ Influx in Tobacco by a Pectate Lyase Isozyme from *Erwinia chrysanthemi*[1]

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ABSTRACT

A purified pectate lyase isozyme derived from *Erwinia chrysanthemi* induced rapid net K^+ efflux and H^+ influx in suspension-cultured tobacco cells. Comparable fluxes of other ions (Na^+, Cl^-) were not observed. The K^+ efflux/H^+ influx response began within 15 minutes after addition of enzyme to cell suspensions and continued for approximately 1 hour after which cells resumed the net H^+ efflux exhibited prior to enzyme treatment. The response was not prolonged by a second enzyme dose 1 hour after the first. The K^+/H^+ response was characterized by saturation at low enzymic activity (2×10^{-3} units per milliliter), and inhibition by the protonophore, carbonyl cyanide *m*-chlorophenylhydrazone, and was not associated with membrane leakiness caused by structural cell wall damage. The total K^+ loss and H^+ uptake induced by enzyme was one-fourth to one-third that induced by *Pseudomonas syringae* pv. *pisi* and did not reduce cell viability. These results indicate that pectate lyase induces a K^+ efflux/H^+ influx response in tobacco similar to but of shorter duration than that induced by *P. syringae* pv. *pisi* during the hypersensitive response. Pectate lyase or other cell wall degrading enzymes may therefore influence the induction of hypersensitivity.

Pectic enzymes are produced by a large number of plant pathogenic bacteria and fungi and appear to play important roles in cell wall penetration and tissue maceration (8). Evidence is growing that these enzymes possess additional activities as elicitors of host defense mechanisms. Synthesis of phytoalexins can be induced in several hosts by pectic enzymes (11, 14, 16, 19, 21, 28) from various sources. A pectic enzyme has also been shown to elicit synthesis of proteinase inhibitor proteins in tomato which are believed to provide protection against insect pests (10, 28). In these cases, elicitor activity resides in oligosaccharides released from the host cell wall by the enzyme. Although an oligosaccharide elicitor has not been implicated, a crude mixture of plant cell wall degrading enzymes has been shown to induce ethylene synthesis in tobacco (1). Other reports have associated ethylene production with pathogen defense reactions (23) and treatment with various polysaccharide elicitors of defense mechanisms (12, 24).

Pectic enzymes may also be involved in both the expression and the suppression of the HR[3]. Other reports have correlated pectate lyase (17) or polygalacturonase (18) activity with hypersensitive-like reactions. However, Azad and Kado (5) found that *Erwinia rubrifaciens* mutants which were unable to induce a HR in tobacco still produced large quantities of pectate lyase. It was concluded that this enzyme was not sufficient to induce a HR. Another complicating factor in the interpretation of these data is the well known ability of pectic enzymes to kill plant cells via cell wall structural damage and subsequent membrane leakiness due to osmotic fragility (7). If pectic enzymes are involved in hypersensitive cell death, this activity must be distinguished from these nonspecific effects of cell wall degradation. Finally, it has been recently reported that low levels of a pectate lyase from *Erwinia chrysanthemi* suppress both the multiplication and HR inducing activity of *Pseudomonas syringae* pv. *pisi* in tobacco. These activities appear to be mediated by a heat-stable factor(s) released from tobacco cell walls by the enzyme (6).

Little is known about the molecular mechanisms by which these various responses to pectic enzymes are triggered. A related question is whether each defense response is triggered independently or whether multiple responses occur as a result of some early common induction process. The elucidation of these questions requires an understanding of very early host responses to pectic enzymes. We have conducted a study of such a response.

It has recently been reported that induction of the HR in tobacco by *P. syringae* pv. *pisi* is associated with the activation of a specific host plasmalemma K^+ efflux/H^+ influx exchange (3). The K^+/H^+ response occurs in both tobacco leaf tissue and suspension-cultured cells. This finding provides an early and specific criterion by which to identify a host response as hypersensitive. We present here our findings on the transient activation of plasmalemma K^+/H^+ exchange in suspension-cultured tobacco cells by low levels of a pectate lyase isozyme from *E. chrysanthemi* and show that this response can be distinguished from nonspecific electrolyte loss resulting from cell wall damage and osmotic fragility. A preliminary report of this work has been given (2).

