Minimal Time Requirement for Lasting Elicitor Effects in Cultured Parsley Cells

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Dedicated to Professor Hans Grisebach on the occasion of his 60th birthday

Non-Toxic Elicitor, Phosphate Uptake, Amino Acid Uptake, Parsley *(Petroselinum hortense)* Cells

Elicitor preparations derived from cell walls of phytopathogenic *Alternaria* spp. rapidly inhibited uptake of both inorganic phosphate and amino acids by cultured parsley cells. The inhibition of phosphate uptake was completely reversed if the elicitor was removed within a time period of approximately 20 min following its addition to the cell cultures. Cells which had been exposed to the elicitor for more than 20 min prior to washing, however, exhibited an increasingly reduced rate of phosphate uptake as compared to non-induced controls. Maximal inhibition of phosphate uptake (about 30 to 40% of the controls) required 40 min or more of elicitor treatment. These results confirm that a minimal time of exposure to elicitor of about 20 min is necessary to induce lasting effects in parsley cells. Moreover, the initial inhibition of amino acid uptake into the parsley cells suggests that the elicitor rapidly decharged the plasmalemma potential of the cells.

Introduction

Phytopathogenic fungi have been shown to induce changes in the plasmalemma of their host cells during infection. For example, regions of the plant plasmalemma in contact with the fungus may lose their ATPase activity [1]. Under such circumstances, plant cells are unable to control the efflux of various solutes to the invading fungus. Biotrophic fungi depend on the solute drain from the host cells and may take up a large percentage of the plant's total assimilates [2]. Very probably, modulation of the host plasmalemma ATPase activity is a key factor in the initial stages of infection by biotrophic as well as by other phytopathogenic fungi.

In addition to various extracellular metabolites, such as toxins [3] or enzymes [4, 5], and mechanical pressure, fungal surface components are supposedly responsible for the disturbance of colonized plant cells' plasmalemma functions [6]. We have previously used fungal cell-wall elicitors and cultured parsley cells as a model system to monitor some of the physiological changes induced upon contact of the fungus with the plant tissue [7, 8]. Within a few minutes, the elicitor induced a pronounced change in the rate of uptake of exogeneously supplied [^{32}P]orthophosphate by the plant cells. This effect was fully reversed if the cells were washed free of elicitor within 10 min of its application. Furthermore, a temporary decrease in the cytoplasmic and vacuolar pH was observed in response to the elicitor treatment, which was accompanied by a shift in the relative proportion of inorganic phosphate in the cytoplasm and in the vacuole. Plant cells usually accumulate phosphate in their vacuoles from where it is available on demand in order to maintain a fairly constant cytoplasmic concentration [9–13]. The flow of phosphate may, however, be reversed from the cytoplasm into the vacuole under stress conditions [14]. On the other hand, the inorganic phosphate concentration may itself regulate cellular transport and metabolic activities ([15–18], see references under [8]). The elicitor-induced synthesis of coumarinic phytoalexins in the parsley cells [7] was ascribed at least in part to the temporarily decreased cytoplasmic phosphate concentration [8].

The mechanism of uptake of inorganic phosphate into plant cells is unknown at present, although a transport protein has recently been identified from a yeast [19]. It appears, however, that plasmalemma transport and transport into mitochondria proceed by similar means [20, 21], and the molecular species taken up is most likely the monoanion [22]. Furthermore, indirect evidence suggests that phosphate

Abbreviations: Ac elicitor, elicitor preparation from cell walls of *Alternaria carthami* Chowdhury; Ad elicitor, elicitor preparation from cell walls of *Alternaria dauci*.

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transport depends on the plasmalemma potential and is linked to an ATPase activity [23–26]. ATPase activity, on the other hand, may be strongly dependent on the intracellular pH, as has been reported for yeast and mammalian cells [27]. A differential inhibition of ATPases in the plasmalemma and the tonoplast of parsley cells could explain the elicitor-induced inhibition of phosphate uptake, as well as the shift in the ratio of vacuolar and cytoplasmic phosphate concentration. Under these conditions, uptake of compounds which rely on proton cotransport, *e.g.* amino acid transport, should also be severely impaired.

The present report describes elicitor-induced changes in the uptake of an amino acid and orthophosphate into cultured parsley cells. Those changes persisting in the cells after removal of the elicitor as well as the partial cell transport recovery in the presence of elicitor were also studied.

Materials and methods

Chemicals

All chemicals were of analytical grade. Sodium dihydrogen [^{32}P]phospate (7.4 TBq/mol) and L[U-^{14}C]asparagine (4.44 TBq/mol) were purchased from Amersham-Buchler, Braunschweig.

Determination of radioactivity

Radioactivity in the cells and in the growth medium was determined by liquid scintillation counting in dioxane containing 100 g naphthalene and 5 g diphenyloxazole $\times$ l^{-1}. Cells were separated and washed twice with fresh growth medium on the filter prior to liquid scintillation counting.

Cell cultures

Parsley *(Petroselinum hortense)* cell suspension cultures were continuously dark grown in B 5 medium [28]. Cells were propagated by transferring about 2.7 g fresh weight of cells into 40 ml of fresh growth medium every 7 days. Cell cultures were used for these studies 8 days after transfer. At this growth stage, a 40-ml culture contained approximately 6 g fresh weight of cells.

Elicitors

Elicitors were prepared from cell walls of either *Alternaria carthami* (Ac elicitor) or *Alternaria dauci* (Ad elicitor) as described previously [8].

Uptake and efflux studies

For each set of experiments several parsley cell cultures of the same age were mixed and the suspension subsequently subdivided into 40-ml portions. Either 18.5 or 37 KBq, of sodium dihydrogen [^{32}P]phosphate or 9.25 KBq of L-[U-^{14}C]asparagine was added to each 40-ml culture. Elicitor (4 or 8 mg dry weight/40 ml culture) was added in 1 ml of water.

Results

Effect of Ad elicitor on the fresh weight increase of cells

In the search for another elicitor exhibiting low toxicity to parsley cells, the effect of Ad elicitor on the fresh weight increase of the cells was studied. Ad elicitor was derived from the cell walls of *Alternaria dauci,* a potential pathogen for several Umbelliferae including parsley [29]. Only high Ad elicitor concentrations caused a slight reduction in cell fresh weight increase with respect to control cultures (Fig. 1), similar to the results obtained previously with Ac elicitor [8]. To ensure maximal elicitor effects with only minimal cell growth inhibition, 0.1 and 0.2 mg/ml, respectively, of either Ac or Ad elicitor were employed in all following experiments.

Effect of *Alternaria* elicitors on uptake of [^{32}P]phosphate

Uptake of [^{32}P]orthophosphate by parsley cells was monitored for 5 h either in the absence of elicitors or in the presence of either Ac elicitor or Ad elicitor

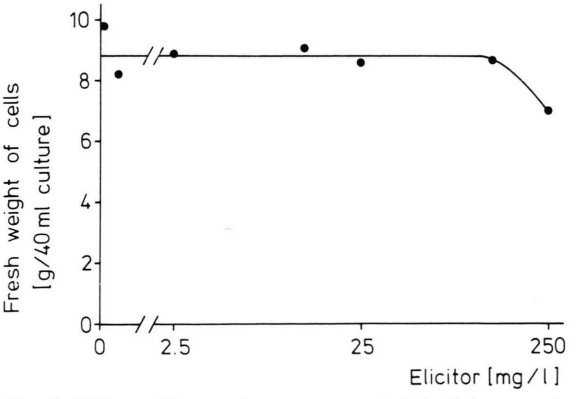

Fig. 1. Effect of increasing amounts of Ad elicitor on the fresh weight increase of parsley cells 13 days after inoculation of the cultures. Portions (2 g fresh weight of cells) of a 7-day-old culture were transferred into fresh growth medium (40 ml) containing the elicitor.