MATERIALS AND METHODS

Characteristics and growth conditions of tobacco (*Nicotiana tabacum*) suspension-cell cultures cv Hicks have been described

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[3] Abbreviations: HR, hypersensitive response; Plc, pectate lyase isozyme derived from the pelC gene from *Erwinia chrysanthemi* CUCPB 1237; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

previously (4). Unless stated otherwise, cells from logarithmically growing cultures were used for experimentation. The cell density of these cultures was approximately 35% (packed cell v/v). Cells were collected by filtration onto Miracloth and washed with assay medium. The standard assay medium contained 0.175 M mannitol, 0.5 mM $CaCl_2$, 0.5 mM K_2SO_4, and 1.0 mM Mes-Tris, pH 6.0. However, mannitol concentration, pH, and ion composition were varied for some experiments as indicated in the text. Washed tobacco cells (0.5 g, fresh weight) were transferred to 50 ml beakers containing 15 ml of the appropriate assay medium. Cell suspensions were preincubated at 27° C on a rotary shaker (180 rpm) for approximately 90 min before enzyme treatment. Purified pectate lyase was added to cell suspensions in 20 to 100 μl aliquots and net ion fluxes were determined for up to 7 h as previously described (3). Briefly, this involved intermittent acid/base titration of cell suspensions to pH 6.0 along with collection of filtrate samples which were later analyzed for K^+ or Na^+ by atomic emission or absorption spectroscopy. Cl^- content of filtrate samples was determined with a chloride specific electrode. To increase sensitivity of the Cl^- assays, $CaCl_2$ content in the assay medium was reduced to 0.1 mM and tobacco cells (1 g) were assayed in 10 ml of this medium.

Inoculation of tobacco cells with *Pseudomonas syringae* pv. *pisi* was performed as previously described (3). The induction of nonspecific electrolyte loss from tobacco cells by pectate lyase was demonstrated in 10 mM Tris-HCl and 0.5 mM $CaCl_2$ (pH 8.5) instead of the standard assay medium. Other assay conditions were as described for pH 6.0 assays except that the 90 min preincubation period prior to enzyme treatment was omitted. Electrolyte loss from tobacco cells was followed by measuring conductivity of the assay medium. K^+, Na^+, and Cl^- were determined as described above. Viability of tobacco cells was determined by Evans blue exclusion (16).

Pectate lyase isozyme Plc (pI 8.3) was obtained from an *Escherichia coli* strain containing a cloned pectate lyase gene (*pelC*) from *Erwinia chrysanthemi* CUCPB 1237 (13). Periplasmic fluids containing the enzyme were obtained by osmotic shock and further purified to electrophoretic homogeneity by granulated gel bed isoelectric focusing. Details of these procedures are published elsewhere (6). Enzyme activity was determined by following the increase in *A* at 230 nm of reaction mixtures. Mixtures contained 2.9 ml of buffer (30 mM Tris-HCl pH 8.5 0.1 mM $CaCl_2$, 0.1% polygalacturonic acid) and 100 μl of enzyme sample.

All experiments were conducted at least twice and representative data are presented.

RESULTS

Enzymic Activity. Plc activity at pH 6.0 was approximately 50-fold less than at the pH optimum of 8.5. All enzyme activities reported here reflect the activity on polygalacturonic acid under the described experimental conditions. Variations in mannitol or K_2SO_4 concentration which were used in some experiments had no detectable effect on enzyme activity at either pH (MM Atkinson, unpublished data).