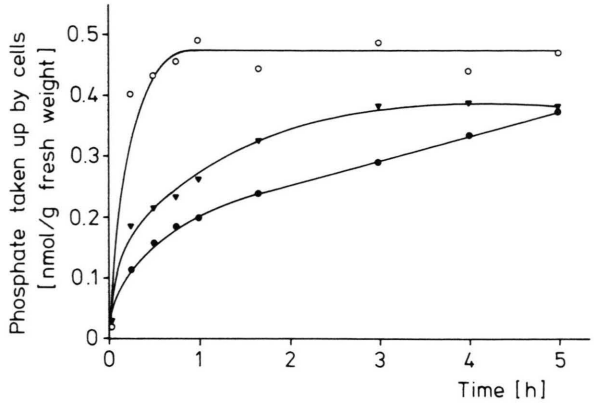

Fig. 2. Inhibition of phosphate uptake into parsley cells by Ac or Ad elicitor. The amount of phosphate incorporated into the cells was monitored for several hours after addition of either 2.5 nmol $NaH_2{}^{32}PO_4$ (○), 2.5 nmol $NaH_2{}^{32}PO_4$ and Ac elicitor (8 mg) (▼), or 2.5 nmol $NaH_2{}^{32}PO_4$ and Ad elicitor (8 mg) (●) to 40 ml cell suspensions derived from an 8-day-old culture. The labelled orthophosphate (7.4 TBq/mol) or phosphate and the respective elicitor were dissolved in 1 ml of water prior to use.

(Fig. 2). At the time of use (8 days after transfer), almost all the phosphate supplied with the growth medium had accumulated in the cells [8]. The plateau in cellular phosphate level reached after 60 min in the absence of elicitor resulted from exhaustion of external phosphate rather than from a change in the rate of uptake, since only about 2.5 nmol [^{32}P]orthophosphate per 40 ml culture had been added. While both the Ac and the Ad elicitor considerably reduced the cellular phosphate accumulation rate, initial inhibition by Ad elicitor appeared to be greater (Fig. 2) as compared to that by an equivalent amount (dry weight) of Ac elicitor.

Reversal of elicitor-induced phosphate uptake inhibition

It has been shown previously that the elicitor-induced phosphate uptake inhibition can be completely reversed by washing out the elicitor shortly after its addition to the parsley cells [8]. A time course study was undertaken in order to determine the maximal time span of elicitor exposure before lasting effects occur in the cells (Fig. 3). Ac elicitor and Ad elicitor were similarly efficient in such studies. Apparently, after at least 20 min of exposure to Ad elicitor the cells begin to suffer in their subsequent uptake of phosphate. This effect is maximal after an additional 20 min of exposure and does not change significantly upon further treatment with the elicitor.

Determination of phosphate efflux from elicitor-treated cells

Parsley cells preloaded with [^{32}P]orthophosphate were carefully washed and resuspended in either regular growth medium, growth medium containing Ac elicitor or growth medium containing Ad elicitor. The cells were subcultured for several hours and leakage of label into the growth medium was monitored (Fig. 4). Whereas during the initial 20 min after washing all cells released some label into the medium (*e.g.* not exceeding 2% of the total phos-

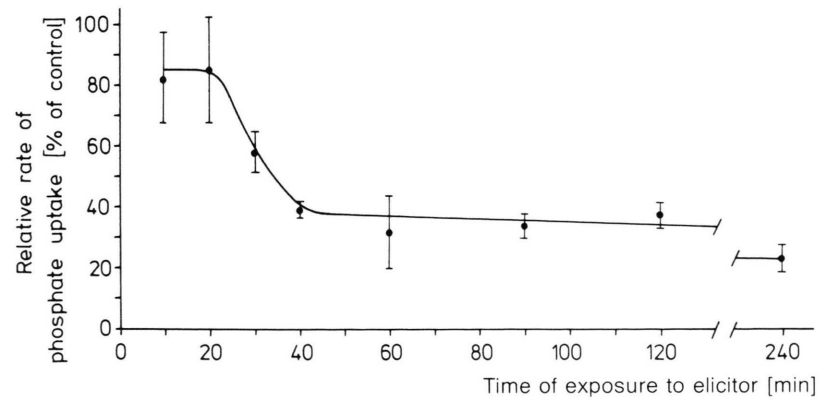

Fig. 3. Relative rate (% of control) of phosphate uptake by parsley cells washed after exposure to Ad elicitor. Either water (1 ml; control) or Ad elicitor (8 mg dissolved in 1 ml water) was added to 40 ml of 8-day-old cell suspensions. After various time periods the cells were harvested by filtration, washed twice with fresh growth medium, and subcultured in fresh growth medium (40 ml) containing $NaH_2{}^{32}PO_4$ (5.0 nmol; 7.4 TBq/mol). The relative rate of phosphate incorporation into the elicitor-treated cells as compared to the controls was monitored for up to 5 h. Each data point represents three determinations of uptake rates.

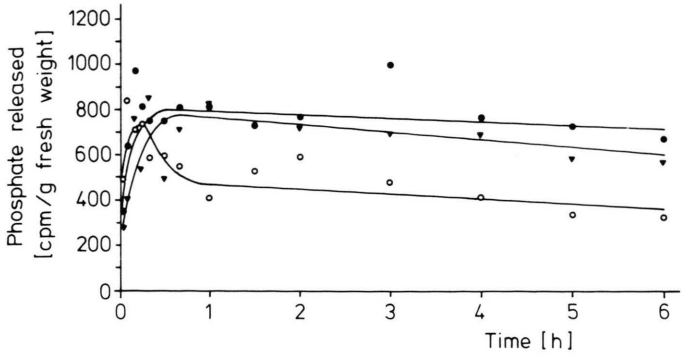

Fig. 4. Efflux of [^{32}P]orthophosphate from elicitor-treated parsley cells. Cell cultures (120 ml; 8-day-old) were subcultured for 30 min in the presence of $NaH_2{}^{32}PO_4$ (about 1.7 nmol; 7.4 TBq/mol). The cells which had accumulated approximately 80% of the total label were harvested and washed twice with fresh growth medium. Portions of these cells were transferred into either fresh growth medium (○), fresh growth medium containing Ac elicitor (8 mg/40 ml) (▼), or fresh growth medium containing Ad elicitor (8 mg/40 ml) (●), and the efflux of radioactivity into the growth medium was monitored for several hours.

phate incorporated), the cells subcultured in the absence of elicitor quickly reabsorbed most of the phosphate. In the presence of either elicitor, however, the cells neither reabsorbed any of the phosphate, nor was additional phosphate released following the initial 20-min period.

Elicitor-induced inhibition of amino acid uptake

The accumulation of L-[U-^{14}C]asparagine in parsley cells was determined over several hours in the presence or absence of Ad elicitor (Fig. 5). The elicitor considerably inhibited the uptake of the amino acid. However, after approximately 60 min in the presence of elicitor the cells partially recovered from the elicitor-inhibition (Fig. 5). This latter phenomenon was repeatedly observed and appeared not to be related to the elicitor concentration employed.

Amino acid efflux from elicitor-treated cells

Parsley cells, preloaded with L-[U-^{14}C]asparagine for 30 min, were carefully washed twice with fresh growth medium. Portions of these cells were resuspended in either regular growth medium, growth medium containing Ac elicitor or growth medium containing Ad elicitor, and the release of label from the cells was determined (Fig. 6). Similar to the situation for orthophosphate, some of the labelled amino acid (roughly 5%) was also released during the initial time period of subculture. Following the initial 60-min period, cells in the absence of elicitor again gradually accumulated the label. In the pre-

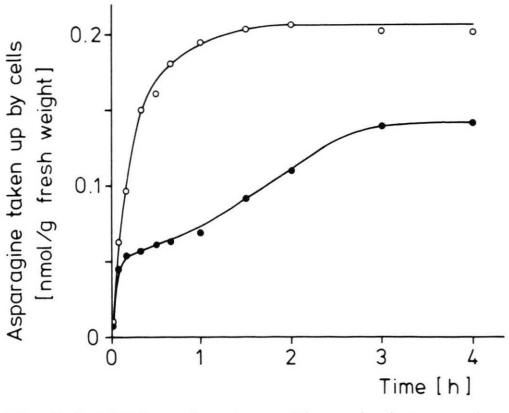

Fig. 5. Inhibition of amino acid uptake into parsley cells by Ad elicitor. The amount of radioactivity incorporated into the cells was monitored for several hours after addition of either 2.1 nmol L-[U-^{14}C]asparagine or 2.1 nmol L-[U-^{14}C]asparagine and Ad elicitor (4 mg) to 40 ml suspensions derived from an 8-day-old culture. The amino acid (4.44 TBq/mol) or the amino acid and the elicitor were dissolved in 1 ml water prior to use.