Experimental Rationale. All experiments involving the K^+/H^+ response were conducted at pH 6.0 to approximate physiological conditions of the plant cell wall environment. This was believed to be especially important since both K^+ and H^+ fluxes are strongly influenced by external pH. Despite the relatively low enzymic activity at pH 6, very small quantities of enzyme were required to induce the K^+/H^+ response. In addition, preliminary experiments indicated that the K^+/H^+ response was strongly inhibited at pH 8.0 or above. Experiments at pH 8.5 deal only with induction of nonspecific electrolyte loss from tobacco cells. This pH was selected because it has typically been used by others to study this phenomenon (7, 8) and because prohibitively large quantities of purified enzyme would have been required to conduct these studies at a significantly lower pH. These experiments were included to provide a comparison by which the K^+/H^+ response could be distinguished from nonspecific electrolyte loss due to structural cell wall damage and subsequent membrane leakiness.

K^+ efflux/H^+ influx response. The K^+/H^+ response occurred at very low enzyme activities and was saturable (Fig. 1). Induction of net K^+ efflux and H^+ influx began at approximately 5×10^{-5} units PLc/ml and reached a near maximum of 7 to 10 μmol/g·h between 2 and 5×10^{-4} units/ml. Further increases in enzymic activity produced very small increases in K^+ loss or H^+ uptake. Under these experimental conditions the stoichiometry of K^+/H^+ fluxes was equal to or slightly greater than 1:1. These enzyme levels did not induce any rapid net fluxes of Na^+ or Cl^-. Heat inactivated enzyme (20 min, 70° C) did not induce a K^+/H^+ response (Table I). Treatment of cells with the protonophore CCCP completely inhibited K^+ efflux indicating that K^+ and H^+ fluxes were closely coupled (Table I). This result also suggests that a plasmalemma H^+ gradient (acid outside) may be required for the response. This was further supported by our observation that tobacco cells did not exhibit a strong K^+/H^+ response unless they were allowed a 60 to 90 min adaptation period in assay buffer prior to enzyme treatment. This adaptation period was also associated with the development of net H^+ efflux capacity as evidenced by acidification of the assay medium.

We observed no signs of cell maceration, protoplast release or cell death for up to 7 h after initial treatment although treated cells developed a slight yellow-brown coloration. Representative viability data was 86.8 ± 3.8% viability of control cells and 87.9 ± 1.0% viability of enzyme treated cells at 2 h after initial treatment. At 7 h control cells exhibited 83.0 ± 0.7% viability as

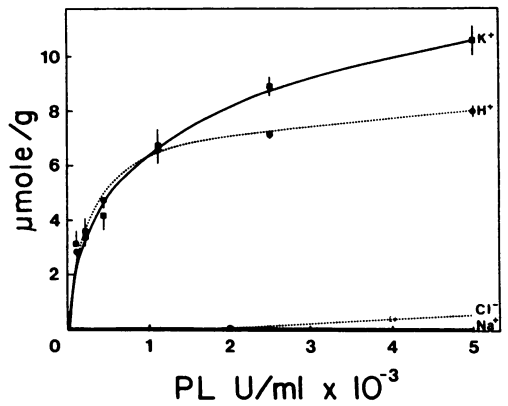

FIG. 1. Dose response: effect of pectate lyase activity on net K^+, Cl^-, and Na^+ loss and H^+ uptake in suspension-cultured tobacco cells at pH 6.0 with 0.175 M mannitol as osmoticum. Measurements were made 1 h after addition of enzyme to cell suspensions. Data are means ± SD of three replicates.

Table I. *Induction of K^+ Efflux and H^+ Influx in Tobacco Cells by Pectate Lyase: Effect of Inhibitors and Heat Treatment of Enzyme*

Measurements were made 60 min after addition of 4×10^{-3} units/ml pectate lyase to cell suspensions. Data represent the difference between fluxes of enzyme-treated and control cells. Means and SD of three replicates are given. Negative values indicate net K^+ influx or H^+ efflux.

Enzyme	Inhibitor	Net K^+ Loss	Net H^+ Uptake
		μmol/g tobacco	
Active	None	3.52 ± 0.81 (100)[a]	3.54 ± 0.61 (100)[a]
Heated	None	0.06 ± 1.13 (1.6)	0.16 ± 0.65 (4.5)
Active	CCCP, 20 μM	−0.79 ± 0.89 (−22)	−0.79 ± 0.31 (−22)

[a] Percent active.

compared to 78.8 ± 5.2% for enzyme-treated cells.

Time course experiments demonstrated that K^+/H^+ exchange began less than 15 min after enzyme addition (Fig. 2). Exchange rates reached a maximum of 5 to 10 μmol K^+ or H^+/g·h within 15 to 30 min and then gradually declined to zero by approximately 1.5 h after enzyme addition. Thereafter, cells resumed a net H^+ efflux equal to or slightly greater than that of untreated cells. Cells did not however, resume the net K^+ influx exhibited prior to enzyme treatment.

The cessation of the initial K^+ efflux H^+ influx response was not caused by a loss of enzyme activity since PL activity declined only slightly during these experiments. In addition, the response could not be prolonged by a second enzyme dose 1 h after the first. Cells remained relatively unresponsive to additional enzyme for at least 5 h after initial treatment. At this point an additional enzyme dose induced a small net K^+ efflux response and little or no change in net H^+ flux. Untreated (control) cells maintained the ability to express the K^+ efflux/H^+ influx response for at least 5 h.

Net H^+ and K^+ fluxes in both control and enzyme treated cells were influenced by osmolarity of the assay medium (Table II). The enzyme-induced K^+/H^+ response, as defined by the difference between net transport in control and enzyme-treated cells, decreased 25 to 43% between 0 and 0.2 M mannitol. Since K^+ efflux apparently occurs down an electrochemical gradient across the plasmalemma, the effect of osmotic pressure on the K^+/H^+ response may reflect its contribution to this gradient. Increasing osmolarity would be expected to reduce the plasmalemma gradient and therefore the rate of K^+ movement.

The K^+/H^+ response induced by live bacteria exhibits maximum ion exchange rates comparable to those induced by enzyme (3) (Fig. 3). The two responses were not additive; the addition of pectate lyase to tobacco cells exhibiting the K^+/H^+ response to bacteria did not increase net H^+ uptake rates (Fig. 3). Similar results were obtained for K^+ efflux (MM Atkinson, unpublished data).

Nonspecific Electrolyte Loss. To distinguish the K^+/H^+ response from nonspecific electrolyte loss due to cell wall breakdown, enzyme dose response, ion specificity and sensitivity to osmolarity were compared for the two phenomena. Because of the relatively high enzymic activity required, nonspecific electrolyte loss was studied at pH 8.5. PLc activities below 1.5×10^{-2}

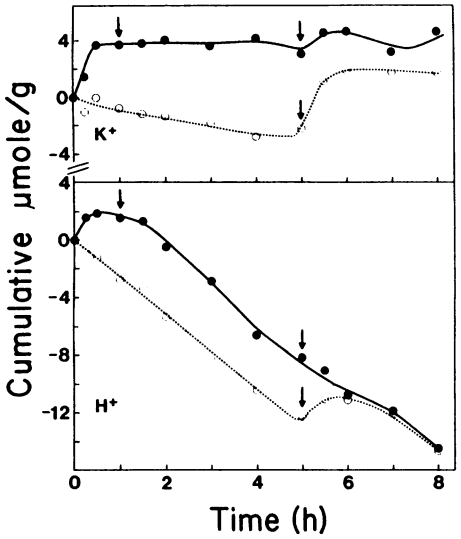

FIG. 2. Effect of pectate lyase on cumulative net K^+ (a) or H^+ (b) transport in suspension-cultured tobacco cells at pH 6.0 pectate lyase (4×10^{-3} units/ml) added to cell suspensions at 0, 1 and 5 h (●); enzyme added at 5 h only (○). Positive values indicate a cumulative loss of K^+ and gain of H^+; negative values indicate a cumulative gain of K^+ and loss of H^+.