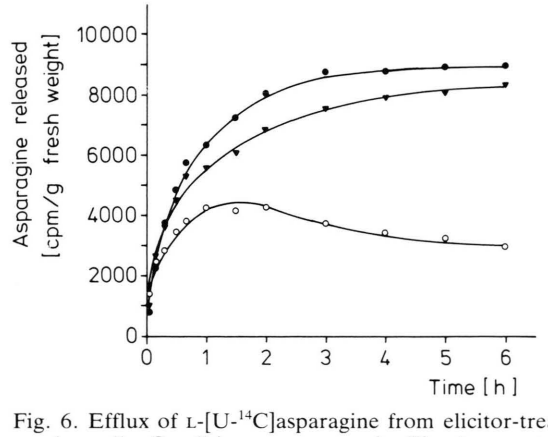

Fig. 6. Efflux of L-[U-^{14}C]asparagine from elicitor-treated parsley cells. Conditions were as under Fig. 4, except that the cells were subcultured for 30 min in the presence of L-[U-^{14}C]asparagine (6.3 nmol/120 ml, 4.44 TBq/mol) instead of labelled phosphate.

sence of either elicitor, however, efflux of label from the cells continued. Within the time span of these experiments (6 h), elicitor-exposed cells lost approximately 10 to 15% of the total previously accumulated label. Upon subsequent gel filtration all the radioactive material released from the cells was found to be of low molecular mass (less than 300 D), suggesting that the amino acid was still present.

Discussion

Amino acid uptake into plant cells is known to proceed via proton cotransport [30] and to depend on carriers [31, 32] as well as on the plasmalemma potential and its ATPase activity [33]. Plant cells may also possess additional regulatory systems for amino acid homeostasis [33, 34]. When parsley cells were treated with elicitors from phytopathogenic *Alternaria* species, not only the uptake of L-asparagine but also the uptake of inorganic phosphate was rapidly inhibited (Figs. 2 and 5) suggesting a change in the plasmalemma structure and/or a strong inhibition of ATPase activity. However, since the effect of elicitor on cell transport properties was fully reversible when the elicitor treatment was short [8] (Fig. 3), a structural modification of the plasmalemma under these conditions appears unlikely.

It has been reported recently that the apparent amino acid accumulation rate of plant cells is based on active uptake and simultaneous passive efflux from the cells [35]. Thus, inhibition of the plasmalemma ATPase would cause the cells to leak their amino acid contents. The large efflux of L-asparagine observed (approximately 10% of total) (Fig. 6) from elicitor treated parsley cells again suggests that the elicitor inhibits the plasmalemma ATPase. It should be noted that under the conditions of the efflux assay a considerable percentage of the amino acid incorporated in the cells was probably no longer available for efflux. Moreover, the elicitor did not induce nonspecific leakiness of the cells since phosphate was not released to the same extent under the conditions of the efflux assay (Fig. 4). Minor efflux observed within the initial 20 min after transfer of cells (Figs. 4 and 6) was due to the damaging washing procedures.

Cells which had been exposed to elicitor for more than 20 min prior to washing showed a lasting reduced rate of phosphate uptake (Fig. 3), and this effect increased with the time of exposure, reaching its maximum at approximately 40 min. The Ac elicitor could fully substitute for The Ad elicitor in such experiments. Obviously, the initial time period of about 20 min is most important for full recognition of the elicitor signal in the parsley cells.

The most probable explanation of our results is that the elicitor reversibly attaches to the surface of the plant cells, thereby inhibiting the plasmalemma ATPase. Moreover, subsequent reactions are initiated which modify the plasmalemma composition within a time period of at least 20 min. According to the fluid mosaic model [36] the plasmalemma arrangement is not static. One might speculate, however, that the elicitor ties down distinct plant plasmalemma components in an order which is later adopted and stabilized by the plant cells even in the absence of elicitor.

Acknowledgements

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