Table II. *Effect of Medium Osmolarity on Pectate Lyase-Induced K^+ Efflux and H^+ Influx at pH 6.0 and Nonspecific Electrolyte Loss at pH 8.5*

Measurements were made 60 min after addition of pectate lyase to cell suspensions. Data represent the difference between fluxes of enzyme-treated and nontreated tobacco cells. Means and SD of three replicates or means and range (two replicates) are given.

Mannitol Concentration	Net Flux		
	K^+ Efflux	H^+ Influx	Electrolyte loss
M	*% control*		
0.0 (control)	100	100	100
0.1	88.6 ± 17.5	96.5 ± 14.8	36.9 (32.0–42.1)
0.2	57.4 ± 7.0	75.2 ± 17.6	12.5 (11.6–13.4)

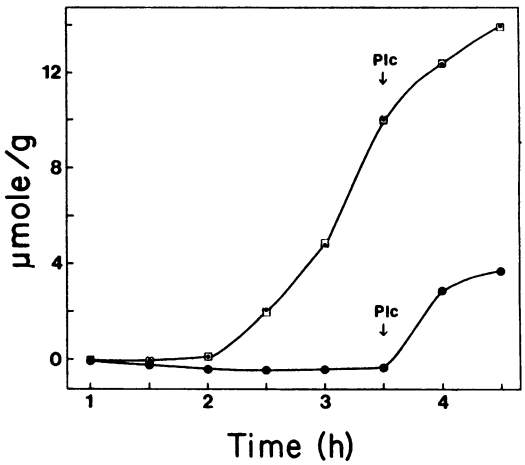

FIG. 3. Effect of pectate lyase on net H^+ uptake in tobacco cells inoculated with *P. syringae* pv. *syringae*. Results are the numerical differences between cells treated with bacteria and/or enzyme and untreated cells. Means of two replicates which did not differ more than 10% are shown. Bacterial inoculation at 0 h only (see text for inoculation details) (◉). Bacterial inoculation at 0 h and pectate lyase addition (4×10^{-3} unit/ml) at 3.5 h (□). Pectate lyase only (●). Data points for the first two treatments are coincident.

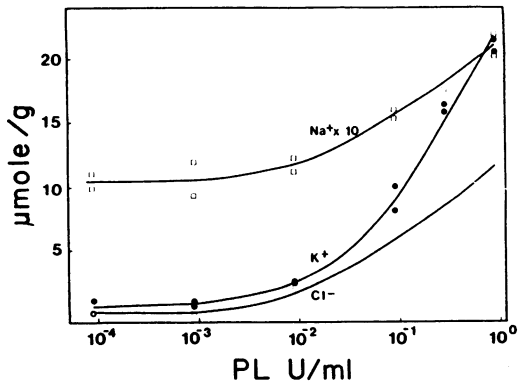

FIG. 4. Dose response: effect of pectate lyase activity on nonspecific electrolyte loss from suspension-cultured tobacco cells at pH 8.5 in the absence of an osmoticum (mannitol). Net K^+, Na^+, and Cl^- effluxes are shown. Measurements were made 1 h after addition of enzyme to cell suspensions. All data points are given for a single representative experiment.

units/ml induced little or no electrolyte loss (Fig. 4) or cell death at pH 8.5 within 1 h. The K^+/H^+ response did not appear to be induced under these alkaline conditions. Enzyme activities greater than this resulted in electrolyte loss and cell death. Enzymic activity of 1.0 unit/ml resulted in 38.9 ± 5.2% nonviable cells within 2 h after initial treatment as compared to 78.7 ± 6.6% nonviability of control cells. Unlike the K^+/H^+ response, a 90 min period of adaptation to assay medium was not required for nonspecific electrolyte loss. Analysis of electrolyte solutions showed that Cl^- and Na^+ in addition to K^+ were lost from tobacco cells. The enzyme dose response was linear over approximately a 20- to 50-fold range of enzymic activity and showed no evidence of saturation.

Nonspecific electrolyte loss at pH 8.5 was highly sensitive to osmolarity and exhibited a 60% decline between 0 and 0.1 M mannitol and a 90% decline at 0.2 M (Table II). Further increases in osmolarity had little effect on the residual electrolyte loss.

DISCUSSION

Our data show that PLc induces net plasmalemma net K^+ efflux and H^+ influx in tobacco cells with a stoichiometry of 1 to 1.5 (K^+:H^+). It should be noted that our data do not distinguish between H^+ influx and OH^- efflux. We have shown by several means (ion specificity, nonlethality, enzyme dose response and degree of sensitivity to osmolarity) that this response is not due to a weakening of cell wall structure and subsequent osmotic fragility of cellular membranes (7, 26). Unlike the K^+/H^+ response, electrolyte loss via this mechanism is lethal, nonspecific, nontransient, and requires approximately 100-fold greater enzymic activity. It appears then that PLc can cause electrolyte loss from tobacco cells by two distinct mechanisms: activation of a specific plasmalemma K^+/H^+ exchange and, as previously recognized, induction of nonspecific electrolyte loss associated with osmotic fragility of cell wall-damaged cells (7).

Although pectic enzymes are known to induce other responses such as the synthesis of phytoalexins, proteinase inhibitors and ethylene (1, 10, 11, 14, 16, 21, 28), the K^+/H^+ response is unique in that it can be detected within a few minutes after cells are treated with enzyme. This result suggests that activation of the K^+/H^+ response may be a very early step in some plant-pathogen interactions. Because of the central role of plasmalemma H^+ transport in regulation of cell membrane potential and active transport (25) and the importance of cytoplasmic pH homeostasis, it is likely that any significant perturbation of transport would be rapidly sensed by the plant cell. The possibility therefore that the K^+/H^+ response may trigger subsequent responses warrants further exploration. For example, we have recently reported the suppression of both hypersensitive necrosis and bacterial multiplication in tobacco by prior treatment of leaf tissue with pectate lyase (6). The results shown here raise the possibility that the transient K^+/H^+ response may be involved in the initiation of this protection. Equally viable alternatives, however, include independent induction of various responses or a common cellular signal not yet described which activates a number of responses.

It has been recently demonstrated that a similar but nontransient K^+/H^+ response occurs during the hypersensitive response of tobacco to *P. syringae* pv. *pisi* (3). Similar maximum exchange rates and the nonadditivity of the two responses suggest, although do not prove, that the mechanism of K^+/H^+ exchange is the same in each case. The transient induction of this response by PLc suggests that this or other host cell wall degrading enzymes may influence HR induction. Under our experimental conditions, this isozyme does not appear to be a HR inducer because it does not kill cells except at activities high enough to cause tissue maceration. It is important to note that tissue maceration is not associated with the HR. However, the possibility that other cell wall degrading enzymes or pectate lyase isozymes may induce a nontransient and potentially lethal K^+/H^+ response warrants further investigation. Such an enzyme might be involved in HR induction. It would be especially interesting to study pectic enzymes from the *P. syringae* pathovars.

We cannot presently explain the transient nature of the K^+/H^+ response. Since the response stops in the presence of active enzyme, an altered host physiology may be responsible. Alternatively, if PLc releases host cell wall fragments of a specific size which elicit the K^+/H^+ response, continued enzymic degradation may destroy elicitor activity and thereby stop the response. This possibility is currently being investigated.

The specificity and rapidity of K^+ and H^+ fluxes suggest that transport requires specific plasmalemma proteins. A pathological alteration of plasmalemma ATPase function may be involved but we have no evidence for or against this. The opening, closing and direction of ion flux through K^+ and other ion channels appears to be regulated by complex factors including ligand binding, cell membrane potential and internal and external membrane environment (9, 20, 27). The data presented here appear consistent with the existence of a K^+ channel which transiently opens during pectate lyase treatment. However, the elucidation of actual molecular events leading to the K^+/H^+ response will require further investigation.

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